Proteomische und genetische Untersuchungen zum Aromaten-Abbau in "*Aromatoleum aromaticum*" Stamm EbN1

Dissertation
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
- Dr. rer. nat. -

dem Fachbereich Biologie/Chemie der
Universität Bremen vorgelegt von

Lars Wöhlbrand
aus Hamburg

Bremen 2008

Die Untersuchungen zu der vorliegenden Doktorarbeit wurden von Januar 2004 bis Januar 2008 am Max-Planck-Institut für Marine Mikrobiologie in Bremen durchgeführt.

1. Gutachter: Prof. Dr. Ralf Rabus
2. Gutachter: Prof. Dr. Friedrich Widdel

Tag des Promotionskolloquiums: 12.06.2008

Inhaltsverzeichnis

Abkürzungen

2DE	Zweidimensionale Gelelektrophorese
AOR	Aldehyd:Ferredoxin Oxidoreduktase
BTEX	Benzol, Toluol, Ethylbenzol und Xylole
CFU	Colony forming units
DIGE	Difference gel electrophoresis
ESI	Electrospray Ionisation
IEP	Isoelektrischer Punkt
GC	Gaschromatographie
log P_{OW}	Logarithmus des Partionskoeffizienten in *n*-Octanol/Wasser
MALDI	Matrix-Assisted-Laser-Desorption/Ionisation
MDR	Multidrug resistance
MS	Massenspektrometrie
PCMH	*p*-Cresol Methylenhydroxylase
PMF	Peptidmassen Fingerabdruck
RND	Resistance-nodulation-cell division
RT-PCR	Reverse Transkription Polymerase Kettenreaktion
TOF	Time of flight

Zusammenfassung

Der aromatische Ring ist nach dem Glykosylrest die häufigste chemische Verbindung in der Natur. In Böden und Sedimenten stellen sich, durch den Sauerstoffverbrauch aerober Bakterien und Pilze, bereits nach kurzer Zeit anoxische Bedingungen ein. Daher kommt dem anaeroben Abbau aromatischer Verbindungen eine wichtige Bedeutung zu. Innerhalb der letzten zwei Dekaden wurde eine Vielzahl solcher anaeroben Abbauer aus kontaminierten und unbelasteten Habitaten isoliert. Viele dieser Bakterien sind Denitrifizierer, die zum *Azoarcus*/*Thauera*-Cluster innerhalb der Betaproteobacteria gehören.

Obwohl einige dieser anaeroben Vertreter zunächst der *Azoarcus*-Gruppe Pflanzen-assoziierter Endophyten und Wurzeloberflächen besiedelnder Bakterien zugeordnet wurden, bilden sie eine distinkte Gruppe innerhalb des Clusters. Phylogenetisch signifikante Unterschiede, auch auf 16S rRNA-Sequenz-Ebene, machten die Beschreibung der neuen Gattung "*Aromatoleum*" notwendig. Im Rahmen dieser taxonomischen Neuordnung sollten sieben phylogenetisch bislang nicht eingeordnete Isolate erstmals beschrieben und *Azoarcus evansii* KB740, *Azoarcus toluvorans* Td-21, *Azoarcus tolulyticus* Tol-4 und *Azoarcus toluclasticus* MF63 in die neue Gattung überführt werden. Klassische physiologische Experimente konnten belegen, dass die anaerobe Verwertung aromatischer Verbindungen eine spezielle Eigenschaft der Gattung "*Aromatoleum*" darstellt. Hingegen können nur wenige "*Aromatoleum*" spp. elementaren Stickstoff fixieren, eine Eigenschaft, die für *Azoarcus* spp. charakteristisch ist. "*Aromatoleum aromaticum*" Stamm EbN1 ist ein metabolisch vielseitiger "*Aromatoleum*" Vertreter, der eine Vielzahl aromatischer Verbindungen unter oxischen und anoxischen Verbindungen abzubauen vermag. Zudem ist er der erste Vertreter der "*Aromatoleum*"/*Azoarcus*/*Thauera* Gruppe dessen Genom sequenziert wurde.

Folgende Schwerpunkte wurden in der vorliegenden Arbeit gesetzt: (**1**) Die Substrat-spezifische Regulation kataboler Wege in "*A. aromaticum*" Stamm EbN1 wurde mit einem differentiell-proteomischen Ansatz untersucht. Inspiriert durch die regulatorischen Muster, konnten (**2**) *p*-Ethylphenol und *p*-Hydroxyacetophenon als neue Substrate für anaerobes Wachstum von Stamm EbN1 nachgewiesen werden. Physiologische und molekulare Methoden ermöglichten die Aufklärung des entsprechenden Abbauwegs, die Identifizierung beteiligter Proteine sowie die Untersuchung ihrer Substrat-spezifischen Regulation. Da Stamm EbN1 bei der Verwertung aromatischer Substrate gleichzeitig deren toxischen Eigenschaften ausgesetzt ist, wurde seine (**3**) Lösungsmitteltoleranz erstmals auf physiologischer/molekularer Ebene untersucht. Die Entwicklung (**4**) eines genetischen Systems für Stamm EbN1 ermöglichte schließlich die Übertragung extrachromosomaler DNA

in Stamm EbN1, sowie die Generierung einer unmarkierten Deletions-Mutante und deren Komplementation.

(1) Die physiologische Anpassung an bestimmte Wachstumsbedingungen spiegelt sich in Veränderungen der Proteinzusammensetzung einer Zelle wider. Mit Hilfe der Difference gel electrophoresis (DIGE)-Technologie, konnten Änderungen der Protein-Zusammensetzung in Stamm EbN1 bei Kultivierung unter 22 verschiedenen Bedingungen quantitativ erfasst werden. Die anschließende MALDI-TOF-MS Analyse ermöglichte es 354 unterschiedliche Proteine auf den 2DE-Gelen zu identifizieren, von denen 199 signifikant veränderte Abundanzen aufwiesen. Anhand der identifizierten, Substrat-spezifisch gebildeten Proteine konnten die meisten genomisch vorhergesagten Abbauwege von Stamm EbN1 bestätigt werden. In drei Fällen war dies jedoch nicht möglich. Allerdings konnten den entsprechenden Abbauwegen auf der Basis spezifischer Regulationsmuster bislang nicht beachtete Proteine zugeordnet werden. Für drei weitere Substrate konnten neue, bislang nicht vorhergesagte Abbauwege vorgeschlagen werden. Die Detektion und Identifizierung von 107 Proteinen unbekannter Funktion belegte erstmalig ihre tatsächliche Synthese. Die spezifische Bildung einiger dieser Proteine unter nur einer der untersuchten Wachstumsbedingungen legt einen entsprechenden Funktionszusammenhang nahe. Insgesamt zeichnet sich für Stamm EbN1 ein fein abgestimmtes regulatorisches Netzwerk hinsichtlich Substrat-spezifischer Abbauwege ab. Hierbei scheint den Redox-Bedingungen eine übergeordnete Rolle zuzukommen. Der globale Vergleich aller identifizierten regulierten und nicht-regulierten Proteine erlaubte erste Einblicke in die regulatorische Hierarchie spezieller Abbauwege im Vergleich zum allgemeinen Stoffwechsel von Stamm EbN1.

(2) *p*-Ethylphenol und *p*-Hydroxyacetophenon konnten als neue anaerobe Wachstumssubstrate von Stamm EbN1 identifiziert werden. Die GC-MS-basierte Identifizierung intermediär gebildeter Metabolite (auch aus deuteriertem *p*-Ethylphenol) erlaubte die Formulierung eines Abbauwegs, der Parallelen zum anaeroben Ethylbenzolabbau aufweist: initiale Hydroxylierung von *p*-Ethylphenol zu 1-(4-Hydroxyphenyl)-ethanol mit anschließender Dehydrogenierung zu *p*-Hydroxyacetophenon. Eine nachfolgende Carboxylierung und thiolytische Spaltung führen zur Bildung von *p*-Hydroxybenzoyl-CoA, welches in den zentralen Benzoyl-CoA-Weg einfließen kann. Mittels differentieller Proteomanalyse konnten Proteine identifiziert werden, die sehr wahrscheinlich die Einzelreaktionen des postulierten Abbauwegs katalysieren: ein *p*-Cresol Methylenhydroxylase-ähnliches Protein (PchCF), zwei vorhergesagte Dehydrogenasen

(ChnA und EbA309), eine Biotin-abhängige Carboxylase (XccAB) und eine Thiolase (TioL). Diese Proteine sind in einer Operon-ähnlichen Struktur auf dem Chromosom von Stamm EbN1 kodiert. Die Gene dieses potentiellen Operons werden koordiniert und spezifisch exprimiert, wahrscheinlich unter der Kontrolle eines Sigma54-abhängenen Regulators (EbA324).

(3) Stamm EbN1 toleriert sowohl beim Wachstum mit Alkylbenzolen als auch bei plötzlichem Schock von Succinat-verwertenden Zellen mit Alkylbenzolen oder (Alkyl)Phenolen hohe Lösungmittelkonzentrationen (z.B. Wachstum mit bis zu 0,48 mM Ethylbenzol). Hohe Lösungsmittelkonzentrationen führten unter beiden Wachstumsbedingungen zu verminderten Wachstumsraten und -erträgen, zu einer Beeinträchtigung der Denitrifikation sowie zu allgemeinen Stressantworten (z.B. Bildung des Chaperons ClpB). Darüber hinaus konnte die Lösungsmittelstress-induzierte Bildung eines bislang unbekannten potentiellen Efflux-Systems nachgewiesen werden, welches Ähnlichkeit zu den RND-Efflux-Systemen von *E. coli* und *Pseudomonas* spp. aufweist. Das Genom von Stamm EbN1 enthält drei strukturell sehr ähnliche Gen-Cluster für das neu entdeckte potentielle Efflux-System. Diese Gen-Cluster befinden sich in direkter chromosomaler Nachbarschaft zu Genen von Abbauwegen aromatischer Verbindungen. Bemerkenswert ist die spezifische und abundante Bildung der Proteinkomponenten dieser Efflux-Systeme in Gegenwart der entsprechenden Wachstumssubstrate.

(4) Die Etablierung von Methoden zur Kultivierung von Stamm EbN1 auf festem Medium bildete die Grundlage für genetische Experimente. Die Generierung einer Streptomycin-resistenten Mutante (Stamm EbN1-SR7) mit den physiologischen Merkmalen des Wildtyps ermöglichte die Gegenselektion auf genetisch veränderte Mutanten. Des Weiteren wurden Protokolle entwickelt, die eine konjugative Übertragung extrachromosomaler DNA vom Donor *E. coli* S17-1 zum Rezipienten Stamm EbN1-SR7 ermöglicht. Mit Hilfe eines konstruierten Suizid-Vektors konnte eine unmarkierte Deletionsmutante (Stamm EbN1-SR7 Δ*ebdC2*) erzeugt und durch die Expression des *ebdC2*-Gens *in trans* komplementiert werden. Das deletierte Gen, *ebdC2*, kodiert die γ-Untereinheit einer paralogen Ethylbenzol Dehydrogenase, deren Funktion bislang unklar ist. Diese Deletion hatte allerdings keinen Einfluss auf bekannte physiologische Eigenschaft von Stamm EbN1, so dass die Funktionalität des kodierten Enzyms weiter unklar bleibt bzw. eher zunehmend in Frage gestellt werden kann.

Teil I: Darstellung der Ergebnisse im Gesamtzusammenhang

A Einleitung

1. Eigenschaften und Vorkommen aromatischer Verbindungen

1.1 Chemische Eigenschaften

1.1.1 Alkylbenzole, (Alkyl)Phenole und andere monoaromatische Verbindungen

Aromatische Verbindungen sind planare, zyklische Moleküle, die ein konjugiertes π-Elektronensystem aufweisen und primär aus Kohlenstoff und Wasserstoff bestehen (z.B. Benzol). Neben den reinen Kohlenwasserstoffen können aromatische Verbindungen auch andere Atome wie Stickstoff (z.B. Pyridin), Sauerstoff (z.B. Furan) oder Schwefel (z.B. Thiophen) enthalten. Letztere werden als Heterocyclen bezeichnet.

Bei Alkylbenzolen trägt das Benzol-Grundgerüst eine oder mehrere Alkyl-Seitenketten (z.B. Ethylbenzol mit einer Ethylgruppe). Im Falle phenolischer Verbindungen ist das Benzol hingegen mit einer oder mehreren Hydroxylgruppen substituiert (z.B. Phenol mit einer Hydroxylgruppe). Alkylphenole sind Derivate des Phenols, welche neben der Hydroxylgruppe noch eine oder mehrere Alkyl-Seitenketten tragen, z.B. Cresole mit einer Methylgruppe, die in *ortho*, *meta* oder *para*-Stellung zur Hydroxylgruppe stehen kann (Abb. 1).

Neben den Alkylbenzolen und (Alkyl)Phenolen gibt es eine Vielzahl weiterer monoaromatischer Verbindungen, die mit funktionellen Gruppen substituiert sind, z.B. aromatische Alkohole (z.B. Benzylalkohol), Aldehyde (z.B. Benzaldehyd), Ketone (z.B. Acetophenon), Carbonsäuren (z.B. Benzoat) oder Amine (z.B. Anilin). Sie können mehrfach mit derselben Gruppe (z.B. 1,4-Phenylendiamin) oder mit verschiedenen Gruppen (z.B. *o*-Aminobenzoat) substituiert sein. Darüber hinaus gibt es eine Vielzahl Halogen-Substituierter Aromaten (z.B. *p*-Chlorphenol), die allerdings anthropogenen Ursprungs sind.

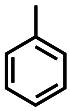
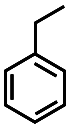
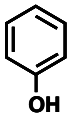
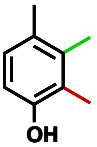
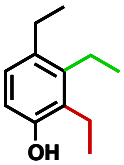
	Toluol	Ethyl-benzol	Phenol	*o*-, *m*-, *p*-Cresol	*o*-, *m*-, *p*-Ethylphenol
					
log P_{OW}	2,7	3,2	1,5	2,0	2,7
Löslichkeit in Wasser (g/l, 20 °C)	0,5	0,2	84,0	20,0	4,9

Abb. 1: Beispiele für Alkylbenzole (Toluol und Ethylbenzol), Phenole (Phenol) und Alkylphenole (*o*-, *m*-, *p*-Cresol und *o*-, *m*-, *p*-Ethylphenol). Die jeweiligen Partitionskoeffizienten in *n*-Octanol/Wasser (log P_{OW}) sowie die Löslichkeit in Wasser sind angegeben (Daten von www.chemdat.merck.de).

1.1.2 Chemische Stabilität und Reaktivität

Aromatische Verbindungen weisen bei moderaten Temperaturen nur eine geringe chemische Reaktivität auf. Die kovalente C–H Bindung ist aufgrund der geringen Differenz der Elektronegativität beider Bindungspartner apolar und daher reaktionsträge. Gleiches gilt für die C–C Bindung. C=C Doppelbindungen sind im Allgemeinen reaktiver. Jedoch besitzt der aromatische Ring ein konjugiertes, Mesomerie-stabilisiertes π-Elektronensystem und damit eine hohe Resonanzenergie (>100 kJ mol^{-1}). Reaktionen am aromatischen Ring erfordern daher erhöhte Aktivierungsenergien (eine Übersicht gibt: Boll et al. 2002). Aliphatische Seitenketten und funktionelle Gruppen beeinflussen die Reaktivität aromatischer Verbindungen sowohl an der Seitenkette selbst als auch die Orientierung von Reaktionen am aromatischen Ring (Breitmaier und Jung 1994). Substituenten können Dipolmomente am aromatischen Ring erzeugen, die bei polaren aromatischen Verbindungen (z.B. Hydroxyl- oder Amino-Substituenten) am stärksten ausgeprägt sind. Jedoch erhöht auch die Methylgruppe des Toluol die Reaktivität im Vergleich zu Benzol (Breitmaier und Jung 1994).

1.2 Toxizität von Alkylbenzolen und (Alkyl)Phenolen

Alkylbenzole und (Alkyl)Phenole sind für Eukaryoten und Prokaryoten cytotoxisch. Die geringe Anzahl funktioneller, polarer Gruppen dieser Verbindungen führt zu einer starken Lipophilie bzw. Hydrophobizität. Die Toxizität eines Lösungsmittels korreliert dabei mit dem Logarithmus seines Partitionskoeffizienten in *n*-Octanol und Wasser (log P_{OW}). Organische

Lösungsmittel mit einem log P_{OW} zwischen 1,5 und 4,0 sind extrem toxisch, da sie bevorzugt in der Cytoplasmamembran akkumulieren (Ramos et al. 2002). Dies führt zur Erhöhung der Membranfluidität und zur Beeinträchtigung ihrer vitalen Funktionen: (i) als Barriere gegenüber der Umwelt, (ii) die Aufrechterhaltung eines chemi-osmotischen Gradienten zur Energiekonservierung und (iii) als Reaktionsmileu Membran-integraler oder -assoziierter Proteine (Sikkema et al. 1995). Die Partitions-Koeffizienten für einige Alkylbenzole und (Alkyl)Phenole sind in Abb. 1 aufgeführt. Bei letalen Konzentrationen kommt es zum unkontrollierten Austritt von Ionen, ATP oder sogar Proteinen sowie zum Zusammenbruch des chemi-osmotischen Gradienten. Die Zelllyse erfolgt jedoch erst bei weiter erhöhten Konzentrationen (Sikkema et al. 1995).

Der Mensch kann Alkylbenzole und (Alkyl)Phenole über die Atemwege, die Haut und den Verdauungstrakt aufnehmen. Die Verteilung der Verbindungen im Organismus wird durch den Lipidgehalt in Geweben und Organen bestimmt. Bevorzugt kommt es zur Akkumulierung in der Leber, Nieren, Herz und Lunge (Rana und Verma 2005). Die Verstoffwechselung dieser Verbindungen findet hauptsächlich in der Leber statt, wobei phenolische Metabolite entstehen. Letztere können unter anderem DNA-Strang Brüche und chromosomale Schäden hervorrufen oder die mitochondriale Spindel zerstören und werden deshalb als cancerogen eingestuft (Brautbar und Williams 2002; Rana und Verma 2005). Da viele Lösungsmittel in der Lage sind die Blut-Hirn-Schranke zu überschreiten, kann eine längere Lösungsmittel-Exponierung (v.a. Toluol) zu Verhaltensänderungen und Einschränkungen der Funktion von Neurotransmitter-Systemen führen (Gralewicz 1999).

Mikroorganismen haben verschiedene Strategien entwickelt, um in Lösungsmittel-Kontaminierten Habitaten zu überleben: (i) die Erhöhung des Anteils *trans*-ungesättigter, gesättigter oder langkettiger Fettsäuren sowie Änderung der Phospholipid-Kopfgruppen Zusammensetzung, (ii) die Bildung Lösungsmittel-Beladener Vesikel und (iii) energie-abhängige Efflux-Pumpen der RND-Familie (resistance-nodulation-cell division), die toxische organische Lösungsmittel aktiv in das externe Medium transportieren (Ramos et al. 2002; Segura et al. 2003). Die Erhöhung des Anteils der *trans*-ungesättigten und gesättigten Fettsäuren erlaubt eine dichtere Packung der Membran und verringert, genau wie die Änderung der Phospholipid-Kopfgruppen, die Membranfluidität.

1.3. Vorkommen aromatischer Verbindungen in der Umwelt

Nach dem Glykosyl-Rest repräsentiert der aromatische Ring die zweithäufigste organische Grundstruktur in der Natur. In der Umwelt können aromatische Verbindungen geologischen, biologischen oder anthropogenen Ursprungs sein.

1.3.1 Geologische Bildung

Alkylbenzole und (Alkyl)Phenole sind signifikante Bestandteile von Erdöl und Kohlenteer (Tissot und Welte 1984; Prince 2002). Erdöl entsteht in mehreren Schritten über geologische Zeiträume (ca. 5-100 Millionen Jahre). Während der so genannten Diagenese wird sedimentiertes organisches Material (v.a. marine Algen) biologisch abgebaut. Das verbleibende organische Material (im so genannten Kerogen) wird kontinuierlich mit neuem Sediment bedeckt. Steigender Druck und Temperaturen >50 °C führen schließlich im Prozess der Katagenese zur Bildung von Erdöl, Kohle und Erdgas aus Kerogen (de Leeuw und Largenau 1993; Prince 2002). Die ältesten Erdöl-Vorkommen sind mehr als 480 Millionen Jahre alt, jüngere erst einige Millionen Jahre. Ungewöhnliche Umweltverhältnisse, wie sie beispielsweise bei den hydrothermalen Quellen des Guyamas Beckens vorherrschen, können jedoch zur Bildung von Erdöl aus Biomasse innerhalb eines viel kürzeren Zeitraumes führen (<5000 Jahre; Prince 2002). Im Durchschnitt enthält Erdöl einen Anteil aromatischer Verbindungen von ~28 %, wobei die absoluten Werte in unterschiedlichen Erdölen stark variieren können (Tissot und Welte 1984).

Erdöl gelangt durch natürliche Sicker-Vorgänge wahrscheinlich seit seiner Entstehung in die Biosphäre und somit in Böden (z.B. Teersände), Wasser und Luft. Der jährliche Eintrag von Erdöl ins Meer beträgt etwa 300 Millionen Liter (Prince 2002). Die stetig wachsende Nutzung von Erdölprodukten durch den Menschen und die damit verbundene Ausbeutung von Erdölvorkommen hat den lokalen Eintrag aromatischer Kohlenwasserstoffe allerdings signifikant erhöht (siehe 1.3.3).

1.3.2 Biologische Bildung

Weltweit betrachtet ist Erdöl jedoch nicht die bedeutendste Quelle aromatischer Verbindungen; der weitaus größte Teil ist biogenen Ursprungs. So ist Phenol als Baustein des Lignins ein essentieller Bestandteil höherer Pflanzen, welche etwa 30 % des gesamten organischen Kohlenstoffs in der Biosphäre repräsentieren (Gibson und Harwood 2002; Boerjan et al. 2003). Darüber hinaus produzieren höhere Pflanzen große Mengen von

aromatischer Sekundär-Metabolite, z.B. Flavonoide oder Terpene (Treutter 2005). Ebenso ist Benzoesäure ein abundanter Bestandteil mancher Harze (z.B. vom Benzoebaum *Styrax torkinensis*) und kommt in einigen Früchten vor (z.B. Pflaumen, Heidel- und Preiselbeeren). Daneben sind auch Tiere (z.B. Naphthalin Synthese durch Termiten) und Prokaryoten in der Lage aromatische Verbindungen zu synthetisieren. So bildet *Tolumonas auensis* anaerob Toluol durch Fermentation von L-Phenylalanin (Fischer-Romero et al. 1996). *p*-Cymol kann hingegen unter methanogenen Bedingungen aus Monoterpenen gebildet werden (Harder und Foss 1999). Neben diesen speziellen Verbindungen sind die aromatischen Aminosäuren L-Phenylalanin, L-Tyrosin und L-Tryptophan essentielle Bestandteile von Proteinen jedes lebenden Organismus und somit ubiquitär und abundant.

1.3.3 Anthropogene Verbreitung

Alkylbenzole und (Alkyl)Phenole werden heutzutage durch die Verkokung von Steinkohle oder direkt aus Erdöl gewonnen und für diverse chemische Synthesen und Produktionsprozesse eingesetzt. Neben ihrer Präsenz in Kraftstoffen, werden Toluol und Ethylbenzol als Lösungsmittel verwendet und auch als Start-Verbindungen für die Herstellung von Styrol (Ethylbenzol) oder Benzol und Xylolen (Toluol) eingesetzt. Im Jahr 2006 wurden weltweit mehr als 23 Millionen Tonnen Toluol produziert (www.icis.com), von denen 50 % für die Synthese von Benzol und Xylolen genutzt wurden. Die Produktion von Ethylbenzol in den USA lag im selben Jahr bei 5,8 Millionen Tonnen (www.icis.com). Phenol hingegen wird hauptsächlich zur Produktion von Bisphenol-A genutzt, welches zur Herstellung von Polycarbonaten, Epoxidharzen oder Alkylphenolen dient. Die globale Phenol Produktion lag im Jahr 2006 bei 8,1 Millionen Tonnen. Die prognostizierte Steigerungsrate von 4 % pro Jahr, geht vor allem auf die wachsende Nachfrage nach optischen Medien (z.B. CDs oder DVDs; www.icis.com) zurück. Durch Unfälle während Produktion, Transport oder Lagerung, gelangen aromatische Lösungsmittel verstärkt in die Umwelt. Tanker-Unglücke haben eine immense lokale Auswirkung. So liefen nach der Havarie der *Exxon Valdez* im Jahr 1989 40 000 t Rohöl in den Prince William Sound, Alaska, und verschmutzten etwa 2000 km Küste (Bragg et al. 1992). Neben diesen Großereignissen führt aber auch die unvollständige Verbrennung oder Verdunstung von Kraftstoffen sowie Leckagen in unterirdischen Tanks zu einer täglichen Emission von Alkylbenzolen und (Alkyl)Phenolen. Vor allem die Kontamination von Grundwasserleitern mit den gut löslichen, toxischen BTEX-Verbindungen (Benzol, Toluol, Ethylbenzol und Xylole) ist von großer ökologischer und ökonomischer Bedeutung.

Neben ihrer Anwendung als Lösungsmittel oder Start-Verbindungen für chemische Synthesen, haben substituierte aromatische Verbindungen große Bedeutung für unser tägliches Leben. So werden beispielsweise (aromatische) Aminosäuren in einer Vielzahl von Nahrungs- und Futtermitteln sowie pharmazeutischen Produkten eingesetzt (Hermann 2003). Im Jahr 2001 belief sich die weltweite Produktion auf mehr als 2 Millionen Tonnen pro Jahr (hauptsächlich L-Glutaminsäure und L-Lysin) mit einem jährlichen Zuwachs von mehr als 10 % (Hermann 2003). Ein weiteres Beispiel quantitativ bedeutsamer aromatischer Verbindungen ist das Benzoat (und die Benzoesäure), welches exklusiv durch die Oxidation von Toluol synthetisiert wird (im Jahr 1998 waren es 698 000 t). Benzoat oder Benzoesäure wird aufgrund seiner bakteriostatischen Wirkung als Konservierungsmittel in Nahrungsmitteln eingesetzt und ist auch in einer Vielzahl anderer Produkte vorhanden, z.B. Zahnpasta, Kosmetika oder Motorfrostschutz (www.inchem.org; Nair 2001).

2. Aerober Abbau aromatischer Verbindungen

2.1 Reaktionen, Gene und Organismen

Viele Bakterien, Pilze und Hefen sind in der Lage in Gegenwart von molekularem Sauerstoff aromatische Verbindungen zu verwerten. Der Sauerstoff dient dabei nicht nur als terminaler Elektronenakzeptor, sondern auch als reaktives Co-Substrat für die Schlüsselreaktionen des Abbaus. Initial erfolgt hierbei eine Funktionalisierung der aromatischen Verbindung durch Mono- oder Dioxygenasen. Monooxygenasen katalysieren den Einbau eines Sauerstoffatoms in das aromatische Substrat, während das andere zu Wasser reduziert wird. Sie sind aber auch an der Aktivierung aliphatischer Kohlenwasserstoffe beteiligt. Dioxygenasen inkorporieren hingegen beide Atome des molekularen Sauerstoffs in das Substrat-Molekül. Um die Reaktion zu ermöglichen, muss der molekulare Sauerstoff zunächst aktiviert werden, da er im Grundzustand (als Triplett) recht reaktionsträge ist. Im reaktiven Zentrum von Mono- und Dioxygenasen erfolgt die reduktive Bildung reaktiver Sauerstoff-Spezies (z.B. Superoxid oder Peroxid) unter Beteiligung eines Metall-Cofaktors (meist Eisen). Dies ermöglicht den Angriff auf die apolaren C–H-Bindungen des aromatischen Rings (Kauppi et al. 1998; Boyd et al. 2001). Die Ringspaltung der zentralen Intermediate Catechol, Protocatechuat oder Gentisat erfolgt schließlich durch Dioxygenasen entweder in ortho- (Intradiol-Spaltung) oder *meta*-Position (Extradiol-Spalung). Der weitere

Abbau mittels β-Oxidation führt anschließend zur Bildung zentraler Metabolite des Intermediärstoffwechsels (Harwood und Parales 1996; Rosenberg 2006).

Der aerobe Abbau aromatischer Verbindungen wurde sowohl für Pilze (Hammel 1995) als auch für Bakterien unterschiedlichster phylogenetischer Gruppen beschrieben. Vertreter Gram-positiver Bakterien wie beispielsweise *Bacillus* oder *Nocardia,* aber auch Vertreter der Alpha- (z.B. *Rhizobium*), Beta- (z.B. *Pseudomonas*) oder Gamma-Untergruppe (z.B. *Acinetobacter*) der Gram-negativen Proteobacteria sind in der Lage, aromatische Verbindungen als Kohlenstoff und Energiequelle zu nutzen (Rosenberg 2006). Entsprechend sind Strukturgene für diese Stoffwechselwege in den Genomen von Organismen dieser Gruppen vorhanden (www.ncbi.nlm.nih.gov).

Am aeroben Abbau von Toluol in *Pseudomonas putida* Stämmen, die das pWW0 Plasmid tragen, sind sowohl Mono- als auch Dioxygenasen beteiligt. Die initiale Reaktion wird durch die Xylol Monooxygenase katalysiert, wodurch ein primärer Alkohol aus der Methylgruppe gebildet wird (Benzylalkohol). Dieser wird anschließend zu Benzoat oxidiert, welches mittels Hydroxylierung und Decarboxylierung in Catechol überführt wird (Abb. 2; Ramos et al. 1997). Die Ring-Spaltung erfolgt schließlich durch eine Dioxygenase und der weitere Abbau über den β-Ketoadipat-Weg (Harwood und Parales 1996; Ramos et al. 1997).

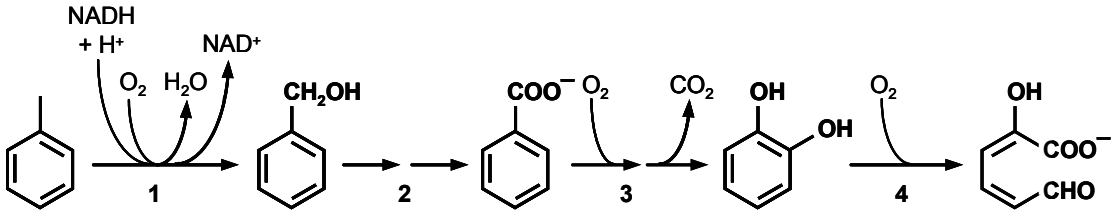


Abb. 2: Aerober Toluol-Abbau durch Mono- und Dioxygenasen in *Pseudomonas putida*. Durch eine Monooxygenase wird ein primärer Alkohol an der Methylseitenkette gebildet (1). Weitere Oxidation (2) führt zur Bildung von Benzoat. Mittels Hydroxylierung und Decarboxylierung (3) wird Catechol gebildet. Die Spaltung des Rings erfolgt durch die Aktivität einer Dioxygenase (4; nach Harwood und Parales 1996; Ramos et al. 1997).

3. Anaerober Abbau aromatischer Verbindungen

Bis vor zwei Jahrzehnten wurde der Abbau aromatischer Kohlenwasserstoffe als strikt aerober Prozess angesehen, da molekularer Sauerstoff unter anoxischen Bedingungen nicht als Co-Substrat zur Verfügung steht. Erste eindeutige Hinweise auf den anaeroben Abbau von Alkylbenzolen lieferten strikt anoxische Anreicherungskulturen und Mikrokosmen (Kuhn et al. 1985; Zeyer et al. 1986; Grbic-Galic und Vogel 1987). Anfang der 1990er Jahre wurde dies durch die Isolierung bisher unbekannter Bakterien mit den entsprechenden Abbaufähigkeiten untermauert (siehe Abschnitt 3.1). Die Isolierung dieser Reinkulturen ermöglichte die detaillierte Untersuchung des anaeroben Aromaten-Abbaus auf biochemischer und molekularer Ebene. Im Folgenden werden die verschiedenen Aspekte des anaeroben Abbaus genauer betrachtet.

3.1 Organismen

Die vollständige Oxidation aromatischer Verbindungen zu CO_2 unter anoxischen Bedingungen scheint nach heutigem Kenntnisstand eine exklusive Eigenschaft der Prokaryoten zu sein. Dabei hängt die generierbare Energie nicht nur vom zu verwertenden Aromaten und dessen Abbauweg ab, sondern auch von den Redoxpotentialen der alternativen Elektronenakzeptoren. Am meisten Energie kann gewonnen werden, wenn Nitrat als terminaler Elektronenakzeptor fungiert. Mangan (IV), Eisen (III) oder Sulfat sind energetisch weniger günstige Elektronenakzeptoren. Die theoretische freie Energie der vollständigen Mineralisation von Toluol ist in Abb. 3 für diese Elektronenakzeptoren angeben.

Alle bislang bekannten Reinkulturen Kohlenwasserstoff-abbauender Bakterien sind den Proteobacteria zuzuordnen und wurden aus unterschiedlichen Habitaten weltweit isoliert (Abb. 4; eine Übersicht bietet Rabus 2005a). Viele denitrifizierende Stämme, die aromatische Kohlenwasserstoffe anaerob verwerten können, gehören der "*Aromatoleum*"/*Azoarcus*/ *Thauera* Gruppe der Betaproteobacteria an (Dolfing et al. 1990; Evans et al. 1991; Schocher et al. 1991; Fries et al. 1994; Anders et al. 1995; Rabus und Widdel 1995; Song et al. 1999; Ehrenreich et al. 2000; Shinoda et al. 2004; Shinoda et al. 2005). Die marinen denitrifizierenden Isolate Col2 und TH1, die beide Toluol vollständig oxidieren, werden der Gamma- bzw. Alpha-Untergruppe zugeordnet (Zengler 1999). Reinkulturen anaerob Aromaten-abbauender Bakterien wurden ebenfalls unter Mangan (IV)-reduzierenden (Langenhoff et al. 1997), Eisen (III)-reduzierenden (Lovley et al. 1989; Lovley und Lonergan 1990), Sulfat-reduzierenden (Rabus et al. 1993; Beller et al. 1996; Galushko et al. 1999;

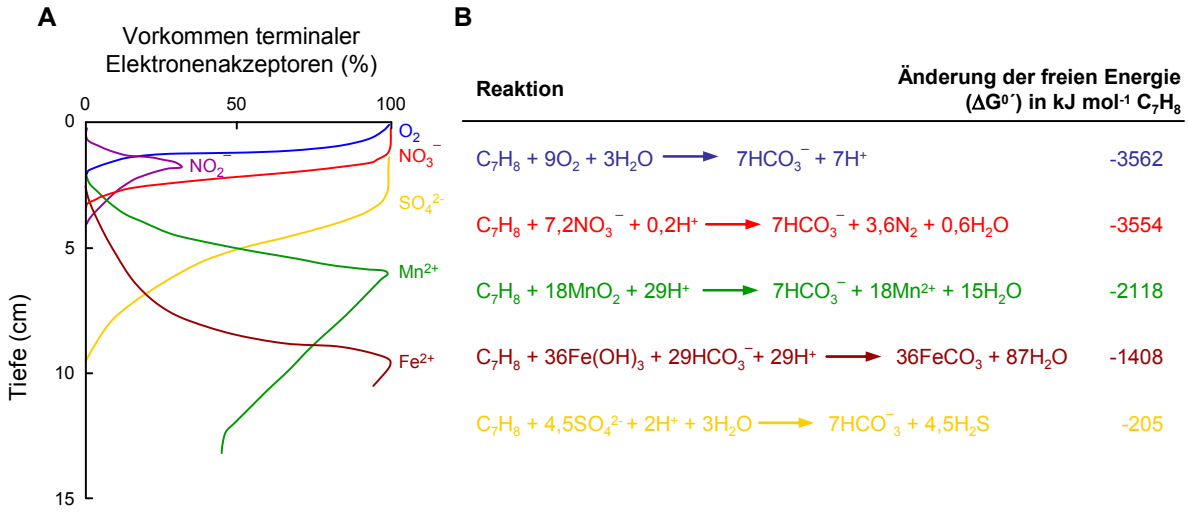


Abb. 3: (A) Vertikalprofile typischer terminaler Elektronenakzeptoren in Frischwasser-Sedimenten (ohne NH_4^+ und CO_2). Das Vorkommen der terminalen Elektronenakzeptoren ist in Prozent vom maximal beobachteten Wert in Süßwasser-Sedimenten angegeben: O_2 300-400 µM, NO_3^- und NO_2^- ~1 µM, Mn^{2+} 50 µM, Fe^{2+} 15 µM, SO_4^{2-} 100-200 µM (verändert nach Nealson 1997).
(B) Freie Energie der vollständigen Oxidation von Toluol, gekoppelt an die Reduktion verschiedener Elektronenakzeptoren (verändert nach Spormann und Widdel 2000).

Harms et al. 1999; Kniemeyer et al. 2003), phototrophen (Zengler et al. 1999) und synthrophen Bedingungen isoliert (Grbic-Galic und Vogel 1987; Ficker et al. 1999). Das Abbauspektrum dieser Stämme umfasst sowohl mono- als auch polycyclische (z.B. Naphthalin) Kohlenwasserstoffe oder *n*-Alkane. Bis heute ist jedoch kein Organismus bekannt, der sowohl *n*-Alkane als auch aromatische Kohlenwasserstoffe abbauen kann. Aufgrund des relativ schnellen Wachstums wurden die Untersuchungen zum anaeroben Kohlenwasserstoff-Abbau bevorzugt mit Nitrat-reduzierenden Bakterien durchgeführt (siehe Kapitel 3.2).

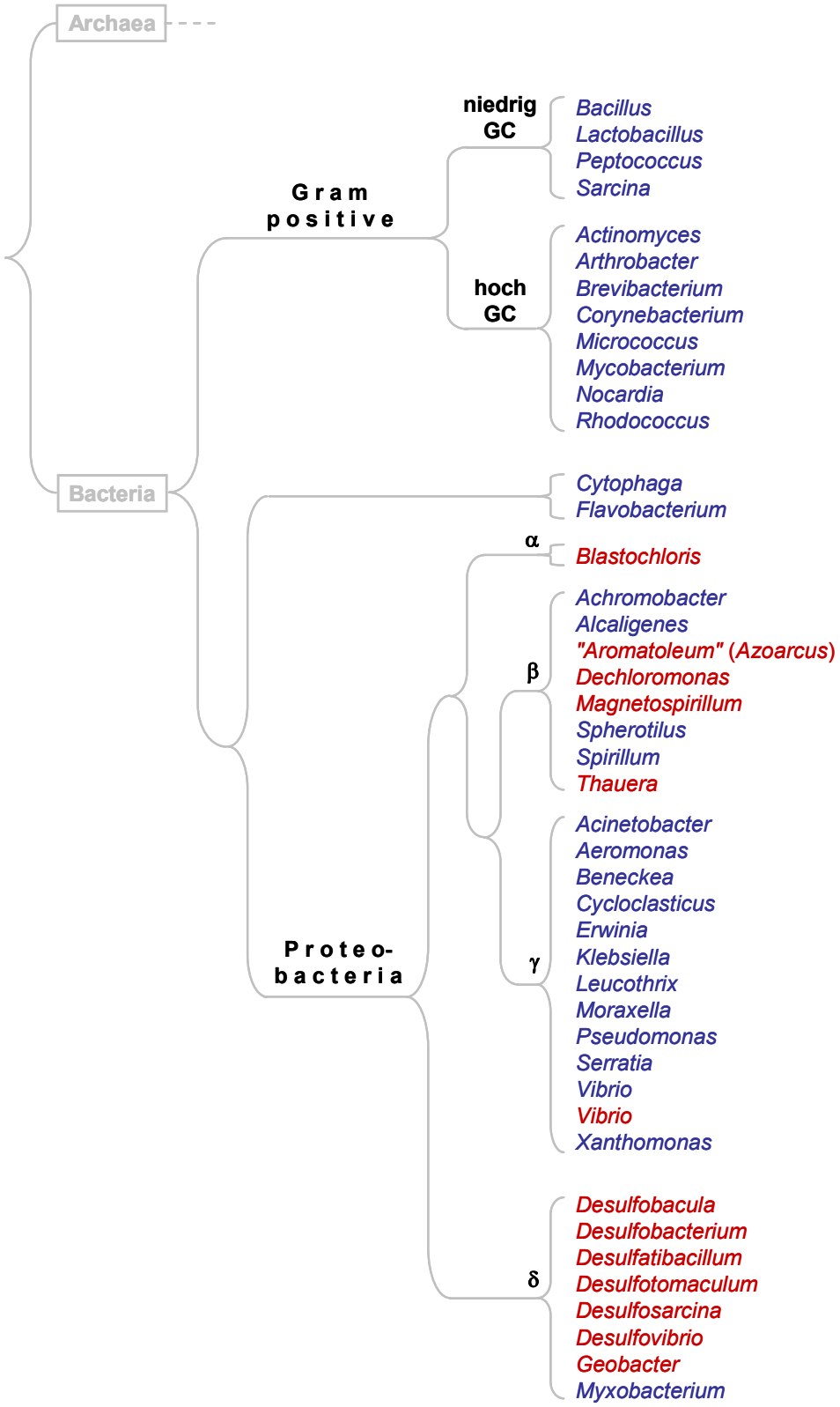


Abb. 4: Diversität und phylogenetische Eingruppierung Kohlenwasserstoff (ohne Methan)-abbauender Bakterien. Blau kennzeichnet aerobe, Rot anaerobe Abbauer. Die Längen der Äste spiegeln keine phylogenetischen Distanzen wieder (verändert nach Widdel und Rabus 2001; Rosenberg 2006; Widdel et al. 2006).

3.2 Reaktionen und Gene

Unter anaeroben Bedingungen werden die meisten aromatischen Verbindungen über den zentralen Benzoyl-CoA-Weg abgebaut. Dies erfolgt über verschiedenste Reaktionssequenzen, die mit einer zumeist Substrat-spezifischen Aktivierungsreaktion initiiert werden (siehe unten). Im Verlauf der Reaktionssequenz wird die CoA-aktivierte Carboxyl-Gruppe von Benzoyl-CoA gebildet. Im Fall von mehrfach substituierten Aromaten muss der zusätzliche Substituent zur Bildung von Benzoyl-CoA entfernt werden; beispielsweise durch reduktive Dehydroxylierung (*p*-Hydroxybenzoat) oder Deaminierung (*o*-Aminobenzoat; Gibson und Harwood 2002).

Aromatische Verbindungen mit zwei oder drei Hydroxylgruppen (z.B. Resorcinol) weisen, im Vergleich zu monosubstituierten Aromaten, eine deutlich verringerte Stabilität des aromatischen Rings auf (durch die ermöglichte Keto-Enol Tautomerie). Daher können diese Verbindungen mittels "gewöhnlicher" Reduktionsmittel (z.B. reduziertem Ferredoxin) reduktiv dearomatisiert werden, ohne die intermediäre Bildung von Benzoyl-CoA (Gibson und Harwood 2002; Boll 2005a).

3.2.1 Fumarat Addition

Die Glycyl-Radikal-abhängige, stereospezifische Addition eines Alkylbenzols oder Cresols an das Co-Substrat Fumarat wurde sowohl für denitrifizierende, Sulfat-reduzierende, Eisen(III)-reduzierende und phototrophe Bakterien als auch methanogene Konsortien beschrieben. Diese Organismen wandeln Toluol, *o*-, *m*-, *p*-Xylol, Ethylbenzol, *m*-, und *p*-Cresol oder 2-Methylnapthalin in das jeweilige Arylsuccinat um. Neben aromatischen Verbindungen können auf diese Weise auch kurz- und langkettige Alkane (C_3-C_{20}) aktiviert werden (Widdel und Rabus 2001; Gibson und Harwood 2002; Heider 2007; Kniemeyer et al. 2007).

Die Bildung von (*R*)-Benzylsuccinat durch Addition von Toluol an Fumarat wird durch die Benzylsuccinat-Synthase (BssABC) katalysiert (Biegert et al. 1996; Beller und Spormann 1997; Leuthner et al. 1998; Rabus und Heider 1998). Zu Beginn des Reaktionszyklus bildet das Adenosylmethionin-abhängige Aktivierungs-Enzym (BssD) ein freies Radikal an einem Glycin-Rest der α-Untereinheit (BssA; Leuthner et al. 1998; Krieger et al. 2001). Durch die Radikal-getriebene Abstraktion eines Wasserstoffatoms von der Methylgruppe des Toluols entsteht ein Benzyl-Radikal, welches an die Doppelbindung des Fumarat addiert. Das gebildete Benzylsuccinyl-Radikal abstrahiert schließlich ein

Wasserstoffatom (welches ursprünglich von der Methylgruppe des Toluols stammt) vom Enzym, wodurch (*R*)-Benzylsuccinat entsteht und das aktive, Enzym-gebundene Radikal regeneriert wird. Mittels einer β-Oxidations-ähnlichen Reaktionssequenz wird Benzylsuccinat anschließend zum zentralen Intermediat Benzoyl-CoA abgebaut (Evans et al. 1992; Leuthner und Heider 2000; Leutwein und Heider 2001; Leutwein und Heider 2002).

Die Gene des anaeroben Toluol-Abbaus sind für mehrere Organismen der Gattungen *Thauera*, "*Aromatoleum*" (*Azoarcus*), *Geobacter* und *Magnetospirillum* beschrieben (teilweise unvollständig). Sie sind in zwei Operons organisiert: (i) das *bss*-Operon, welches die Benzylsuccinat-Synthase kodiert und (ii) das *bbs*-Operon, welches die Enzyme für die Umwandlung von Benzylsuccinat in Benzoyl-CoA kodiert. In Stamm EbN1 und *G. metallireducens* sind beide Operons in direkter Nachbarschaft auf dem Chromosom kodiert (6,5 bzw. 4,6 kb Abstand). Alle bekannten Gen-Produkte dieser Operons weisen eine sehr hohe Aminosäuresequenz Identität auf (70-98 %).

3.2.2 Dehydrogenierung

Die Sauerstoff-unabhängige Methylhydroxylierung von *p*-Cresol zu *p*-Hydroxybenzylalkohol und die anschließende Oxidation zu *p*-Hydroxybenzoat wurde bereits Ende der 1970er Jahre für *Pseudomonas putida* gezeigt (Hopper 1976). Später wurde diese Initialreaktion des *p*-Cresol Abbaus auch in anaeroben Bakterien gefunden (Bossert et al. 1989; Hopper et al. 1991; Rudolphi et al. 1991). Für die denitrifizierenden Stämme EbN1 und EB1 wurde ein weiterer auf Dehydrogenierung beruhender Mechanismus zur Aktivierung von Alkylbenzolen beschrieben: die Einführung einer Hydroxylgruppe in die Alkyl-Seitenkette des Ethylbenzols, unter Bildung von (*S*)-1-Phenylethanol. Diese Reaktion wird durch die periplasmatische Ethylbenzol Dehydrogenase (EbdABC) katalysiert (Johnson et al. 2001; Kniemeyer und Heider 2001a). Das Enzym enthält einen Molybdän Cofaktor sowie Eisen-Schwefel Zentren und gehört zur Familie der DMSO Reduktasen. Das Enzym von Stamm EbN1 wurde erst kürzlich auf Ebene der Kristallstruktur (Kloer et al. 2006) und kinetisch/mechanistischer Ebene untersucht (Szaleniec et al. 2007). Die Polarisierung der C–C-Bindung der Ethyl-Seitenkette durch Aminosäure-Reste des aktiven Zentrums ermöglicht die Abstraktion eines Hydrids vom C_1 durch den Molybdän-Oxo-Komplex. Das entstandene Carbokation abstrahiert sofort die Molybdän(VI)-gebundene Hydroxylgruppe, wodurch Molybdän(IV) entsteht. Elektronen, die bei der Re-Oxidation des Molybdän Cofaktors entstehen, werden über die Fe-S Cluster auf Häm *b* übertragen. Nachfolgend

werden die Elektronen vermutlich auf periplasmatisches Cytochrom *c* übertragen (Szaleniec et al. 2007).

Ethylbenzol Dehydrogenase kodierende Gene (*ebdABC*) wurden für die denitrifizierenden Stämme EbN1 und EB1 beschrieben (Johnson et al. 2001; Rabus et al. 2002). Beide Sequenzen sind sehr ähnlich und unterscheiden sich lediglich in vier Nukleotiden (Rabus et al. 2002). In Stamm EbN1 sind die *ebdABC* Gene zusammen mit *ebdD* (Chaperon) und *ped* ((*S*)-1-Phenylethanol Dehydrogenase) in einem Operon organisiert, welches somit beide Enzyme des oberen Ethylbenzol Abbauwegs kodiert (von Ethylbenzol bis Acetophenon). Das Operon für den unteren Abbauweg (von Acetophenon bis Benzoyl-CoA) liegt in direkter chromosomaler Nachbarschaft des Ethylbenzol Dehydrogenase Operons (~7 kb upstream). Diese "Lücke" enthält Gene für zwei Zwei-Komponenten Sensor-Regulator Systeme (Tcs1/Tcr1 und Tcs2/Tcr2), die vermutlich die Transkription beider Operons Substrat-spezifisch kontrollieren (Rabus et al. 2002).

Interessanterweise wurden im Genom des Sulfat-reduzierenden Bakteriums *Desulfococcus oleovorans* Hxd3 Ethylbenzol Dehydrogenase kodierende Gene annotiert (dole_0194-dole_0197), die eine Aminosäuresequenz Identität von 30-52 % aufweisen (www.jgi.doe.gov). Allerdings konnte diese Abbauleistung bisher nicht für *D. oleovorans* Hxd3 gezeigt werden (Rueter et al. 1994; Aeckersberg et al. 1998). Der Abbau von Ethylbenzol unter Sulfat-reduzierenden Bedingungen konnte bisher nur für Stamm EbS7 mittels Fumarat-abhängiger Bildung des Arylsuccinats demonstriert werden (Kniemeyer et al. 2003).

3.2.3 Carboxylierung

Im anaeroben Phenol-Abbau ist die Carboxylierung des aromatischen Rings in *para*-Position der eigentliche, funktionalisierende Schritt. Diese Reaktion wird durch die Phenylphosphat Carboxylase (PpcABCD) katalysiert. Untersuchungen in *Thauera aromatica* K172 zeigten jedoch, dass im initialen Schritt zunächst die Hydroxylgruppe phosphoryliert wird (Phenylphosphat Synthase; PpsAB), um die Carboxylierung zu ermöglichen (Lack und Fuchs 1992; Lack und Fuchs 1994; Schühle und Fuchs 2004). Eine entsprechende Carboxylierung in *para*-Position wird für den anaeroben Abbau von Anilin im Sulfat-reduzierden Bakterium *Desulfobacterium anilini* angenommen (Schnell et al. 1989).

Ein Gen-Cluster für den anaeroben Phenol-Abbau wurde erstmals für *T. aromatica* beschrieben (Breinig et al. 2000) und nachfolgend auch im Genom von Stamm EbN1 gefunden (Rabus et al. 2005). Das Genom von *G. metallireducens* enthält ebenfalls Gene für

alle Untereinheiten der Phenylphosphat Synthase und zwei Untereinheiten der Phenylphosphat Carboxylase (*ppcBC*; www.jgi.doe.gov).

Die Acetophenon Carboxylase stellt eine weitere Carboxylase im anaeroben Aromaten-Stoffwechsel dar. Im denitrifizierenden Stamm EbN1 katalysiert sie die Carboxylierung der Methylgruppe von Acetophenon unter Bildung von Benzoylacetat und agiert somit nicht wie die Phenylphosphat Carboxylase direkt am aromatischen Ring. Nach Aktivierung zu Benzoylacetyl-CoA erfolgt eine thiolytische Spaltung in die zentralen Intermediate Acetyl-CoA und Benzoyl-CoA (Champion et al. 1999; Rabus et al. 2002). Acetophenon Carboxylase kodierende Gene (*apc1*-5) wurden bisher lediglich für Stamm EbN1 beschrieben (Rabus et al. 2002). Sie sind in einem Operon zusammen mit der vorhergesagten Benzoylacetat CoA-Ligase (*bal*) sowie hypothetischen Proteinen organisiert und liegen in direkter chromosomaler Nachbarschaft zu den Genen des oberen Ethylbenzol Abbauwegs (siehe 3.2.2).

3.2.4 Reduktive Dearomatisierung

Die entscheidende Reaktion im zentralen Benzoyl-CoA-Weg ist die reduktive Dearomatisierung. Sie führt zur Aufhebung des aromatischen Systems und ermöglicht damit alle nachfolgenden Abbauschritte über konventionelle Reaktionen. Die reduktive Dearomatisierung wird durch die Benzoyl-CoA Reduktase (BcrCABD) katalysiert und wurde sehr gut in denitrifizierenden und phototrophen Bakterien untersucht. In *T. aromatica* wird der Transfer von zwei Elektronen von reduziertem Ferredoxin unter Bildung von Cyclohex-1,5-dien-carbonyl-CoA an die stöchiometrische Hydrolyse von 2 ATP gekoppelt (Boll 2005a). Die initiale Thioester-Bildung ist hierbei notwendig, da sie den ersten Elektronentransfer durch die intermediäre Bildung eines relativ stabilen Ketylradikal-Anions ermöglicht (Boll 2005a). Alle bekannten Benzoyl-CoA Reduktasen sind Heterotetramere ($\alpha\beta\gamma\delta$) mit einer modularen Zusammensetzung: die $\alpha\delta$-Untereinheiten bilden das Modul in dem die Elektronen ATP-abhängig aktiviert werden und die $\beta\gamma$-Untereinheiten enthalten das aktive Zentrum, in dem das aromatische Substrat reduktiv dearomatisiert wird (Boll 2005b). Anschließende Oxidationsreaktionen und die Ring-Öffnung bilden 3-Hydroxypimelyl-CoA, welches mittels β-Oxidation zu 3 Acetyl-CoA und CO_2 abgebaut wird (Boll 2005b). Aufgrund der limitierten Energie-Ausbeute Sulfat- oder Eisen-reduzierender aber auch synthropher Bakterien wird für diese Gruppe anaerober Abbauer ein anderer Mechanismus der Dearomatisierung angenommen (Gibson und Harwood 2002). In der Tat haben erste

Untersuchungen gezeigt, dass das Wachstum von *Desulfococcus multivorans* und *G. metallireducens* mit Benzoat ein bislang unbekanntes Molybdän- und Selenocystein enthaltendes Protein involviert (Peters et al. 2004; Wischgoll et al. 2005). Entsprechend weist die Genomsequenz von *G. metallireducens* keine homologen Gene zur Benzoyl-CoA Reduktase auf (Wischgoll et al. 2005).

Gene, die die strukturellen Untereinheiten der Benzoyl-CoA Reduktase kodieren, wurden in *T. aromatica*, *R. palustris* und *A. evansii* identifiziert und sind in den sequenzierten Genomen weiterer Proteobacteria (hauptsächlich *Thauera* spp. und "*Aromatoleum*" spp. (*Azoarcus*)) nachgewiesen worden (Song und Ward 2005). Die Gene der "*Aromatoleum*-" (*Azoarcus*-) bzw. *Thauera* Gruppe weisen dabei eine hohe Ähnlichkeit untereinander auf, unterscheiden sich aber von den Enzymen der anderen Gruppe. Interessanter Weise enthält das Chromosom von Stamm EbN1 zwei verschiedene Gen-Cluster, die Benzoyl-CoA Reduktasen kodieren (Rabus et al. 2005). Es wird angenommen, dass eines (*bcrCABD*) für die reduktive Dearomatisierung von Benzoyl-CoA verantwortlich ist, und das Zweite (*ebA742-748*) für *m*-Hydroxybenzoyl-CoA, wie es für den *m*-Hydroxybenzoat Abbau in *T. aromatica* beschrieben wurde (Laempe et al. 2001; Rabus et al. 2005).

3.3 Ökophysiologie des anaeroben Abbaus

Bei einem Überangebot organischen Materials in Sedimenten und Böden kommt es durch aerobe Atmungsprozesse zu einer Sauerstoffzehrung. Letztere führt zu einer raschen Einstellung anoxischer Bedingungen und Verwendung alternativer Elektronenakzeptoren (z.B. Nitrat oder Sulfat; siehe Abb. 3; Spormann und Widdel 2000). Aufgrund des schnellen Sauerstoff-Verbrauchs in vielen Erdöl-kontaminierten, Grundwasser-leitenden Gesteinsschichten (Aquifer) herrschen auch dort zumeist anoxische Bedingungen vor (Lovley 1997). Der Abbau der Kohlenwasserstoffe in solchen Habitaten konnte sowohl in Labor-Mikrokosmen als auch *in situ* durch das Einbringen von Nitrat oder Sulfat stimuliert werden (Cunningham et al. 2000; Pelz et al. 2001). Entsprechend wurde die Expression von *bssA* Genen, welche die katalytische Untereinheit der Benzylsuccinat Synthase kodieren, in kontaminierten Aquifers nachgewiesen wie auch Arylsuccinate (z.B. Benzylsuccinat) als korrespondierende Abbau-Intermediate (Beller 2002; McKelvie et al. 2005; Gödeke et al. 2006; Winderl et al. 2007). Untersuchungen über das Vorkommen Kohlenwasserstoff-abbauender Bakterien im natürlichen Habitat wurden mittels Hybridisierung der 16S rRNA mit Oligonukleotid-Sonden durchgeführt. Diese haben zeigen können, dass Proteobacteria (hauptsächlich Angehörige der Beta- und Gamma-Untergruppe) einen signifikanten Anteil

der Gesamt-Bakterienpopulation in kontaminierten Aquifer-Mikrokosmen ausmachen (Hess et al. 1997; Winderl et al. 2007). Die Abundanz von Aromaten-abbauenden "*Aromatoleum*" (*Azoarcus*) ähnlichen spp. (1-6 % der Gesamtpopulation) deutet auf eine signifikante Rolle dieser Bakteriengruppe im anaeroben Aromaten bzw. Kohlenwasserstoff-Abbau hin (Hess et al. 1997; Pelz et al. 2001).

3.4 Stamm EbN1 als Modellorganismus

Der Modellorganismus der vorliegenden Arbeit, das denitrifizierende Bakterium Stamm EbN1, wurde unter Nitrat-reduzierenden Bedingungen mit Ethylbenzol als einziger Kohlenstoff- und Energiequelle aus Schlämmen verschiedener Gräben und der Weser in Bremen isoliert (Rabus und Widdel 1995). Phylogenetisch gehört Stamm EbN1 zum "*Aromatoleum*"/*Azoarcus*/*Thauera* Cluster der Betaproteobacteria. Aufgrund ihrer anaeroben Abbau-Fähigkeiten und ihrer geographischen Verteilung, gewinnen die Vertreter dieses Clusters zunehmend an Interesse. Als bisher einziger bekannter Organismus ist Stamm EbN1 in der Lage neben Ethylbenzol ebenfalls Toluol als Substrat zu verwerten. Beide Verbindungen können gleichzeitig verwertet werden, auch direkt aus Rohöl (Rabus und Widdel 1995; Rabus und Widdel 1996; Kühner et al. 2005). Darüber hinaus ist Stamm EbN1 in der Lage eine Vielzahl weiterer aromatischer und aliphatischer Säuren, Ketone und Alkohole (z.B. Phenylacetat, Benzaldehyd oder Propanol) unter anaeroben Bedingungen als Substrat zu nutzen. Auch das potentiell toxische Aceton kann anaerob von Stamm EbN1 abgebaut werden (Rabus und Widdel 1995). Einige aromatische und aliphatische Verbindungen können ebenfalls aerob komplett oxidiert werden (z.B. Benzoat oder Ethanol).

Aufgrund seiner hohen metabolischen Vielfalt und der interessanten biochemischen Reaktionen der Abbauwege (siehe 3.2) wurde die vollständige Genom Sequenz von Stamm EbN1 als erstem Vertreter der "*Aromatoleum*"/*Azoarcus*/*Thauera*-Gruppe bzw. eines anaeroben Kohlenwasserstoff-Abbauers bestimmt (Rabus et al. 2005). Die Genom-basierte metabolische Rekonstruktion bestätigte allerdings nicht nur bekannte Abbauleistungen. Genetische Hinweise auf weitere, bislang nicht für Stamm EbN1 bekannte aromatische Substrate für anaerobes Wachstum, konnten experimentell bestätigt werden (z.B. anaerober Abbau von Phenol oder *p*-Cresol). Neben einem klassischen Denitrifikationssystem (wie von *Pseudomonas* bekannt) verfügt Stamm EbN1 auch über respiratorische Komplexe unterschiedlicher Sauerstoff-Affinitäten. Das Vorkommen von Genen für eine Vielzahl regulatorischer Proteine weist auf ein komplexes regulatorisches Netzwerk hin, welches

Stamm EbN1 erlauben sollte sich optimal auf die fluktuierende Verfügbarkeit von Kohlenstoffquellen und Elektronenakzeptoren einzustellen (Rabus et al. 2005).

4. Regulation des Aromaten-Abbaus

Eine große Vielfalt regulatorischer Systeme ist bekannt, die die Expression von Genen kataboler Enzyme kontrolliert. Spezifische Proteine, die den Abbau aromatischer Verbindungen regulieren, gehören zu allen bedeutenden Klassen prokaryotischer Regulatoren und folgen den allgemeinen Mechanismen der jeweiligen Regulator-Familie (Shingler 2003; Tropel und van der Meer 2004). Die Regulation des aeroben Aromaten-Abbaus ist in *Pseudomonas* spp. sowie ihrer assoziierten Plasmide am besten verstanden. Meist sind Zwei-Komponenten Sensor-Regulator-Systeme bei Bakterien verantwortlich für die Erkennung von Umweltsignalen und ihrer Übersetzung in differentielle Genexpression (Tropel und van der Meer 2004). Dabei wird das Signal von einer Senor-Histidin-Kinase wahrgenommen und es kommt zu deren Autophosphorylierung. Mittels eines Phosphoryltransfers wird das Signal auf die Regulator-Komponente übertragen, wodurch diese aktiviert wird (Tropel und van der Meer 2004). In *P. putida* F1 und DOT-T1 wird auf diese Weise die Expression des *tod*-Operons, welches die Proteine des aeroben Toluol-Abbaus kodiert, durch das TodST-System Toluol-spezifisch aktiviert (Lau et al. 1997; Mosquéda et al. 1999). Neben den Zwei-Komponenten-Systemen sind aber auch viele Regulations-Systeme bekannt, die aus nur einer Komponente bestehen. So vereinigen z.B. Sigma54-abhängige Regulatoren die Signalerkennung und die spezifische Bindung an regulatorische Elemente der DNA in einem Molekül. Der aktivierte Regulator bindet an "upstream activating sequences" der DNA, die normalerweise mehr als 100 bp upstream der RNA-Polymerase Bindestelle liegen. Durch Krümmung der DNA kommt es zur Interaktion zwischen DNA-gebundenem Regulator und dem Sigma54-enthaltenden Holokomplex der RNA-Polymerase. Diese Interaktion führt letztlich zur Initiation der Transkription (Morett und Segovia 1993). Interessanter Weise wird in *P. putida* mt-2 die Transkription der auf dem TOL Plasmid kodierten Gene des "oberen" Abbauwegs von Toluol (sowie *m*- und *p*-Xylol) durch den Sigma54-abhängigen Regulator XylR aktiviert (Ramos et al. 1997). Das Beispiel des aeroben Toluol-Abbaus in *Pseudomonas* spp. verdeutlicht, dass gleiche (oder ähnliche) Abbauwege keineswegs durch gleichartige Regulations-Systeme kontrolliert werden müssen (Shingler 2003).

Auch in anaerob Aromaten-abbauenden Bakterien, konnte die Substrat-spezifische Bildung von Proteinen der jeweiligen Abbauwege gezeigt werden; z.B. in *T. aromatica* (für Toluol, Phenol und andere Aromaten; Heider et al. 1998) oder Stamm EbN1 (für Toluol,

Ethylbenzol und Acetophenon; Champion et al. 1999). Die *bss*-Gene des anaeroben Toluol Abbaus in *T. aromatica* T1 und K172 sowie "*Aromatoleum*" (*Azoarcus*) sp. Stamm T und EbN1 befinden sich in unmittelbarer chromosomaler Nachbarschaft zu Genen (*tdiSR*) für ein Zwei-Komponenten-System (Coschigano und Young 1997; Leuthner und Heider 1998; Achong et al. 2001; Kube et al. 2004). Das TdiSR-System wird für die Kontrolle der Transkription des *bss*-Operons verantwortlich gemacht (Coschigano et al. 1998; Hermuth et al. 2002; Kube et al. 2004). Erst kürzlich konnte mittels physiologischer, proteomischer und transkriptomischer Experimente gezeigt werden, dass die Gene des anaeroben Toluol-Abbaus (*bss* und *bbs*) in Stamm EbN1 koordiniert und Substrat-spezifisch reguliert werden (Kühner et al. 2005). Im Gegensatz dazu werden die Gene des anaeroben Ethylbenzol-Abbaus sequenziell reguliert: der "obere" Teil des Abbauwegs (von Ethylbenzol zu Acetophenon) wird durch das Substrat Ethylbenzol, nicht aber durch das Intermediat Acetophenon induziert. Der "untere" Teil des Abbauwegs (von Acetophenon zu Benzoyl-CoA) wird jedoch, aufgrund der intermediären Bildung von Acetophenon, auch während des Wachstums mit Ethylbenzol exprimiert (Kühner et al. 2005). Diese Substrat-spezifischen Regulationsprozesse werden vermutlich von zwei Zwei-Komponenten-Systemen reguliert, die zwischen den beiden katabolen Gen-Clustern kodiert sind (Rabus et al. 2002).

Im natürlichen Habitat ist es für Bakterien nicht nur erforderlich einzelne, katabole Wege in Abhängigkeit von der Verfügbarkeit des jeweiligen Substrates zu kontrollieren. Vielmehr muss die Regulation einzelner Stoffwechselwege unter Berücksichtigung anderer relevanter *in situ* Bedingungen erfolgen. Eine sich dynamisch verändernde Vielfalt von Umweltreizen muss integriert und Reizantworten auf hierarchische, den jeweiligen ökophysiologischen Erfordernissen angepasste Weise erfolgen (Cases und de Lorenzo 2005). So müssen unter oxischen Bedingungen nicht nur die zur Substrat-Verwertung benötigten katabolen Enzyme gebildet werden, sondern auch Proteine zum Schutz vor reaktiven Sauerstoffspezies (z.B. Katalase oder Superoxid-Dismutase; Bauer et al. 1999). Andererseits ist die Bildung von Enzymen der entsprechenden anaeroben Abbauwege unökonomisch, insbesondere wenn diese Sauerstoff empfindlich sind (z.B. die Benzylsuccinat Synthase des anaeroben Toluol-Abbaus). Entsprechend wurden diverse übergeordnete Regulationssysteme identifiziert (z.B. SoxR oder Fnr), die unterschiedliche Sensitivität gegenüber Sauerstoff besitzen und die differentielle Kontrolle der Zielgene in Bezug auf veränderte Sauerstoff Partialdrücke ermöglichen (Bauer et al. 1999). So ist der globale Regulator Fnr in *E. coli* für die Kontrolle von mehr als 120 Genen unter aeroben bzw. anaeroben Bedingungen

verantwortlich (Sawers et al. 1988). Unter anoxischen Bedingungen unterdrückt Fnr die Expression aerober Respirationsenzyme (z.B. Cytochrom Oxidase oder NADH Dehydrogenase) und aktiviert die Expression von Genen deren Produkte die Reduktion alternativer Elektronenakzeptoren erlaubt (Bauer et al. 1999).

5. Umweltmikrobiologie im Zeitalter von Genomik und Proteomik

Seit dem Beginn der Genom-Ära mit der Sequenzierung des ersten bakteriellen Genoms von *Haemophilus influenzae* Rd (Fleischmann et al. 1995) steigt die Zahl mikrobieller Genomprojekte exponentiell an. Aktuell sind 866 bakterielle Genome vollständig sequenziert, während 2654 weitere prokaryotische Genome sich bereits im Sequenzierungsprozess befinden (Stand Januar 2008; www.genomesonline.org). Erst kürzlich gelang es in einer metagenomischen Studie mittels Shotgun-Sequenzierung von Wasserproben aus der Sargassosee mehr als 1 Milliarde Basenpaare nicht-redundanter Sequenzinformation zu produzieren (Venter et al. 2004). Ermöglicht wurde dieser dramatische Anstieg an DNA Sequenzierleistung durch die Entwicklungen der Labor-Automatisierung und der Computer-basierten Prozessierung der generierten Daten. Die neueste Entwicklung der so genannten Pyrosequenzierung mit hohem Durchsatz, 454 Sequenzierung, erlaubt die Produktion von mehr als 100 Millionen Basenpaaren innerhalb von 7,5 h (Margulies et al. 2005). Diese technische Innovation wird Sequenzierungsprojekte, die sowohl einzelne Stämme als auch Konsortien und komplexe Umweltproben umfassen, weiter vorantreiben. Wurden zunächst hauptsächlich pathogene Mikroorganismen sequenziert, stellen Umweltmikroorganismen heute mehr als die Hälfte aller Sequenzierungsprojekte (www.genomesonline.org). In der Tat plant die Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) innerhalb der nächsten Dekade alle Genome von Typstämmen in der Kultursammlung zu bestimmen (www.dsmz.de).

Die Genomsequenz ermöglicht einen Einblick in das genetische Potential eines Organismus. Der tatsächliche Funktionsnachweis erfordert jedoch die Analyse der Genexpression bzw. Proteinbildung, die die Dynamik physiologischer und zellulärer Abläufe widerspiegeln. Besonders im Fall hypothetischer Gene/Proteine stellt die so genannte funktionelle Genomanalyse die einzige Möglichkeit dar eine erste Vorstellung von ihrer Funktion zu erhalten, basierend auf dem physiologischen Zusammenhang ihrer Bildung.

Die Transkriptomanalyse zielt ab auf die quantitative Erfassung aller Transkripte (mRNAs), die eine Zelle unter definierten experimentellen Bedingungen bildet (Velculescu et al. 1997). Dies erlaubt eine Unterscheidung zwischen konstitutiv exprimierten, Kontext-abhängig induzierten und stillen Genen. Eine vielfach angewandte Technik stellen DNA-Microarrays dar. Sie erlauben die globale Analyse der Genexpression mit Hilfe immobilisierter Gen-Sensoren (Oligonukleotide oder PCR-Produkte). Allerdings finden auch während des Informationsflusses von der mRNA zum Protein noch eine Vielzahl regulatorischer Prozesse statt. Die verschiedenen mRNA-Spezies werden unterschiedlich schnell abgebaut und mit unterschiedlicher Effizienz translatiert. Daher ist eine direkte Korrelation zwischen mRNA und Proteinmenge kaum möglich (Gygi et al. 1999; Greenbaum et al. 2003). Ebenso vermag die RNA-Analyse keine Aussage über posttranslationale Modifikationen von Proteinen zu treffen (z.B. Glykosilierung, Methylierung, Phosphorylierung oder proteolytische Prozessierung), welche jedoch für die biologische Funktion der Proteine essentiell sein können.

Proteine sind die katalytisch aktiven Moleküle, die eine Zelle zum Leben erwecken. Das Proteom umfasst "die Gesamtheit aller von der Zelle zu einem bestimmten Zeitpunkt und unter definierten Bedingungen synthetisierten Proteine" (Wasinger et al. 1995). Im Gegensatz zum Genom, ist das Proteom hoch dynamisch, da nur solche Proteine gebildet werden, deren Funktion für das Überleben bzw. Wachstum unter den aktuellen Bedingungen notwendig ist. Umweltbedingungen können darüber hinaus auch die Lokalisation, Stabilität und Quantität von Proteinen sowie das Vorkommen posttranslationaler Modifikationen bestimmen. Somit korrespondiert eine Vielzahl von Proteomen zu nur einem Genom. Die Proteomik untersucht daher die Dynamik der Proteinzusammensetzung eines Organismus und erfasst dabei die Abundanz, postranslationale Modifikation und sub-zelluläre Lokalisation der Proteine. Dies ermöglicht einen Einblick in physiologische und zelluläre Prozesse sowie regulatorische Netzwerke auf Proteinebene (Görg et al. 2004).

Mit Hilfe differentieller Proteomik kann die relative Häufigkeit einzelner Proteine (oder ihrer Untereinheiten) quantitativ erfasst werden. Dieser Ansatz kann einen innovativen Beitrag zur Aufklärung von u.a. Stoffwechselwegen und regulatorischen Prozessen in Abhängigkeit von veränderten Umweltbedingungen leisten. Proteomische Methoden befassen sich mit der Separierung komplexer Proteingemische sowie der nachfolgenden Proteinidentifizierung. Die am weitesten verbreitete Technik zur Proteinerfassung stellt die zweidimensionale Gelelektrophorese (2DE) dar. Komplementär wird zunehmend

Flüssigchromatographie eingesetzt (Zieske 2006). Unabhängig von der verwendeten Methode zur Proteinseparierung werden vor allem massenspektrometrische Verfahren zur Proteinidentifikation eingesetzt (de Hoog und Mann 2004).

Die Separierung komplexer Proteingemische mittels der 2DE beruht auf zwei unterschiedlichen physikochemischen Eigenschaften von Proteinen: (i) dem isoelektrischen Punkt (IEP) und (ii) der molekularen Masse. Der IEP eines Proteins entspricht dem pH-Wert, an dem seine Nettoladung null ist und es nicht mehr im elektrischen Feld wandert. In der ersten Dimension der 2DE werden Proteine in einem immobilisierten pH-Gradienten gemäß ihres IEPs aufgetrennt. Da Proteine unterschiedlicher Masse denselben IEP besitzen können, erfolgt in der zweiten Dimension, einer Natriiumdodecylsulfat-Polyacryalmid-Gelelektrophorese (SDS-PAGE), eine Auftrennung gemäß der Proteinmasse. Die Kombination dieser zwei Trennprinzipien ermöglicht es, komplexe Proteingemische schnell und hochauflösend zu trennen und gleichzeitig auf einem Gel darzustellen. Heute ist es mit dieser Technik möglich Proteine mit IEPs von pH 2,5 – 12 und einer molekularen Masse von 10 – 200 kDa aufzulösen (Görg et al. 2004). Allerdings lassen sich hydrophobe Proteine (z.B. Proteine mit mehreren transmembran Domänen) meist nicht oder nur eingeschränkt in immobilisierten pH-Gradienten auftrennen. Es wird angenommen, dass bis zu 25 % aller Proteine eines Bakteriums integrale Membranproteine sind (Wallin und von Heijne 1998), während die verbleibenden 75 % einen IEP zwischen pH 3 – 13 besitzen (Himmelreich et al. 1996).

Im Gegensatz zu klassischen, postelektrophoretischen Färbemethoden (z.B. Silbernitrat oder Coomassie Brilliant Blau) werden bei der DIGE-Technologie (difference gel electrophoresis) Fluoreszenzfarbstoffe (CyDyes™) bereits vor der Separierung kovalent an die ε-Aminogruppe von Lysin-Resten der Proteine gebunden (Ünlü et al. 1997). Wichtige Eigenschaften der drei verfügbaren Farbstoffe (Cy2, Cy3 und Cy5) sind die niedrige Detektionsgrenze, ein breiter dynamischer Bereich (4 Größenordnungen), gleichartiger Einfluss auf die Migrationseigenschaften der zu separierenden Proteine (so genannte "Ladungs- und Massen-Abstimmung") sowie distinkte, nicht überlappende Absorptions- und Emissionsmaxima. Zusammengenommen erlauben diese Eigenschaften bis zu drei verschiedene Proteinproben gleichzeitig in einem 2DE-Gel aufzutrennen (Multiplexing). Durch anschließendes, wiederholtes Scannen mit Cy-Farbstoff-spezifischen Anregungs-wellenlängen, werden digitale Gelbilder für die einzelnen Proben erzeugt (Gade et al. 2003; Görg et al. 2004). Die Software-basierte (DeCyder™) Analyse der Proteinmuster (~1000-

2000 Proteinspots pro Gel) erlaubt die Erkennung der Proteinspots sowie die Erfassung quantitativer Unterschiede durch Zuordnung gleicher Spots in parallelen Gelen.

Die Massenspektrometrie (MS) stellt heute eine der bedeutendsten analytischen Methoden der Biowissenschaften dar. Die effizienten und zugleich schonenden Ionisierungstechniken Matrix-Assisted-Laser-Desorption/Ionisation (MALDI) und Electrospray-Ionisation (ESI) ermöglichen die Bestimmung molekularer Massen von großen Biomolekülen. Sie zählen damit zu den wichtigsten Techniken der routinemäßigen Peptid- und Proteinanalytik (de Hoog und Mann 2004) und ersetzen die langsamere und arbeitsaufwändigere N-terminale Sequenzierung (Edman-Abbau). Zur Identifizierung werden ausgewählte Proteinspots zunächst aus dem 2DE Gel ausgestochen und anschließend mittels sequenzspezifischer Proteasen (z.B. Trypsin) fragmentiert. Dadurch entsteht ein Protein-spezifisches Gemisch aus Peptiden definierter Längen und Aminosäuresequenzen. Während der MALDI-TOF-MS Analyse werden die Peptide (eines Proteins) ionisiert und in die Gasphase überführt. Im elektrischen Feld werden die generierten Peptidionen dann mit gleicher kinetischer Energie beschleunigt und im Hochvakuum innerhalb einer feldfreien Driftstrecke aufgrund ihrer Flugzeit voneinander getrennt. Schwere Peptidionen fliegen dabei langsamer als leichte und erreichen daher erst später den Detektor. Auf diese Weise wird für jedes Protein ein charakteristischer Satz von Peptidmassen bestimmt; die so genannten Peptidmassen Fingerabdrücke (PMF). Zur Identifizierung werden diese experimentell generierten Massenleitern mit *in silico* erzeugten PMFs von in Datenbanken hinterlegten Proteinsequenzen verglichen. Dies erlaubt in den meisten Fällen die Zuordnung eines kodierenden oder homologen Gens zu einem Protein und somit eine eindeutige Identifizierung bzw. funktionelle Zuweisung. Je höher die Übereinstimmung (Score oder Coverage) zwischen experimentell bestimmtem und *in silico* berechnetem PMF ist, desto verlässlicher ist die Identifizierung. Allerdings ist dieses Verfahren nur möglich, wenn eine entsprechende DNA- oder Proteinsequenz vorliegt. Mittlerweile erlaubt eine nochmalige Fragmentierung von Peptidionen mittels Tandem-MS (MS-MS) aber auch eine *de novo* Sequenzierung.

Während die 2DE als Standardtechnik in viele Bereiche biologischer Forschung Einzug gehalten hat (meist mit Silber-Färbung), ist der Einsatz der modernen DIGE-Technologie noch nicht so weit verbreitet. Bei proteomischen Untersuchungen zur Physiologie des marinen Planktomyceten *Rhodopirellula baltica* (Gade et al. 2005a; Gade et al. 2005b) sowie

zur Regulation des anaeroben Alkylbenzol-Abbaus in "*A. aromaticum*" EbN1 (Kühner et al. 2005) kam die DIGE Technologie erstmals bei Umweltmikroorganismen zur Anwendung. In jüngster Zeit wurden DIGE-basierte proteomische Studien auch mit Cyanobakterien durchgeführt (Slabas et al. 2006; Hongsthong et al. 2007). Andere quantitative proteomische Untersuchungen von Umweltmikroorganismen nutzen meist LC-MS basierte Methoden oder die Quantifizierung Silber-gefärbter Gele (z.B. Kim et al. 2006).

6. Zielsetzung der Arbeit

Zu Beginn der vorliegenden Arbeit waren der denitrifizierende Stamm EbN1 sowie weitere Denitrifizierer mit der Fähigkeit zum anaeroben Aroamten-Abbau der Gattung *Azoarcus* zugeordnet. *Azoarcus senso strictu* umfasst N_2-fixierende Endophyten oder Wurzel-Oberflächen kolonisierende Bakterien. Diese Eigenschaft fehlt allerdings den mit Stamm EbN1 verwandten Aromaten-Abbauern. Umgekehrt sind die Pflanzen-assoziierten *Azoarcus* spp. sind nicht in der Lage Aromaten anaerob abzubauen. Daher sollten Wachstumsversuche und molekulare Untersuchungen durchgeführt werden, um eine physiologische Basis für die taxonomische Abgrenzung beider Bakteriengruppen zu ermöglichen.

Hauptziel dieser Arbeit war die proteomische Rekonstruktion der genomisch vorhergesagten anaeroben und aeroben Abbauwege aromatischer Wachstumssubstrate von Stamm EbN1. Durch quantitative, DIGE-basierte Erfassung Substrat-spezifischer Subproteome sollte darüber hinaus erstmals ein Einblick in die regulatorischen Hierarchien eines denitrifizierenden Aromaten-Abbauers erzielt werden. Schließlich sollten Substrat-spezifische Proteinmuster auch als Inspiration für die Suche nach bislang unbekannten Abbauleistungen dienen: Beispielhaft für die erfolgreiche Umsetzung dieser Strategie sei die Entdeckung des anaeroben *p*-Ethylphenol-Abbaus genannt.

Das Wachstum von Stamm EbN1 mit toxischen Substanzen (z.B. Alkylbenzole) legt die Fähigkeit zur Anpassung an Lösungsmittel-Stress nahe. In aktuellen Untersuchungen wurde bereits die Stressantwort von Stamm EbN1 auf semi-inhibitorische (d.h. ~50 % Wachstumshemmung) Konzentrationen von Toluol und Ethylbenzol untersucht. Dabei wurden zwei Kultivierungsstrategien verfolgt: in einem Fall waren die entsprechenden Abbauwege operativ, im anderen Fall nicht. In der vorliegenden Arbeit sollte die Stressantwort von Stamm EbN1 auf plötzlichen Schock mit Phenol bzw. *p*-Cresol untersucht werden.

Die Genomsequenz von Stamm EbN1 enthält zahlreiche Gene, deren Funktion bislang durch rein bioinformatische Analyse nicht geklärt werden konnte. Proteomische

Untersuchungen dieser und vorheriger Arbeiten konnten erstmals die Substrat-spezifische und abundante Bildung einiger dieser hypothetischen Proteine belegen. Als Grundlage für eine weiterführende Funktionsaufklärung sollte im Rahmen dieser Arbeit ein genetisches System für Stamm EbN1 etabliert werden. Hierzu sollten zunächst Methoden zur effizienten Kultivierung auf festem Medium sowie zum Transfer von mobilen DNA-Elementen (Plasmide) entwickelt werden. Schließlich sollte ein Gen des anaeroben Aromaten-Abbaus deletiert und die generierte Mutante *in trans* komplementiert werden.

B Ergebnisse und Diskussion

Im Folgenden werden die Ergebnisse der Publikationen/Manuskripte übergreifend im Zusammenhang dargestellt. An dieser Stelle werden allerdings nur besondere Aspekte der Veröffentlichungen hervorgehoben. Für eine detaillierte Betrachtung aller Einzelergebnisse dieser Arbeit sei auf die angefügten Veröffentlichungen verwiesen (Teil II).

1. Taxonomische Beschreibung der Gattung "*Aromatoleum*" gen. nov.

Die Mehrzahl der bisher isolierten denitrifizierenden Bakterien mit der Fähigkeit zum anaeroben Aromaten-Abbau lässt sich zwei phylogenetischen Clustern innerhalb der Betaproteobacteria zuordnen. Das eine Cluster entspricht der Gattung *Thauera* (Macy et al. 1993) mit dem gut untersuchten *T. aromatica* K172 (Anders et al. 1995) sowie weiteren anaeroben Aromaten- (z.B. *T. aminoaromatica* S2^T und *T. chlorobenzoica* 3CB 1^T; Song et al. 2001; Mechichi et al. 2002) und Monoterpen-abbauenden Vertretern (*T. linaloolentis* 47Lol^T und *T. terpenica* 58Eu^T; Foss und Harder 1998). Das andere Cluster wurde bislang der Gattung *Azoarcus* zugeordnet. Mitglieder von *Azoarcus senso strictu* sind jedoch stickstofffixierende Endophyten oder Wurzeloberflächen von Kallar Gras kolonisierende Bakterien, wie z.B. *A. indigens* VB32^T und *A. communis* SWub3^T (Reinhold-Hurek et al. 1993b). Der erste taxonomisch beschriebene anaerobe Aromaten-Abbauer des *Azoarcus* Clusters war *A. evansii* KB740^T (Anders et al. 1995). Spätere Studien beschrieben weitere anaerobe Aromaten-Abbauer, die taxonomisch dem Genus *Azoarcus* zugeteilt wurden: *A. buckelii* U120^T (Mechichi et al. 2002), *A. anaerobius* LuFRes1^T (Springer et al. 1998), *A. tolulyticus* Tol-4^T (Zhou et al. 1995), *A. toluvorans* Td-21^T und *A. toluclasticus* MF63^T (Song et al. 1999). Weitere sieben Isolate, die Stämme EbN1^T, ToN1^T, PbN1^T, pCyN1, EB1, T^T und 22Lin^T gehören zur *A. evansii* Verwandtschaftsgruppe und wurden bislang noch nicht taxonomisch beschrieben. Eine frühere vergleichende 16S rRNA Gensequenz-Analyse wies bereits darauf hin, dass die Gruppe der "Abbauer" ebenso nah verwandt mit den Pflanzen-assoziierten *Azoarcus* spp. wie mit den Spezies der Gattung *Thauera* ist (Reinhold-Hurek und Hurek 2000), so dass eine Neuordnung des *Azoarcus*/*Thauera* Clusters geboten scheint.

Zur taxonomischen Einordnung der sieben unbeschriebenen Isolate und der Differenzierung der anaerob Aromaten-abbauenden von den Pflanzen-assoziierten Stämmen, mussten auch die bereits beschriebenen sechs Stämme mit einbezogen werden. Der

16S rRNA-basierte phylogenetische Baum der *Thauera* und *Azoarcus* zugeordneten Stämme weist drei distinkte Cluster innerhalb der *Rhodocyclus*-Gruppe der Betaproteobacteria auf (Abb. 5). Daher wurde die neue Gattung "*Aromatoleum*" vorgeschlagen, um der phylogenetischen Aufspaltung dieser drei Gruppen Rechnung zu tragen. Der Gattungsname nimmt dabei Bezug auf die Vielzahl aromatischer Verbindungen und Rohöl-Komponenten die von dieser Gruppe anaerob abgebaut werden kann.

Zur Unterstützung der phylogenetischen Aufspaltung wurden Unterschiede hinsichtlich allgemeiner Physiologie, Substratverwertung und Zusammensetzung der Membranfettsäuren herausgearbeitet. Im Rahmen dieser Arbeit wurden klassische Wachstumsexperimente durchgeführt, in denen alle 13 "*Aromatoleum*" spp., sowie die Pflanzen-assoziierten *Azoarcus* spp. *A. indigens* VB32^T, *A. communis* SWub3^T und *Azoarcus* sp. BH72 auf Verwertung diverser aromatischer, aliphatischer und anderer Verbindungen (z.B. Kohlenhydrate) getestet wurden (siehe Tabelle 2 in Publikation 5). Diese Ergebnisse zeigten, dass die Eigenschaft des anaeroben Abbaus aromatischer und anderer chemisch reaktionsträger Verbindungen allein den Vertretern der "*Aromatoleum*"-Gruppe zu eigen ist. Darüber hinaus können innerhalb der "*Aromatoleum*"-Gruppe einzelne Isolate anhand der jeweiligen Substratspektren differenziert werden. So sind die Stämme EbN1^T, ToN1^T, pCyN1^T, und T^T alle in der Lage anaerob Toluol abzubauen, aber lediglich Stamm EbN1^T verwertet anaerob auch Ethylbenzol. Andererseits kann nur Stamm pCyN1^T *p*-Ethyltoluol und *p*-Cymol anaerob verwerten. Interessanter Weise kann kein "*Aromatoleum*"-Vertreter unter oxischen Bedingungen Kohlenwasserstoffe abbauen.

Die Fähigkeit der Stickstofffixierung scheint in der "*Aromatoleum*"-Gruppe selten vorzukommen, da lediglich bei wenigen Vertretern (Stamm 22Lin^T, Tol-4^T und Td-21^T) *nifH* Gene identifiziert werden konnten, welche die Eisen-enthaltende Untereinheit der Nitrogenase kodieren. Entsprechend bilden nur diese Stämme Ethylen in Weichagarkulturen, während diese Fähigkeit ein charakteristisches Merkmal aller Pflanzen-assoziierten *Azoarcus* spp. darstellt (Reinhold-Hurek und Hurek 2000).

Der Vergleich der bisher sequenzierten Genome von "*A. aromaticum*" EbN1^T (Rabus et al. 2005) und *Azoarcus* sp. BH72 (Krause et al. 2006) unterstreicht die taxonomische Unterscheidung beider Gruppen auf genomischer Ebene. Beide Genome weisen nur einen geringen Grad von Syntenie auf. Das Chromosom von *Azoarcus* sp. BH72 kodiert mehrere Proteinkomponenten für Oberflächenassoziation, die wahrscheinlich an der Pflanzen-assoziierten Lebensweise beteiligt sind (Krause et al. 2006). Das Genom von Stamm EbN1^T weist diese Gene entweder gar nicht auf, oder die Gene von *Azoarcus* sp. BH72 zeigen höhere

Sequenzähnlichkeit zu Genen Pflanzen-assoziierter oder pathogener Bakterien. Umgekehrt sind im Genom von Stamm BH72 keine Gene vorhanden, die der Denitrifikation oder dem anaeroben Aromaten-Abbau zugeordnet werden können. Darüber hinaus lässt die Vielzahl mobiler Elemente im Genom von Stamm EbN1 (z.B. mehr als 200 Transposasen) auf eine hohe Genomplastizität schließen, welche vorteilhaft für den horizontalen Transfer von Abbauleistungen wäre (Rabus et al. 2005). Das Genom von Stamm BH72 weist hingegen nur wenige mobile Elemente auf, welches als Ausdruck der relativ stabilen Lebensumgebung in Pflanzen angesehen wird (Krause et al. 2006).

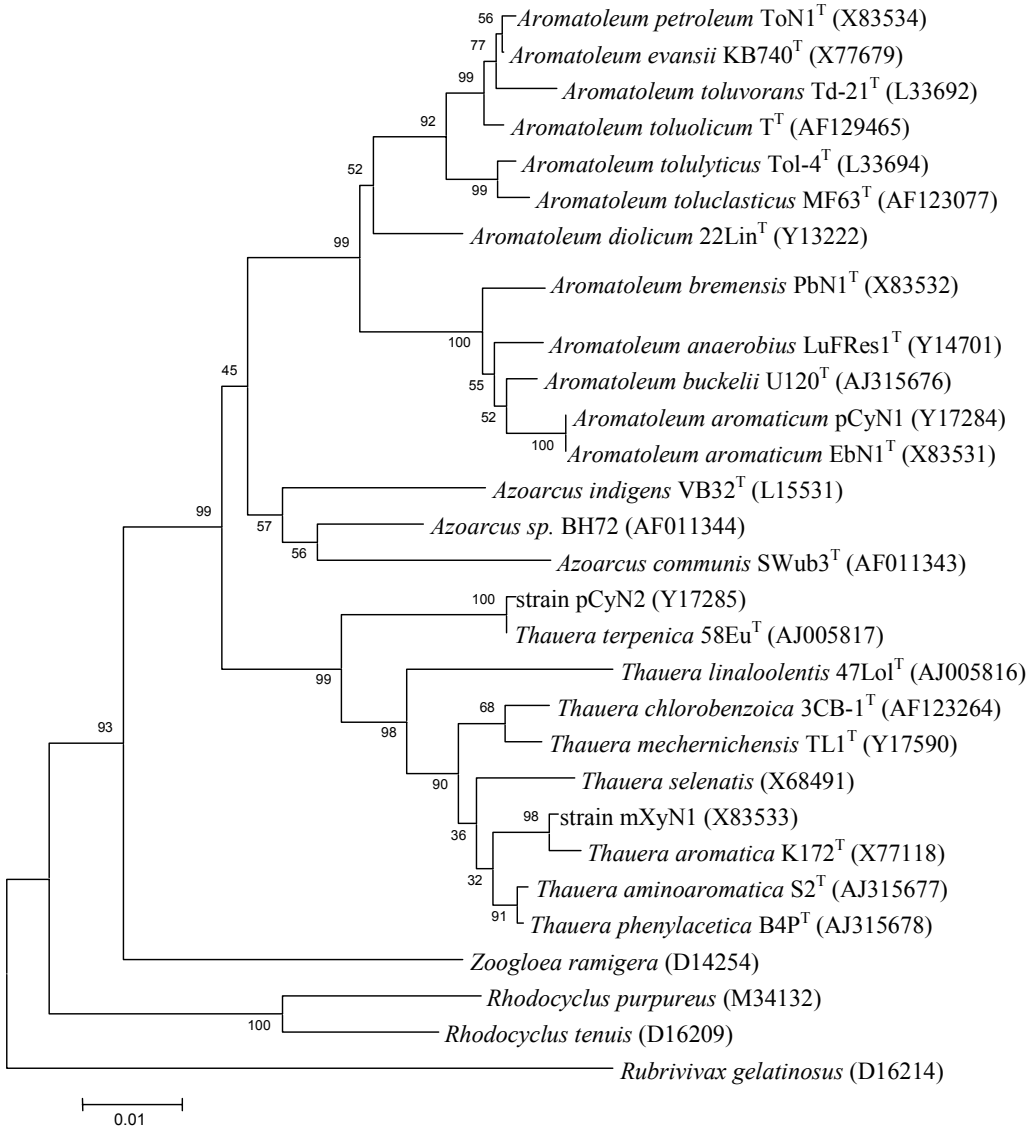


Abb. 5: Der phylogenetische Baum zeigt deutlich die Cluster der Gattungen "*Aromatoleum*", *Azoarcus* und *Thauera* innerhalb der *Rhodozyclus* Gruppe der Betaproteobacteria. Der Baum wurde mit der Neighbor-Joining-Methode errechnet.

2. Untersuchung Substrat-abhängiger Regulation aerober und anaerober Abbauwege mittels differentieller Proteomik

Die komplette Genomsequenz von "*A. aromaticum*" EbN1 bildete die Grundlage für die umfangreiche proteomische Untersuchung dieses Organismus, da sie die routinemäßige Identifizierung von 2DE-separierten Proteinspots mittels ihres PMF ermöglicht. Das 4,3 Mb umfassende zirkuläre Chromosom und die beiden Plasmide (0,21 bzw. 0,22 Mb) kodieren 4603 vorhergesagte Proteine (Rabus et al. 2005). Mittels Sequenzvergleichen mit bereits bekannten Proteinen konnte 2772 Genen eine Funktion zugewiesen werden, während die der restlichen vorhergesagten Proteine (1831) unbekannt ist. Auf diese Weise konnten zehn anaerobe und vier aerobe Abbauwege aromatischer Verbindungen vorhergesagt werden (Abb. 6), deren Gene meist in Clustern auf dem Chromosom angeordnet sind. Zusätzlich konnten die zentralen Stoffwechselwege rekonstruiert werden, z.B. der Citratzyklus oder die Gluconeogenese (Rabus et al. 2005). Die Vielzahl im Genom kodierter regulatorischer Proteine weist auf eine fein abgestimmte Regulation kataboler Wege hin. Die Präsenz globaler Regulatoren im Genom (z.B. 7 Gene der Fnr/Crp-Familie) deutet darüber hinaus auf ein übergeordnetes, Redox-spezifisches Regulationsnetzwerk hin. Die Verfügbarkeit der Genomsequenz in Kombination mit differentieller Proteomanalyse (2D DIGE) und MS-basierter Proteinidentifizierung, ermöglichten erstmals die globale Untersuchung aerober und anaerober Abbauwege.

Grundlage für diese umfangreiche Studie bildeten Kulturen von Stamm EbN1, die an das Wachstum mit 17 verschiedenen aromatischen und aliphatischen Verbindungen unter anoxischen oder oxischen Bedingungen angepasst waren. Insgesamt wurden die löslichen Proteome von 22 verschiedenen Anpassungsbedingungen untersucht. Diese 22 Wachstums-bedingungen lassen sich drei Gruppen zuordnen: (i) anaerobes Wachstum mit aromatischen Verbindungen, (ii) aerobes Wachstum mit aromatischen Verbindungen und (iii) anaerobes Wachstum mit aliphatischen Alkoholen, Ketonen und Carbonsäuren (Abb. 6 und 7). In allen drei Fällen führen die jeweiligen Abbauwege zur Bildung zentraler Intermediate: Benzoyl-CoA während des anaeroben Aromaten-Abbaus und Acetyl-CoA im Fall des aeroben Aromaten- und anaeroben Aliphaten-Abbaus. Die direkten Vorläufer dieser Verbindungen (Benzoat bzw. Acetat) wurden entsprechend für Referenz-Anzuchten bzw. als Referenz-Zustände eingesetzt, um bei den DIGE-Analysen Proteine zu entdecken, die spezifisch für das

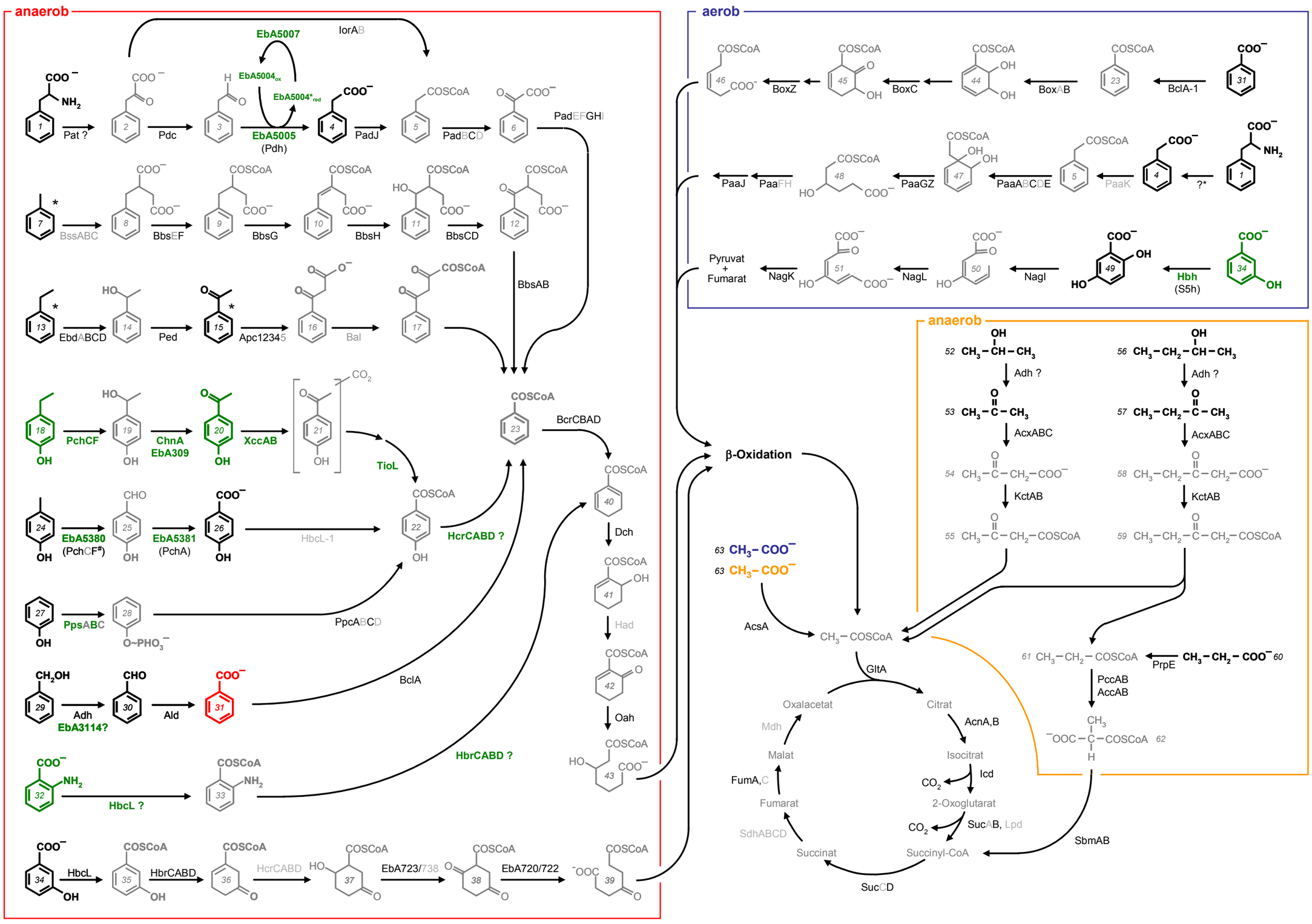

anaerob
EbA5007
IorAB
EbA5004ox
EbA5004*red
EbA5005
(Pdh)
Pat ?
Pdc
PadJ
PadBCD
PadEFGHI
BssABC
BbsEF
BbsG
BbsH
BbsCD
BbsAB
EbdABCD
Ped
Apc12345
Bal
PchCF
ChnA
EbA309
XccAB
TioL
BcrCBAD
EbA5380
(PchCF#)
EbA5381
(PchA)
HbcL-1
HcrCABD ?
PpsABC
PpcABCD
Adh
EbA3114?
Ald
BclA
HbrCABD ?
HbcL ?
HbcL
HbrCABD
HcrCABD
EbA723/738
EbA720/722
Dch
Had
Oah
aerob
BoxZ
BoxC
BoxAB
BclA-1
PaaJ
PaaFH
PaaGZ
PaaABCDE
PaaK
?*
Pyruvat
+
Fumarat
NagK
NagL
NagI
Hbh
(S5h)
β-Oxidation
anaerob
Adh ?
AcxABC
KctAB
AcsA
GltA
Oxalacetat
Citrat
AcnA,B
Isocitrat
Icd
2-Oxoglutarat
SucAB, Lpd
Succinyl-CoA
SbmAB
SucCD
Succinat
SdhABCD
Fumarat
FumA,C
Malat
Mdh
PrpE
PccAB
AccAB

Abb. 7. Rekonstruktion kataboler Wege in "*A. aromaticum*" EbN1 basierend auf der differentiellen Proteom-Analyse (2D DIGE). Die farbigen Umrandungen kennzeichnen anaerobes Wachstum mit aromatischen Verbindungen (Rot), anaerobes Wachstum mit aliphatischen Verbindungen (Orange) und aerobes Wachstum mit aromatischen Verbindungen (Blau). Die Wachstumssubstrate der Referenz-Zustände sind in entsprechender Farbe (Rot, Orange oder Blau) gekennzeichnet. Grün markierte Substanzen wurden in dieser Arbeit erstmals als Wachstumssubstrate von Stamm EbN1 identifiziert. (*) Die Substrat-abhängige Regulation wurde bereits untersucht (Kühner et al. 2005).

Namen der Verbindungen: (*1*) Phenylalanin, (*2*) Phenylpyruvat, (*3*) Phenylacet-aldehyd, (*4*) Phenylacetat, (*5*) Phenylacetyl-CoA, (*6*) Phenylglyoxylat, (*7*) Toluol, (*8*) (*R*)-Benzylsuccinat, (*9*) (*R*)-Benzylsuccinyl-CoA, (*10*) (*E*)-Phenylitaconyl-CoA, (*11*) 3-Hydroxybenzoylsuccinyl-CoA, (*12*) Benzoylsuccinyl-CoA, (*13*) Ethylbenzol, (*14*) (*S*)-1-Phenylethanol, (*15*), Acetophenon, (*16*) Benzoylacetat, (*17*) Benzoylacetyl-CoA, (*18*) *p*-Ethylphenol, (*19*) 1-(4-Hydroxyphenyl)-ethanol, (*20*) *p*-Hydroxyacetophenon, (*21*) unbekanntes Carboxylierungsprodukt, (*22*) *p*-Hydroxybenzoyl-CoA, (*23*) Benzoyl-CoA, (*24*) *p*-Cresol, (*25*) *p*-Hydroxybenzaldehyd, (*26*) *p*-Hydroxybenzoat, (*27*) Phenol, (*28*) Phenylphosphat, (*29*) Benzylalkohol, (*30*) Benzaldehyd, (*31*) Benzoat, (*32*) *o*-Aminobenzoat, (*33*) *o*-Aminobenzoyl-CoA, (*34*) *m*-Hydroxybenzoat, (35) *m*-Hydroxybenzoyl-CoA, (*36*) 2-Ene-5-oxo-cyclohex-1-carbonyl-CoA, (*37*) 2-Hydroxy-5-oxo-cyclohex-1-carbonyl-CoA, (*38*) 2,5-Dioxo-cyclohex-1-carbonyl-CoA, (*39*) 4-Oxopimelyl-CoA, (*40*) Cyclohex-1,5-dien-1-carbonyl-CoA, (*41*) 6-Hydroxycyclohex-1-en-1-carbonyl-CoA, (*42*) 6-Oxocyclohex-1-en-1-carbonyl-CoA, (*43*) 3-Hydroxypimelyl-CoA, (44) 2,3-Dihydroxy-4,6-dien-cyclohexylcarbonyl-CoA, (*45*) 2-Oxo-3-hydroxy-5-en-cyclohexylcarbonyl-CoA, (*46*) β-En-adipyl-CoA, (*47*) *cis*-Dihydrodiol Derivat von Phenylacetyl-CoA, (*48*) β-Hydroxyadipyl-CoA, (*49*) Gentisat, (*50*) Maleylpyruvat, (*51*) Fumarylpyruvat, (*52*) 2-Propanol, (*53*) Aceton, (*54*) 3-Oxo-butyrat, (*55*) 3-Oxo-butyryl-CoA, (*56*) 2-Butanol, (*57*) 2-Butanon, (*58*) 3-Oxo-valerat, (*59*) 3-Oxo-valeryl-CoA, (*60*) Propionat, (*61*) Propionyl-CoA, (*62*) Methylmalonyl-CoA, (*63*) Acetat, (*64*) Acetyl-CoA,

Enzymnamen (in alphabetischer Reihenfolge): Farbkennzeichnung: schwarz, identifiziert; grau, nicht identifiziert; grün, identifiziert und dem Abbauweg aufgrund der differentiell-proteomischen Daten neu zugeordnet. Enzymnamen in Klammern wurden *in silico* vorhergesagt. Die Funktion von mit (?) gekennzeichneten Enzymen konnte in dieser Arbeit weder bestätigt noch widerlegt werden. (AceA) Isocitrat Lyase, (AcnA, AcnB) Aconitase Isoenzym, (AscA) Acetyl-CoA Synthetase, (AcxABC) Aceton Carboxylase, (Adh) Alkohol Dehydrogenase, (Ald) Aldehyd Dehydrogenase, (BclA) Benzoat CoA-Ligase, (BclA-1) ″aerobe″ Benzoat CoA-Ligase, (BcrCBAD) Benzoyl-CoA Reduktase, (BoxAB) Benzoyl-CoA Oxidationskomplex, (BoxC) Enoyl-CoA Hydratase/Isomerase, (BoxZ) Aldehyd Dehydrogenase, (ChnA) vorhergesagte Cyclohexanol Dehydrogenase, (Dch) Cyclohex-1,5-diene-1-carbonyl-CoA Hydratase, (EbA309) vermutliche Dehydrogenase, (EbA720/722) vermutliche 3-Oxo-5-ene-cyclohexylcarbonyl-CoA Hydratase und 3,5-Dioxo-cyclohexylcarbonyl-CoA Hydrolase, (EbA723/738) vermutliche 3-Oxo-5-hydroxy-cyclohexylcarbonyl-CoA Dehydrogenase, (EbA5004) Eisen-Schwefel Cluster Bindeprotein, ähnlich zu 4Fe-4S Ferredoxin, (EbA5005) vorhergesagte Aldehyde:Ferredoxin Oxidoreduktase, (EbA5007) Ferredoxin:NADH Oxidoreduktase, (EbA3118) Zn-enthaltende Alkohol

Dehydrogenase, (EbA5380) vermutliche *p*-Cresol Methylhydroxylase, (FumA, FumC) Fumarase Isoenzyme, (GlcB) Malate Synthase, (GltA) Citrat synthase, (Had) 6-hydroxycyclohex-1-en-1-carbonyl-CoA Dehydrogenase, (HbcL) *m*-Hydroxybenzoat CoA-Ligase, (HbcL-1) *p*-Hydroxybenzoat CoA-Ligase, (Hbh) Hydroxybenzoat Hydroxylase, (HbrCBAD) *m*-Hydroxybenzoyl-CoA Reduktase, (HcrCAB) *p*-Hydroxybenzoyl-CoA Reduktase, (Icd) Isocitrat Dehydrogenase, (IorAB) Indolpyruvat:Ferredoxin Oxidoreduktase, (KctAB) Succinyl-CoA:3-Ketoacyl CoA-Ligase, (Mdh) Malat Dehydrogenase, (NagI) Gentisat-1,2-dioxygenase, (NagK) Fumarylpyruvat Hydrolase, (NagL) Maleylpyruvat Isomerase, (Oah) 6-Oxocyclohex-1-en-1-carbonyl-CoA Hydrolase, (PaaABCDE) Phenylacetyl-CoA (di)oxygenase, (PaaGZ) Ring öffnendes Enzym, (PaaG) Enoyl-CoA Hydratase/Isomerase, (PaaZ) Aldehyd Dehydrogenase, (PaaFHJ) beteiligt an β-Oxidation via β-Ketoadipyl-CoA, (PaaK) Phenylacetat CoA-Ligase, (PadBCD) Phenylacetyl-CoA:Acceptor Oxidoreduktase, (PadEFGHI) Phenylglyoxylat:Acceptor Oxidoreduktase, (PadJ) Phenylacetat CoA-Ligase, (Pat) Phenylalanin Aminotransferase, (PccAB, AccBC) Propionyl-CoA Carboxylase, (PchA) *p*-Hydroxybenzaldehyd Dehydrogenase, (PchCF[#]; EbB97/EbA3165) *p*-Cresol Methylhydroxylase, (PchCF; EbA300/301) *p*-Cresol Methylenhydroxylase ähnliches Protein, (Pdc) Phenylpyruvat Decarboxylase, (Pdh) Phenylacetaldehyd Dehydrogenase oder vermutliche *p*-Hydroxybenzaldehyd Dehydrogenase, (PpcABCD) Phenylphosphat Carboxylase, (PpsABC) Phenylphosphat Synthetase, (PrpE) Propionat CoA-Ligase, (SbmAB) Methylmalonyl-CoA Mutase, (SdhABCD) Succinat Dehydrogenase, (SucAB, Lpd) 2-Oxoglutarat Dehydrogenase-Komplex, (SucCD) Succinyl-CoA Synthetase, (TioL) vorhergesagte Thiolase, (XccAB) Biotin-abhängige Carboxylase (verändert nach Rabus et al. 2005).

untersuchte Wachstumssubstrat bzw. den Abbauweg sind. Acht der 17 untersuchten Verbindungen sind nicht nur Wachstumssubstrate von Stamm EbN1, sondern stellen auch Abbauintermediate anderer Substrate dar (z.B. Phenylacetat im anaeroben Phenylalanin-Abbau). Somit konnten auch die Subproteome sequenzieller Abbauwege (z.B. von Phenylalanin zu Phenylacetat und von Phenylacetat zu Benzoyl-CoA; Abb. 6) differenziert und in ihrer Substrat-spezifischen Regulation untersucht werden. Das experimentelle Vorgehen bei dieser Studie lässt sich in folgende Schritte untergliedern (Abb. 7): (i) Extraktion löslicher Proteine von verschieden adaptierten Zellen, (ii) Kontrolle der Qualität der Proteinextrakte mit Hilfe Silber-gefärbter 2DE-Gele, (iii) Quantitatives, differentielles Proteinprofiling (DIGE) zur Detektion und Selektion von regulierter Proteinspots für die MS-basierte Identifizierung, (iv) Herstellung preparativer Coomassie-gefärbter 2DE-Gele zur Bereitstellung ausreichender Proteinmenge für die MS-Analyse, (v) Protein-Identifizierung

durch PMF-mapping und (vi) proteogenomische Rekonstruktion des katabolen Netzwerks von Stamm EbN1.

Es sei an dieser Stelle darauf hingewiesen, dass die Signifikanz der Abundanz-Änderung eines Proteins durch das untersuchte biologische System bestimmt wird. In dieser Studie wurde ein stringenter Signifikanz-Grenzwert einer >|2,5|-fachen Abundanz-Änderung angelegt, da die Studie hauptsächlich auf katabole, d.h. erwartungsgemäß abundante Proteine ausgerichtet war. Zudem sollten Abundanz-Unterschiede ein hohes Maß an Konfidenz aufweisen. Zur Vereinfachung und Anschaulichkeit, wird die verringerte Abundanz eines Proteins als negative Änderung angegeben (z.B. -3-fach bei einer Verringerung um zwei Drittel; also eigentlich eine 0,3-fache Änderung). Proteine, die eine Änderung von 1,0 oder -1,0 aufweisen, zeigen somit überhaupt keine Änderung in ihrer relativen Häufigkeit.

Insgesamt konnten in dieser Studie 369 unterschiedliche Protein-Spezies mit hoher Zuverlässigkeit identifiziert werden. Diese Proteine können verschiedenen funktionalen Kategorien zugeordnet werden: anaerober Aromaten-Abbau (48), aerober Aromaten-Abbau (17), anaerober Aliphaten-Abbau (14), allgemeiner Metabolismus (77), Biosynthese (56), zelluläre Funktionen (50) und Proteine unbekannter Funktion (107). Besonders die Identifizierung von 37 hypothetischen Proteinen ist hervorzuheben, da ihre tatsächliche Bildung zum ersten Mal gezeigt werden konnte. Die spezifische Co-Regulation einiger Proteine unbekannter Funktion lässt erste Rückschlüsse auf ihre Funktion zu (siehe unten).

2.1 Spezifität und Ausmaß der Regulation

Von den 369 identifizierten verschiedenen Proteinen, waren 213 unter mindestens einem Wachstumszustand differentiell reguliert, während die verbleibenden 156 Proteine eine unveränderte Abundanz aufwiesen. Einen Überblick über das Ausmaß der maximalen Änderung der Abundanz aller identifizierten Proteine bietet Abb. 8. Die überwiegende Mehrzahl der regulierten Proteine zeigte eine 2,5- bis 50-fach verstärkte Abundanz. Hingegen wiesen nur wenige Proteine eine verringerte Abundanz auf. Die größte Anzahl regulierter Proteinspots wurde während des anaeroben Wachstums mit aromatischen Verbindungen detektiert (z.B. mehr als 200 mit Phenol; Referenz Benzoat). Berücksichtigte man, dass mehr als 120 Proteinspots spezifisch während des anaeroben Wachstums mit Benzoat gebildet werden (Referenz Acetat), läge diese Zahl noch höher. Hingegen wurden nicht mehr als 115 Proteinspots während des aeroben Aromaten-Abbaus (z.B. m-Hydroxybenzoat) und nur 70 während des anaeroben Aliphaten-Abbaus reguliert (Referenz jeweils Acetat). Diese hohe

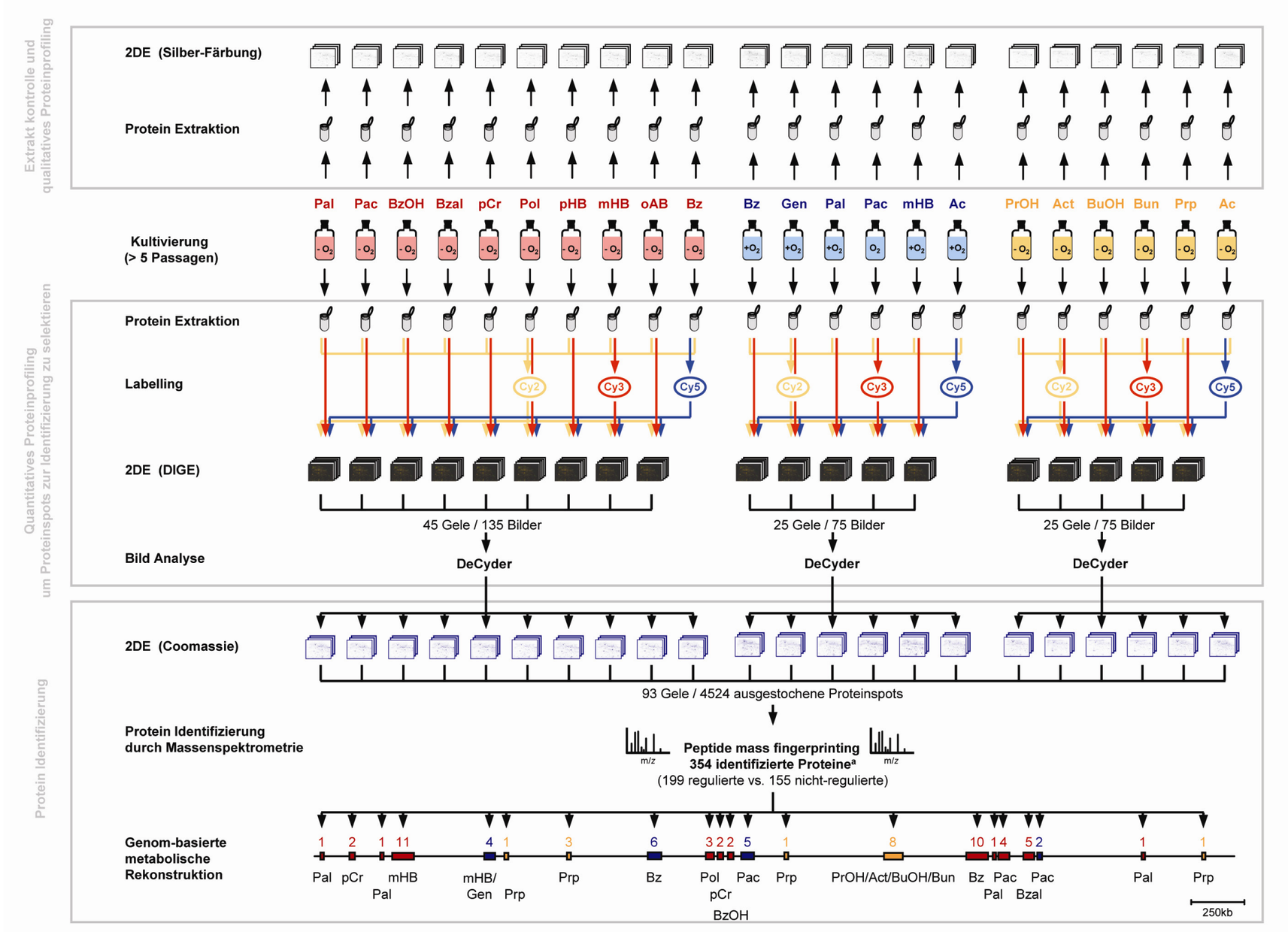


Abb. 7: Überblick des experimentellen Vorgehens zur Untersuchung Substrat-spezifischer Regulation in "*A. aromaticum*" EbN1. Vor der proteomischen Analyse wurden die Zellen über mindestens 5 Passagen an das aerobe (Blau) oder anaerobe (Rot und Orange) Wachstum mit den jeweiligen Substraten angepasst. Substratnamen: Pal, Phenylalanin; Pac, Phenylacetat; BzOH, Benzylalkohol; Bzal, Benzaldehyd; pCr, *p*-Cresol; Pol, Phenol; pHB, *p*-Hydroxybenzoat; mHB, *m*-Hydroxybenzoat; oAB, *o*-Aminobenzoat; Bz, Benzoat; Gen, Gentisat; Ac, Acetat; PrOH, 2-Propanol; Act, Aceton; BuOH, 2-Butanol; Bun, 2-Butanon; Prp, Propionat. Die Qualität der präparierten Proteinextrakte wurde mittels Silber-gefärbter Gele überprüft (oben). Die Anwendung der DIGE-Technologie erlaubte die quantitative Analyse unabhängiger Proteinextrakte (Mitte). Das Gesamtprojekt war in drei Arbeitspakete aufgeteilt: anaerobes Wachstum mit aromatischen Verbindungen, aerobes Wachstum mit aromatischen Verbindungen und anaerobes Wachstum mit aliphatischen Carbonsäuren, Ketonen und Alkoholen. Die Bild-Analyse wurde mit der DeCyder-Software durchgeführt. Ausgewählte regulierte und nicht-regulierte Proteinspots wurden aus separaten Coomassie-gefärbten Gelen ausgestochen und mittels ihres PMF identifiziert.

[a] Die identifizierten Proteine des *p*-Ethylphenol Abbauwegs sind nicht enthalten, so dass in dieser Arbeit insgesamt 369 verschiedene Proteine identifiziert wurden.

Zahl regulierter Proteine während des anaeroben Aromaten-Abbaus könnte primär die komplexen und diversen biochemischen Reaktionen reflektieren.

Neben Proteinen mit zugewiesener Funktion in den Abbauwegen, wurden mehrere weitere Proteine Kontext-spezifisch gebildet. Darunter befanden sich Proteine mit zugewiesener, aber Abbauweg-unabhängiger und mit unbekannter Funktion (Abb. 9). So wurden beispielsweise drei hypothetische Proteine (EbA4705, EbA4712 und EbA4715) spezifisch während des anaeroben Wachstums mit Ketonen gebildet. Auf dem Chromosom von Stamm EbN1 liegen die kodierenden Gene dieser drei Proteine zwischen denen der Aceton Carboxylase (*acxABC*) und der Succinyl-CoA:3-Ketoacyl-CoA Ligase (*kctAB*), den ersten beiden Enzymen des anaeroben Ketonabbaus (Abb. 3 in Publikation 1). Die starke Hochregulierung (bis zu 750-fach) und die sehr hohe Abundanz dieser Proteine (bis zu 10 % aller 2DE separierten Proteine) lassen eine Funktion im anaeroben Ketonabbau vermuten. Regulationsmuster dieser Art deuten darauf hin, dass manche Abbauwege bzw. Wachstumszustände mehr Proteine involvieren als bisher angenommen.

Der Gesamtdatensatz dieser Studie erlaubt eine erste Korrelation zwischen Anpassungszustand und Spezifität der Regulation. So wurde im Bereich des anaeroben Aromaten-Abbaus der höchste Grad an Substrat-Spezifität der differentiellen Proteinprofile beobachtet. Dies trifft auch auf modulare Abbauwege zu. So induzierte Phenylacetat unter anoxischen Bedingungen lediglich die Bildung von Proteinen des Phenylacetat-Abbaus. Die Bildung der Proteine, die an der Umsetzung von Phenylalanin zu Phenylacetat beteiligt sind (Abb. 6) wurde jedoch nicht induziert. Einzig Proteine, die nicht katabolen Reaktionssequenzen zugeordnet werden (z.B. das Bindeprotein eines ABC-Transporters, EbA1033) zeigten bei Wachstum mit verschiedenen aromatischen Substraten eine erhöhte Abundanz. Für den anaeroben Abbau aliphatischer Alkohole, Ketone und Carbonsäuren wurden ebenfalls hochspezifische Regulationsmuster festgestellt. So wurde die Propionyl-CoA Carboxylase (PccA) des Methylmalonyl-CoA Wegs nur während des Wachstums mit Propionat, 2-Butanol und 2-Butanon gebildet. Nur bei Verwertung dieser drei Aliphaten ist der Methylmalonyl-CoA Weg erforderlich (Abb. 6). Im Gegensatz zum anaeroben Katabolismus, wurde im aeroben Aromaten-Abbau ein gewisses Ausmaß von Kreuzregulierung festgestellt. So wurden die Proteine eines Abbauwegs nicht nur in Gegenwart ihres Abbau-Substrats gebildet, sondern auch in Gegenwart anderer Substrate, wenngleich in geringerem Ausmaß (siehe auch Abb. 4 in Publikation 1). Interessanter Weise führte das aerobe Wachstum mit Substraten, die auch anaerob verwertet werden können (z.B.

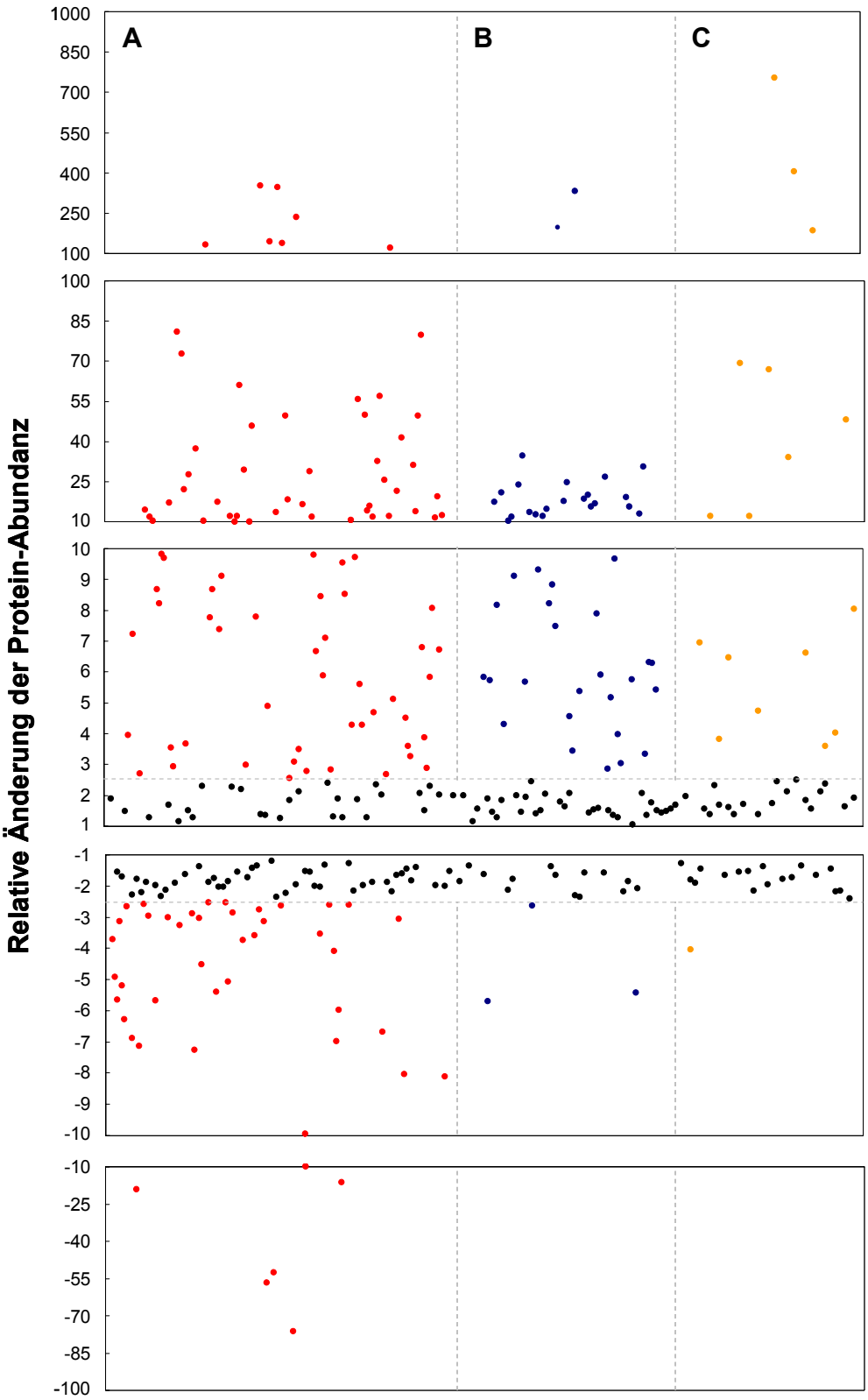


Abb. 8: Relative Änderung der Protein-Abundanz aller identifizierter Proteine. Proteine, die keine signifikante Änderung der Abundanz aufwiesen sind schwarz markiert. Proteine, die spezifisch während des anaeroben Wachstums mit aromatischen Verbindungen reguliert waren, sind in Rot gezeigt (A). Blaue Punkte repräsentieren regulierte Proteine während des aeroben Wachstums mit aromatischen Verbindungen (B) und Proteine, die spezifisch während des anaeroben Wachstums mit aliphatischen Verbindungen reguliert waren sind in Orange gezeigt (C).

Benzoat), zur geringfügigen Bildung der Proteine des anaeroben Abbaus. In ähnlicher Weise wurde in T. aromatica eine geringfügige Expression der Benzoyl-CoA Reduktase während des aeroben Wachstums mit aromatischen Verbindungen beobachtet (Heider et al. 1998). Ungeachtet dieser geringen Abweichungen von Kontext-spezifischer Regulation, scheint auch in Stamm EbN1 Sauerstoff eine übergeordnete regulatorische Rolle einzunehmen.

2.2 Konstitutiv gebildete Proteine

Das konstitutive Proteom umfasst alle Proteine, die unter den untersuchten Bedingungen keine signifikante Abundanz-Änderung aufwiesen (d.h. eine Abundanz-Änderung <|2,5|-fach). Insgesamt konnten ~92 % aller detektierten Proteinspots dieser Gruppe zugeordnet werden. Die Meisten dieser Proteine haben vorhergesagte Funktionen im allgemeinen Stoffwechsel (z.B. Citratzyklus oder Pyruvat Metabolismus), in der Biosynthese (z.B. Aminosäure- oder tRNA-Synthese) oder im allgemeinen Zellstoffwechsel (z.B.

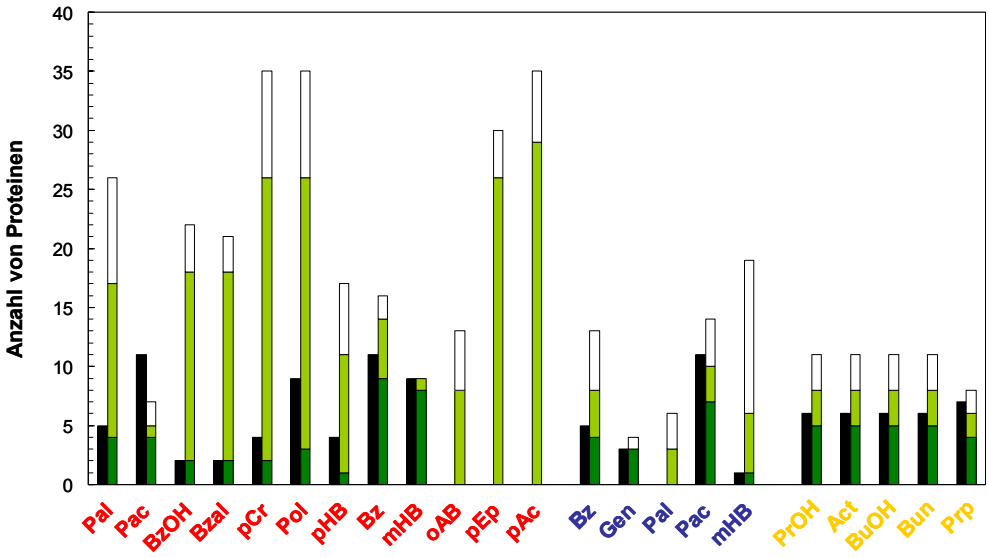


Abb. 9: Anzahl von Proteinen mit genomisch vorhergesagter Funktion in den untersuchten Abbauwegen im Vergleich zu proteomisch identifizierten und Substrat-spezifisch regulierten Proteinen. Proteine mit genomisch vorhergesagter Funktion im jeweiligen Abbauweg sind in Schwarz gezeigt. Spezifisch regulierte und in dieser Studie identifizierte Proteine mit einer zugewiesenen Funktion im vorhergesagten Abbauweg (Dunkelgrün), mit anderer vorhergesagter Funktion (Hellgrün) oder mit unbekannter Funktion (Weiß) sind gezeigt. Die Abkürzungen der Wachstumssubstrate entsprechen denen in Abb. 7 angegebenen: pAc, *p*-Hydroxyacetophenon; pEp, *p*-Ethylphenol. Rot kennzeichnet anaerobes und Blau aerobes Wachstum mit aromatischen Verbindungen, während anaerobes Wachstum mit aliphatischen Alkoholen, Ketonen und Carbonsäuren in Orange dargestellt ist.

Energiekonservierung, Detoxifizierung oder Transkription und Translation). Interessanter Weise lag die durchschnittliche Abundanz-Änderung der identifizierten, konstitutiven Proteine bei ~|1,3|-fach (Standardabweichung ± 0,30). Somit wurde ihre Bildung unter keiner der getesteten 22 Wachstumsbedingungen maßgeblich beeinflusst. Ein prominentes Beispiel bildet das universelle Chaperon GroEL, dessen Abundanz-Änderung in allen 22 untersuchten Zuständen zwischen 1,07 und 1,16 variiert (siehe auch Abb. 5 in Publikation 1). Im marinen Bakterium *Rhodopirellula baltica* wurden ebenso geringe Abundanz-Änderungen von Enzymen des Citratzyklus beobachtet (Gade et al. 2005a).

2.3 Bestätigung genomisch vorhergesagter Abbauwege

Die Mehrzahl genomisch vorhergesagter Abbauwege konnte mit Hilfe des differentiell proteomischen Ansatzes bestätigt werden. Einen Überblick über die metabolische Rekonstruktion der Abbauwege in Stamm EbN1 gibt Abb. 6. In etlichen Fällen konnten Untereinheiten der meisten (bis aller) Enzyme eines Abbauwegs auf den 2DE-Gelen identifiziert und ihre Abundanz-Änderung über verschiedene Wachstumsbedingungen vergleichend verfolgt werden. Beispielhaft sei hier der anaerobe Phenol-Abbau vorgestellt. Durch Genomanalyse wurde für Stamm EbN1 ein Abbauweg über Phenylphosphat und *p*-Hydroxybenzoat vorgeschlagen (Rabus et al. 2005), wie er für *T. aromatica* beschrieben wurde (Lack und Fuchs 1992; Lack und Fuchs 1994; Breinig et al. 2000). Die Abundanz von Untereinheiten der beiden vorhergesagten initalen Enzyme Phenylphosphat Synthase (PpsB, 3,6-fach) und Phenylphosphat Carboxylase (PpcAC, 81,0- bzw. 120,3-fach) war während des anaeroben Wachstums mit Phenol spezifisch erhöht (siehe auch Abb. 4 in Publikation 1), welches ihre vorhergesagte Funktion unterstützt. Interessanter Weise ist an einer anderen Stelle des Chromosoms eine paraloge Phenylphosphat Synthase kodiert (*ebA5781* und *ebdA5783* bei ~3,4 Mb; *ppsABC* und *ppcABCD* bei ~1,9 Mb). Während des anaeroben Wachstums mit Phenol war die Abundanz der identifizierten B-Untereinheit, EbA5783, allerdings nicht erhöht (1,9-fach). Lediglich während des anaeroben Wachstums mit *p*-Cresol war die EbA5783-Abundanz erhöht (19,3-fach). Dieses Regulationsmuster sowie das Fehlen der C-Untereinheit im paralogen Gen-Cluster lassen eine essentielle Rolle des paralogen Enzyms im anaeroben Phenol-Abbau unwahrscheinlich erscheinen.

2.4 Identifizierung neuer Abbau-Substrate und Vorhersage neuer Abbauwege

Anhand der Genomsequenz von Stamm EbN1 wurde über die Möglichkeit eines aeroben Abbaus von *o*-Aminobenzoat unter Beteiligung einer *o*-Aminobenzoat Monooxygenase spekuliert. Jedoch ist Stamm EbN1 nicht in der Lage dieses Substrat aerob zu verwerten. Interessanter Weise konnte in dieser Arbeit erstmals die anaerobe Verwertung von *o*-Aminobenzoat gezeigt werden. Ein anaerober Abbauweg über CoA-Aktivierung und reduktive Deaminierung zu Benzoyl-CoA wurde für *T. aromatica* beschrieben (Lochmeyer et al. 1992). Kodierende Gene des deaminierenden Enzyms sind jedoch noch unbekannt. Die Aktivierung von *o*-Aminobenzoat in *T. aromatica* erfolgt durch die Benzoyl-CoA Ligase (Schühle et al. 2003). Monohydroxybenzoat Analoga werden hingegen von anderen spezifischen CoA-Ligasen aktiviert (Biegert et al. 1993; Laempe et al. 2001). Während des anaeroben Wachstums von Stamm EbN1 mit *o*-Aminobenzoat konnte die differentielle Proteom-Analyse die spezifische Bildung der *m*-Hydroxybenzoat CoA-Ligase (HbcL; 17,2-fach erhöhte Abundanz) und eine verringerte Abundanz der Benzoyl-CoA Ligase (BclA; -4,5-fach) zeigen. Dieses regulatorische Muster deutet darauf hin, dass die CoA-Aktivierung von *o*-Aminobenzoat in Stamm EbN1 durch HbcL anstatt BclA erfolgt. Zusammen mit HbcL, wurden zwei Untereinheiten der Hydroxybenzoyl-CoA Reduktase (HbrCABD) in anaerob mit *o*-Aminobenzoat gewachsenen Zellen verstärkt gebildet (23,4- bzw. 82,1-fach erhöht), was eine Beteiligung an der reduktiven Dearomatisierung vermuten lässt. Da Proteine der weiteren Oxidation des *m*-Hydroxybenzoat-Wegs nicht induziert wurden (z. B. EbA720 -1,5-fach). ist anzunehmen, dass der nachfolgende Abbau über den anaeroben Benzoat-Weg zu 3-Hydroxypimelyl-CoA verläuft (siehe Abb. 6). Neben *o*-Aminobenzoat (anaerob), konnten *m*-Hydroxybenzoat und Phenylalanin als neue aerobe Substrate von Stamm EbN1 identifiziert werden. Aufgrund der differentiell proteomischen Daten konnte ihre Einbettung in das Katabole-Netzwerk vorgeschlagen werden (siehe Abb. 6 und Publikation 1).

2.5 Katabole Funktionen bislang nicht beachteter Proteine

Für die anaeroben Wachstumssubstrate Phenylalanin, Benzylalkohol und *p*-Cresol konnten die vorgeschlagenen Abbauwege bzw. die beteiligten Enzyme nicht bestätigt werden. Anhand der Substrat-spezifischen Proteinmuster konnten jedoch andere, bisher nicht in Betracht gezogene Proteine den jeweiligen Abbauwegen zugewiesen werden. Dies soll im Folgenden am Beispiel des anaeroben *p*-Cresol-Abbaus dargestellt werden.

Der anaerobe *p*-Cresol-Abbau wurde für *Pseudomonas putida* sowie einige *Thauera* und "*Aromatoleum*" (*Azoarcus*) Stämme beschrieben (Hopper und Taylor 1977; Rudolphi et al. 1991). Initiale Hydroxylierung und Oxidierung der Methylgruppe führt zur Bildung von *p*-Hydroxybenzaldehyd. Durch nachfolgende reduktive Dehydroxylierung entsteht das zentrale Intermediat Benzoyl-CoA (Abb. 6). Gene der beteiligten Enzyme wurden auf dem Chromosom von Stamm EbN1 annotiert. Eine Untereinheit der initialen *p*-Cresol Methylhydroxylase (PchF; EbA3165) sowie das zweite Enzym des Abbauwegs, die *p*-Hydroxybenzaldehyd Dehydrogenase (PchA), wurden in der vorliegenden Arbeit auf 2DE-Gelen identifiziert. Überraschender Weise zeigten beide Proteine keine erhöhte Abundanz in *p*-Cresol gewachsenen Zellen (1,1- bzw. 1,2-fach erhöht), jedoch in Zellen, die an das anaerobe Wachstum mit Benzylalkohol oder Benzaldehyd angepasst waren (z.B. PchA 5,3- bzw. 7,8-fach erhöht). Aufgrund dieser Befunde erscheint es unwahrscheinlich, dass diese beiden Proteine eine Rolle im anaeroben *p*-Cresol Katabolismus spielen. Hingegen zeigte eine vorhergesagte FAD-abhängige Oxidase (EbA5380) die höchste Abundanz in *p*-Cresol angepassten Zellen (352,2-fache Erhöhung). Die Oxidase besitzt eine hohe Aminosäuresequenz Identität (jeweils 60 %) zu *p*-Cresol Methylenhydroxylasen von *P. putida* und *Nitrosomonas eutropha* und könnte somit die initial Reaktion katalysieren. Das kodierende Gen (*ebA5380*) bildet zusammen mit den Genen für eine vorhergesagte Phenylacetaldehyd Dehydrogenase (*pdh* bzw. *ebA5381*) und ein regulatorisches Protein (*ebA5384*) eine Opern-ähnliche Struktur auf dem Chromosom von Stamm EbN1. Die vorgesagte Funktion von EbA5381 im anaeroben Phenylalanin-Abbau konnte aufgrund der spezifischen Bildung in Gegenwart von *p*-Cresol (7,4-fach; lediglich -1,4-fach mit Phenylalanin) nicht bestätigt werden (siehe unten). EbA5381 gehört zu einer Gruppe von Aldehyd Dehydrogenasen, die ein breit gefächertes Spektrum aliphatischer und aromatischer Substrate oxidieren. Aufgrund des genetischen Zusammenhangs und der spezifischen Bildung in Gegenwart von *p*-Cresol ist es wahrscheinlich, dass EbA5381 an der Oxidation der Aldehydgruppe des *p*-Hydroxybenzaldehyd und nicht der des Phenylacetaldehyd beteiligt ist.

Diese Hypothese wird weiter unterstützt durch die spezifische Bildung einer Aldehyd:Ferredoxin Oxidoreduktase (AOR; EbA5005) während des anaeroben Wachstums mit Phenylalanin (9,7-fach erhöhte Abundanz). EbA5005 könnte die ursprünglich der Pdh zugeordnete Funktion der Phenylacetaldehyd Oxidation übernehmen. Das kodierende Gen ist in einer Operon-ähnlichen Struktur mit einem 4Fe-4S Ferredoxin-ähnlichen Protein (*ebdA5004*) und einer Ferredoxin:NADH Oxidoreduktase (*ebA5007*) organisiert. Beide Proteine zeigen ebenfalls erhöhte Abundanz während des anaeroben Wachstums mit

Phenylalanin (49,7- bzw. 6,0-fach erhöht). Enzyme der AOR-Familie katalysieren die Oxidation einer Vielzahl aliphatischer und aromatischer Aldehyde zu den korrespondierenden Carbonsäuren, wobei Ferredoxin als Elektronenakzeptor genutzt wird (Roy und Adams 2002; Hagedoorn et al. 2005). Die Oxidation von Phenylacetaldehyd durch AOR wurde bereits für *Thermococcus* Stamm ES-1 und *Eubacterium acidaminophilum* gezeigt (Heider et al. 1995; Rauh et al. 2004). Somit könnte die AOR EbA5005 die Oxidation von Phenylacetaldehyd katalysieren und die frei werdenden Elektronen auf EbA5004 übertragen. Das dritte Protein des Gen-Clusters, die Ferredoxin:NADH Oxidoreduktase EbA5007, könnte schließlich an der Regenerierung des Ferredoxin Cofaktors beteiligt sein (Abb. 6).

2.6 Anaerober Abbau von *p*-Ethylphenol in Stamm EbN1

Während des anaeroben Wachstums mit *p*-Cresol wurden Proteine einer paralogen Ethylbenzol Dehydrogenase, die allerdings keine Funktion im anaeroben Ethylbenzol Abbau hat, verstärkt gebildet. Inspiriert durch die Analogie zur Induktion des anaeroben Ethylbenzol Abbaus durch das strukturell verwandte Toluol (Kühner et al. 2005), wurden *p*-Ethylphenol und auch *p*-Hydroxyacetophenon erstmals als anaerobe Wachstumssubstrate von Stamm EbN1 erkannt. Eine anaerobe Verwertung von *p*-Ethylphenol ist bislang nur für den verwandten Stamm EB1 bekannt (Ball et al. 1996), wobei der zu Grunde liegende Abbauweg noch unbekannt ist. Die Sauerstoff unabhängige Hydroxylierung von *p*-Ethylphenol wurde für den aeroben Abbau in *Pseudomonas putida* JD1 beschrieben (Darby et al. 1987). Da nachfolgende Reaktionen allerdings Monooxygenase-katalysierte Reaktionen involvieren (Tanner und Hopper 2000), muss in Abwesenheit von Sauerstoff ein anderer Abbauweg vorliegen.

2.6.1 Aufklärung des Abbauwegs

Mit Hilfe vergleichender GC-MS Analysen konnten *p*-Hydroxyacetophenon, *p*-Hydroxybenzoat und Benzoat als Intermediate des anaeroben *p*-Ethylphenol Abbaus in Stamm EbN1 identifiziert werden. Dieser Befund wurde durch die Identifizierung entsprechender 2,3,5,6-d_4-Isotopomere während des Wachstums mit 2,3,5,6-d_4-*p*-Ethylphenol zusätzlich unterstützt. Entsprechend konnten *p*-Hydroxybenzoat und Benzoat als Intermediate des anaeroben *p*-Hydroxyacetophenon-Abbaus nachgewiesen werden.

Diese chemisch-analystischen Ergebnisse erlaubten den Vorschlag eines anaeroben Abbauwegs von *p*-Ethylphenol, welcher Analogien zum anaeroben Ethylbenzol-Abbau

aufweist: Nach initialer Hydroxylierung zu 1-(4-Hydroxyphenyl)-ethanol führt eine Dehydrogenierung zur Bildung von *p*-Hydroxyacetophenon. Nachfolgende Carboxylierung und thiolytische Spaltung ergeben *p*-Hydroxybenzoyl-CoA, welches durch reduktive Dehydroxylierung zum zentralen Intermediat Benzoyl-CoA umgesetzt wird (Abb. 10B).

2.6.2 Identifizierung beteiligter Proteine und kodierender Gene

Die löslichen Subproteome von Zellen, die über mindestens 5 Passagen an das anaerobe Wachstum mit *p*-Ethylphenol, *p*-Hydroxyacetophenon oder Benzoat (Referenz) angepasst waren, wurden mittels differentieller zweidimensionaler Gelelektrophorese (2D DIGE) untersucht. Ziel war es, Substrat-spezifische Proteine als Enzymkandidaten für die untersuchten Abbauwege zu identifizieren. Neunundzwanzig Proteine wurden spezifisch während des Wachstums mit *p*-Ethylphenol und *p*-Hydroxyacetophenon gebildet (bis zu >250-fach erhöhte Abundanz; Abb. 10D). Zwölf von ihnen konnten erstmals dem anaeroben *p*-Ethylphenolabbau zugeordnet werden. Ihre genomisch vorhergesagten Funktionen sind: Untereinheit eines *p*-Cresol Methylenhydroxylase-ähnlichen Proteins (PchF), Thiolase (TioL), Cyclohexanol Dehydrogenase (ChnA), putative Dehydrogenase (EbA309), FAD-abhängige Oxidase (EbA310), Zucker Phosphatase (EbA312), Biotin-abhängige Carboxylase (XccAB) und vier hypothetische Proteine (EbA318, EbA329, EbA332 und EbA335).

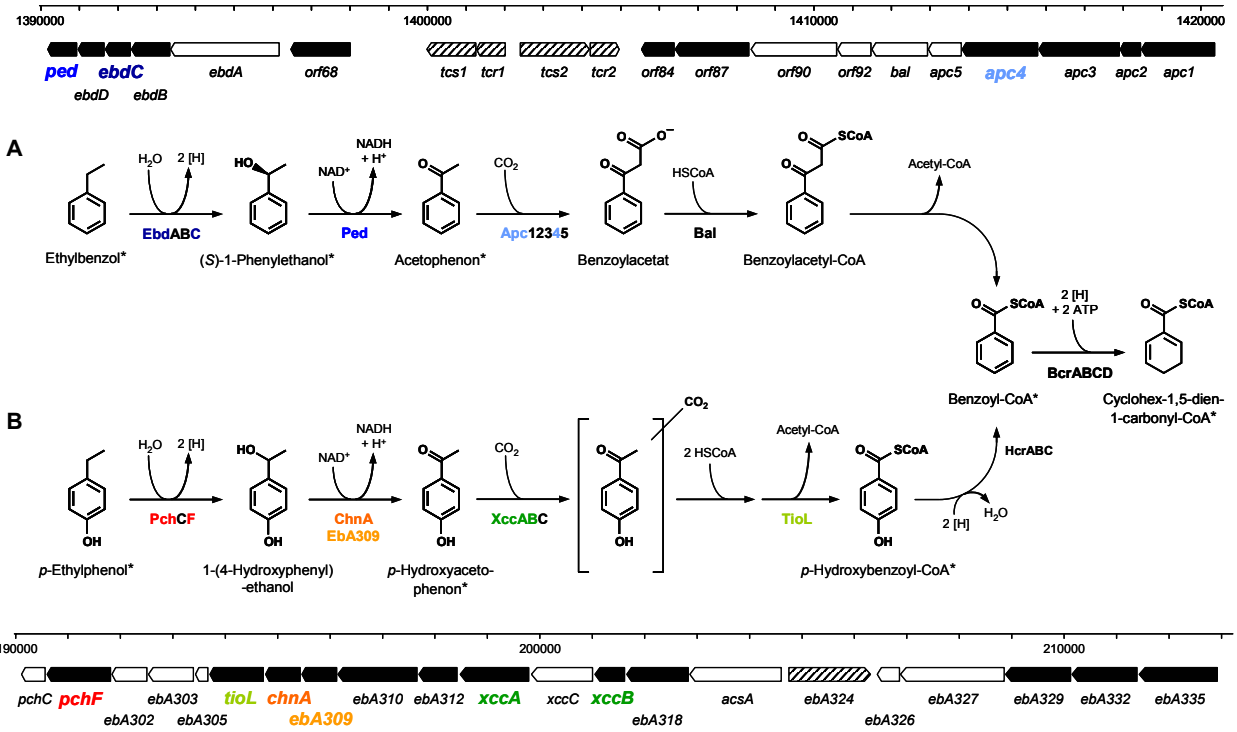
Alle genannten Proteine sind in einer großen Operon-ähnlichen Struktur (~15 kb) auf dem Chromosom von Stamm EbN1 kodiert. Lediglich die hypothetischen Proteine EbA329, EbA332 und EbA335 sind in einer zweiten Operon-ähnlichen Struktur 2,5 kb upstream des 15 kb Operons kodiert (Abb. 10B). Interessanter Weise liegt zwischen den beiden "Operons" ein Gen für einen Sigma54-abhängigen Regulator (*ebA324*) *in trans*, der wahrscheinlich an der Regulation der Gene des *p*-Ethylphenol Abbauwegs beteiligt ist (siehe Abschnitt 2.6.3).

Aufgrund ihrer vorhergesagten Funktionen können die Meisten der spezifisch gebildeten Proteine einzelnen Reaktionen im vorgeschlagenen Abbauweg zugeordnet werden. Die initiale Dehydrogenierung von *p*-Ethylphenol könnte durch das *p*-Cresol Methylenhydroxylase-ähnliche Protein (PchCF) katalysiert werden. PchF von Stamm EbN1 weist 31 % Aminosäuresequenz Identität mit der *p*-Cresol Methylenhydroxylase (PCMH) von *Pseudomonas putida* NCIMB 9866 auf, welche ebenfalls *p*-Ethylphenol umsetzt (McIntire et al. 1985). Die *para*-ständige Hydroxylgruppe des *p*-Ethylphenols ermöglicht die intermediäre Bildung eines Quinon-Methids, weshalb das Redox-Potential des Flavin-Cofaktors (+280 mV) der PCMH für die initiale Dehydrogenierung wahrscheinlich ausreichend ist. Im Gegensatz dazu, erfordert die initiale Oxidation des chemisch ähnlichen, jedoch

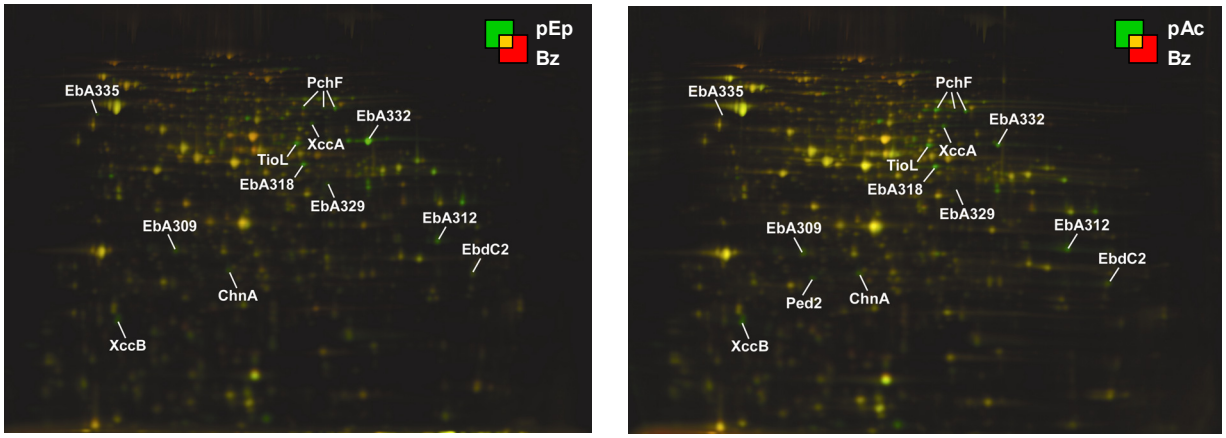
reaktionsträgeren Ethylbenzols durch die Ethylbenzol Dehydrogenase einen Molybdän-Cofaktor sowie einen Elektronenakzeptor mit höherem Redox-Potential (+380 mV) um signifikante Oxidationsraten zu erhalten (Kniemeyer und Heider 2001a). Die Oxidation des ersten Intermediates 1-(4-Hydroxyphenyl)-ethanol zu *p*-Hydroxyacetophenon wird wahrscheinlich durch eine der vorhergesagten Dehydrogenasen (ChnA und EbA309) katalysiert. Beide Proteine besitzen eine hohe Aminosäuresequenz Identität (33 % bzw. 36 %) zur (*S*)-1-Phenylethanol Dehydrogenase (Ped) von Stamm EbN1, welche die analoge Reaktion im anaeroben Ethylbenzol-Abbauweg katalysiert (Abb. 10A; Kniemeyer und Heider 2001b). Die Katalyse dieser zweiten Reaktion ebenfalls durch die PCMH von Stamm EbN1 erscheint unwahrscheinlich. Das orthologe Enzym von *P. putida* bildet in der initialen Reaktion bevorzugt das *R*(+)-Enantiomer. Letzteres wird allerdings nur sehr langsam von der gereinigten PCMH zu *p*-Hydroxyacetophenon umsetzt (Reeve et al. 1990).

Die anschließende Carboxylierung von *p*-Hydroxyacetophenon in Stamm EbN1 wird wahrscheinlich von der Biotin-abhängigen Carboxylase XccAB katalysiert. Biotin-abhängige Carboxylasen sind weit verbreitete Enzyme einer sehr diversen Gruppe, die einen zweigeteilten Reaktionsmechanismus aufweisen: (i) initiale Carboxylierung des Biotin, gefolgt von (ii) der Transcarboxylierung vom Biotin auf das jeweilige Akzeptor-Molekül (Cronan und Waldrop 2002). Interessanter Weise zeigt XccAB keine Ähnlichkeit zur Acetophenon Carboxylase (Apc1-5), welche die analoge Reaktion beim anaeroben Abbau von Ethylbenzol katalysiert (Abb. 10A). Ebenso konnte keine Ähnlichkeit zur Aceton Carboxylase (AcxABC) oder der Phenylphosphat Carboxylase (PpcABCD) von Stamm EbN1 festgestellt werden. Somit stellt die Biotin-abhängige Carboxylase des anaeroben *p*-Ethylphenol Abbauwegs eine neue Art von Carboxylasen in Stamm EbN1 dar. Es ist zu erwarten, dass diese einen anderen katalytischen Mechanismus aufweist, als die Carboxylasen der anderen Gruppen. Zurzeit ist nicht bekannt, an welcher Stelle des *p*-Hydroxyacetophenon Moleküls die Carboxylierung erfolgt.

Eine CoA-aktivierung könnte durch die kodierte Acetoacetyl-CoA Synthetase (AcsA) katalysiert werden und die anschließende thiolytische Spaltung des unbekannten Carboxylierungsprodukts zur Bildung von *p*-Hydroxybenzoyl-CoA könnte von der vorhergesagten Thiolase (TioL) durchgeführt werden. Bemerkenswerter Weise weist AcsA keine Ähnlichkeit zur Benzoylacetat-CoA Ligase (Bal) des anaeroben Ethylbenzol-Wegs auf. Dies könnte als Hinweis auf ein signifikant anderes Carboxylierungsprodukt gedeutet werden. Die abschließende reduktive Dehydroxylierung zu Benzoyl-CoA könnte durch die vorhergesagte Hydroxybenzoyl-CoA Reduktase (HcrABC) katalysiert, wie es für den


1390000
1400000
1410000
1420000
ped
ebdC
ebdA
orf68
ebdD
ebdB
tcs1
tcr1
tcs2
tcr2
orf84
orf87
orf90
orf92
bal
apc5
apc4
apc3
apc2
apc1
A
Ethylbenzol*
EbdABC
(S)-1-Phenylethanol*
Ped
Acetophenon*
Apc12345
Benzoylacetat
HSCoA
Bal
Benzoylacetyl-CoA
Acetyl-CoA
Benzoyl-CoA*
2 [H] + 2 ATP
BcrABCD
Cyclohex-1,5-dien-1-carbonyl-CoA*
B
p-Ethylphenol*
PchCF
1-(4-Hydroxyphenyl) -ethanol
ChnA
EbA309
p-Hydroxyaceto-phenon*
XccABC
2 HSCoA
TioL
p-Hydroxybenzoyl-CoA*
HcrABC
190000
200000
210000
pchC
pchF
ebA303
tioL
chnA
ebA310
ebA312
xccA
xccC
xccB
acsA
ebA324
ebA327
ebA329
ebA332
ebA335
ebA302
ebA305
ebA309
ebA318
ebA326

C


pEp
Bz
EbA335
PchF
EbA332
XccA
TioL
EbA318
EbA329
EbA312
EbA309
EbdC2
ChnA
XccB
pAc
Bz
EbA335
PchF
EbA332
XccA
TioL
EbA318
EbA329
EbA312
EbA309
EbdC2
Ped2
ChnA
XccB

D

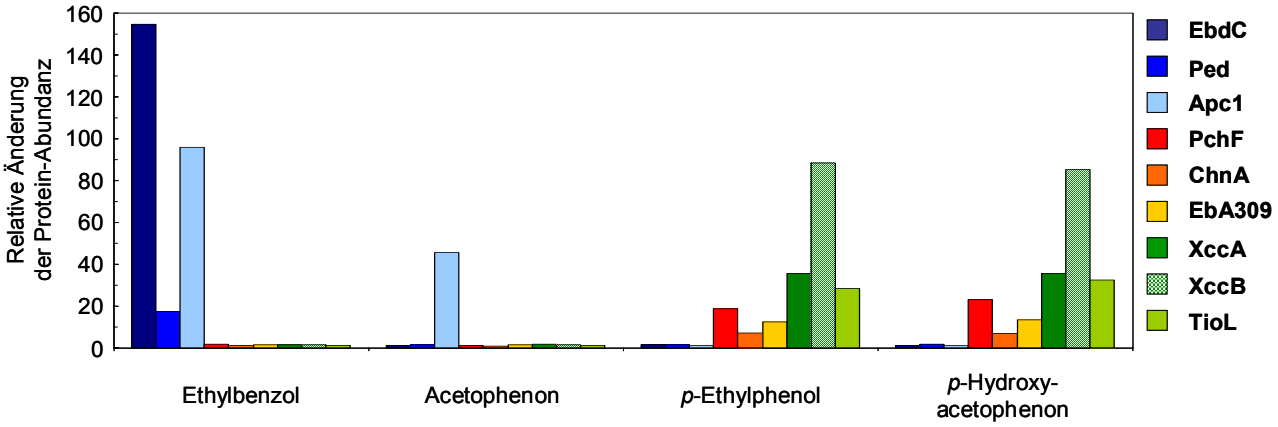

Relative Änderung der Protein-Abundanz
160
140
120
100
80
60
40
20
0
Ethylbenzol
Acetophenon
p-Ethylphenol
p-Hydroxy-acetophenon
EbdC
Ped
Apc1
PchF
ChnA
EbA309
XccA
XccB
TioL

Abb. 10: Anaerobe Abbauwege von Ethylbenzol (A) und *p*-Ethylphenol (B) in "*A. aromaticum*" EbN1. Die beiden chemisch analogen Initialreaktionen werden von unterschiedlichen Enzymen katalysiert. Beide Abbauwege laufen auf der Stufe von Benzoyl-CoA zusammen. Die Enzymnamen der angegebenen Genprodukte sind: Apc1-5, Acetophenon Carboxylase; Bal, Benzoylacetat CoA-Ligase; BcrABCD, Benzoyl-CoA Reduktase; ChnA, vorgeschlagene Cyclohexanol Dehydrogenase; EbA309, mögliche Dehydrogenase; EbdABCD, Ethylbenzol Dehydrogenase; HcrABC, *p*-Hydroxybenzoyl-CoA Reduktase; Ped, (*S*)-1-Phenylethanol Dehydrogenase; PchCF, *p*-Cresol Methylenhydroxylase ähnliches Protein; TioL, vorgeschlagene Thiolase; XccABC, Biotin-abhängige Carboxylase. Für jeden Abbauweg ist die Organisation der kodierenden Gene dargestellt. Der Maßstab gibt die Lokalisierung der Gene auf dem Chromosom an (Nukleotid-Positionen). Die Gene von identifizierten Proteinen sind schwarz gekennzeichnet. Vorgeschlagene Intermediate, die mittels GC-MS-Analyse identifiziert wurden, sind markiert (*). (C) 2D DIGE Gelbilder *p*-Ethylphenol adaptierter (pEp, links) und *p*-Hydroxyacetophenon adaptierter (pAc, rechts) Zellen (Referenz Benzoat, Bz). Identifizierte Proteine des *p*-Ethylphenol Abbauwegs sind annotiert. (D) Die Änderung der Abundanz von Proteinen beider Abbauwege in Substrat-angepaßten Zellen wurde mittels 2D DIGE bestimmt. Protein-Extrakte von anaerob mit Benzoat gewachsenen Zellen wurden als Referenz genutzt, da die aktivierte Form, Benzoyl-CoA, das erste gemeinsame Intermediat beider Abbauwege darstellt. Einzelne Enzyme (oder ihre Untereinheiten; siehe A und B) sind durch einen Farbcode markiert.

anaeroben Abbau von Phenol und *p*-Cresol vorhergesagt wurde (Abb. 10B; Rabus et al. 2005). Allerdings konnte keine Untereinheit dieses Proteins im Rahmen der vorliegenden Arbeit auf den 2DE-Gelen identifiziert und keine signifikante Expression des *hcrB* Gens festgestellt werden. Dies trifft auch für Zellen zu, die an das anaerobe Wachstum mit *p*-Hydroxybenzoat angepasst waren. Daher ist der letzte Schritt des anaeroben Abbaus von *p*-Ethylphenol sowie von Phenol und *p*-Cresol in Stamm EbN1 bislang noch nicht vollständig verstanden.

2.6.3 Substrat-spezifische Regulation des Abbauwegs

Die Regulation des anaeroben *p*-Ethylphenol-Abbaus wurde in Substrat-adaptierten Zellen von Stamm EbN1 untersucht. Anpassungssubstrate waren *p*-Ethylphenol, *p*-Hydroxyacetophenon, Ethylbenzol und Acetophenon. Regulationsmuster wurden vergleichend auf mRNA (real-time RT-PCR) und Protein Ebene (2D DIGE) untersucht. Als Referenz-Zustand diente in allen Fällen anaerobes Wachstum mit Benzoat. Im Fall der Transkriptions-Analysen wurde *bcrC* (Untereinheit der Benzoyl-CoA Reduktase) als

Referenzgen verwendet, da die Abbauwege für *p*-Ethylphenol und Ethylbenzol auf der Stufe des Benzoyl-CoA zusammenfließen (Abb. 10A und B).

Die genomische Organisation aller Gene des anaeroben *p*-Ethylphenol-Abbaus in einer Operon-ähnlichen Struktur lässt eine koordinierte Regulation vermuten. Dies steht im Gegensatz zur sequentiellen Regulation des zweigeteilten Abbauwegs für Ethylbenzol. In Abb. 10D sind die relativen Häufigkeiten der identifizierten Proteine beider Abbauwege in Abhängigkeit der Anpassungssubstrate dargestellt. Obwohl die Substrate und bislang bekannten Intermediate der beiden Abbauwege sich lediglich durch eine Hydroxylgruppe unterscheiden, folgt die Bildung der jeweiligen Enzyme einer hohen Substrat-Spezifität. Die Abundanzprofile der Proteine des *p*-Ethylphenol Abbauwegs sind in *p*-Ethylphenol und *p*-Hydroxyacetophenon adaptierten Zellen ausgesprochen ähnlich. So ist beispielsweise die Abundanz der Flavin-Untereinheit der PCMH (PchF) beim Wachstum mit *p*-Ethylphenol 18,9-fach und mit *p*-Hydroxyacetophenon 23,1-fach erhöht. Dieses gleichartige regulatorische Muster konnte durch das ähnliche Expressions-Level von *pchF* und *xccA* ebenfalls auf transkriptoineller Ebene gezeigt werden (140- bzw. >140-fach erhöht in *p*-Ethylphenol-adaptierten Zellen und 107- bzw. 97-fach erhöht in *p*-Hydroxyacetophenon-adaptierten Zellen). Darüber hinaus konnte gezeigt werden, dass beide Gene als ein polycistronisches Transkript abgelesen werden, welches die Hypothese der koordinierten Regulation weiter untermauert.

Diese transkriptionelle Regulation des *p*-Ethylphenol Abbauwegs wird möglicherweise durch den Sigma54-abhängigen Regulator EbA324 vermittelt. Sowohl das Operon der katabolen Enzyme, als auch das benachbarte Operon der hypothetischen Proteine weisen eine -12/-24 Konsensus-Sequenz (5´-TGGC-N_7-TTGCA-3´; Morett und Segovia 1993) Sigma54-abhängiger Promotoren ~100 bp stromaufwärts des Translationsstarts von *acsA* und *ebA335* auf. Sigma54-abhängige Regulatoren sind an der transkriptionellen Kontrolle einer Vielzahl physiologischer Funktionen beteiligt, einschließlich des Aromaten-Abbaus (Abril et al. 1989). Da die Expression der Gene des *p*-Ethylphenol-Abbaus während des Wachstums mit *p*-Ethylphenol und *p*-Hydroxyacetophenon in Stamm EbN1 gleichermaßen induziert wurde, könnte man spekulieren, dass beide Verbindungen vom Regulator EbA324 erkannt werden. Die chemisch verwandten Verbindungen Ethylbenzol und Acetophenon scheinen jedoch nicht zum Effektor-Spektrum des Regulators EbA324 zu gehören. Darüber hinaus konnte die Bildung der Enzyme des anaeroben *p*-Ethylphenol-Abbaus auch nicht während des anaeroben Wachstums mit anderen aromatischen oder aliphatischen Substraten gezeigt werden (Wöhlbrand et al. 2007), was auf eine sehr stringente

Regulation hindeutet. Die Integration von Effektor-Sensierung und Transkriptions-Initiation in einem Molekül (wie bei EbA324) sollte eine Kreuz-Aktivierung durch einen anderen Sensor ausschließen. Letzteres wurde im Fall des Response-Regulators Tcr2 des Ethylbenzol Abbauwegs durch den Toluol-Sensor TdiS diskutiert (Kühner et al. 2005).

3. Physiologische und proteomische Antwort auf Lösungsmittelstress

3.1 Relevanz der eingesetzten Lösungsmittelkonzentrationen

"*A. aromaticum*" EbN1 sowie die anderen Mitglieder der "*Aromatoleum*"/*Thauera* Verwandtschaftsgruppe anaerober Kohlenwasserstoff-Abbauer wurden aus kontaminierten und unbelasteten Böden oder Süßwassersedimenten isoliert. Die Kohlenwasserstoff-konzentration in diesen Habitaten kann aufgrund verschiedener Umwelteinflüsse (z.B. unterschiedlicher Eintrag in den Boden oder Änderung der Strömungsverhältnisse) variieren. Ethylbenzol- und Toluolkonzentrationen von 1 mg/L (d.h. 9,4 µM bzw. 10,9 µM) sind charakteristisch für Benzin-kontaminierte Grundwasserleiter (Gulensoy und Alvarez 1999). Des Weiteren wurden in industriellen Abwässern Phenol- und Alkylphenol-Gesamtkonzentrationen von bis zu 68 g/L nachgewiesen (Bérne und Cordonnier 1995). Als Reaktion auf die toxischen Eigenschaften von Lösungsmitteln bilden aerobe Mikroorganismen Efflux-Pumpen, Hitzeschock-Proteine und verändern die Struktur ihrer Membranphospholipide (Isken und de Bont 1998; Heipieper et al. 2007). Die Anpassung anaerob Kohlenwasserstoff-abbauender Bakterien an Lösungsmittelstress ist bisher allerdings kaum untersucht und somit nicht verstanden.

In vorangegangenen sowie dieser Arbeit wurden umfangreiche physiologische und proteomische Untersuchungen zur Anpassung an Lösungsmittelstress unter anaeroben Bedingungen durchgeführt. Die Reaktion von Stamm EbN1 auf experimentell bestimmte, semi-inhibitorische (d.h. ~50 % Wachstumsinhibierung) Konzentrationen aromatischer Wachstumssubstrate wurde unter zwei unterschiedlichen Bedingungen untersucht: (i) während des anaeroben Wachstums mit 0,32 mM Ethylbenzol oder 0,74 mM Toluol und (ii) während des anaeroben Wachstums mit Succinat und plötzlicher Exposition (Schock) gegenüber 0,5 mM Ethylbenzol, 1,2 mM Toluol, 3,0 mM *p*-Cresol und 6,5 mM Phenol als Einzelverbindungen oder als Lösungsmittel-Gemisch mit einer Gesamtkonzentration von 2,7 mM. Im ersten Fall sind die katabolen Abbauleistungen vorhanden, während Sie im zweiten Fall, bei den reinen Schock-Experimenten, nicht operativ sind.

Unter allen getesteten Bedingungen konnte eine Lösungsmittelkonzentrations-abhängige Beeinträchtigung des Wachstums von Stamm EbN1 beobachtet werden: sowohl die Wachstumsrate als auch die maximal erreichte optische Dichte waren signifikant reduziert. Die bestimmten semi- und vollständig-inhibitorischen Lösungsmittel-konzentrationen (siehe Abb. 11A) belegen, dass Ethylbenzol für Stamm EbN1 die höchste Toxizität und Phenol die geringste besitzt. Dieser Befund korreliert gut mit den Partitions-Koeffizienten ($\log P_{OW}$) der getesteten Lösungsmittel (siehe Abb. 1). Interessanter Weise liegen die tolerierten Lösungsmittelkonzentrationen deutlich höher als die in der Umwelt bekannten. So kann Stamm EbN1 beispielsweise mit Ethylbenzolkonzentrationen von bis zu 0,48 mM wachsen, während in der Umwelt bislang nur 9,6 µM als Höchstwert nachgewiesen wurde. Daher kann man annehmen, dass Stamm EbN1 in den meisten kontaminierten Habitaten überleben und sich vermehren kann. Bemerkenswerter Weise lagen die semi-inhibitorischen Alkylbenzol-Konzentrationen für Succinat-verwertende Zellen mindestens 2,5-fach höher als kürzlich für andere anaerobe Abbauer (z.B. *T. aromatica* oder *Geobacter sulfurreducens*) berichtet wurde (Duldhardt et al. 2007). In der Tat ähnelt die Ethylbenzol-Toleranz von Stamm EbN1 eher der des aeroben Abbauers *P. putida* (Duldhardt et al. 2007).

3.2 Physiologische und molekulare Anpassung an Lösungsmittelstress

In Gegenwart semi-inhibitorischer Lösungsmittelkonzentrationen war neben der Wachstumsrate auch die Denitrifikation deutlich beeinträchtigt. Sowohl während des anaeroben Wachstums mit Alkylbenzolen als auch beim Lösungsmittelschock Succinat-verwertender Kulturen war der Nitrat-Verbrauch verlangsamt und die intermediäre Akkumulierung von Nitrit reduziert und verlängert. Während des Wachstums mit hohen Alkylbenzolkonzentrationen (0,21 und 0,32 mM Ethylbenzol bzw. 0,74 mM Toluol) kam es zum Übergang in die stationäre Phase wenn Nitrat verbraucht, aber Nitrit noch in maximaler Konzentration vorhanden war. Succinat-verwertende, Lösungsmittel-exponierte Zellen setzten das Wachstum jedoch nach dem Verbrauch von Nitrat fort. Die proteomischen Analysen zeigten, dass verschiedene Proteine der Denitrifikation unter allen Lösungsmittelstress Bedingungen hochreguliert wurden. So war die Abundanz der periplasmatischen Nitrit-Reduktase (NirS) beim Wachstum mit 0,32 mM Ethylbenzol und 0,74 mM Toluol >3-fach erhöht. Eine erhöhte NirS-Abundanz wurde bereits während des anaeroben Wachstums mit anderen aromatischen Verbindungen beobachtet (z.B. >6 fach mit Phenol oder m Hydroxybenzoat; Wöhlbrand et al. 2007). Dies könnte auf eine generelle Beeinträchtigung der

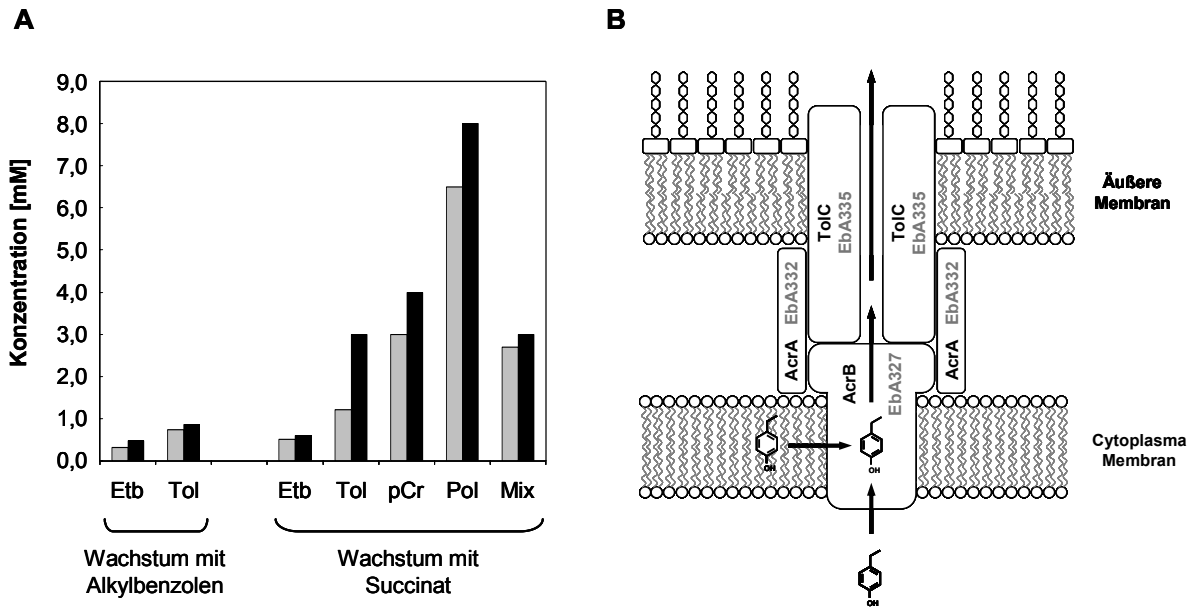


Abb. 11: Untersuchungen zum Lösungsmittelstress bei "*A. aromaticum*" EbN1. (A) Semi- (Grau) und vollständig–inhibierende (Schwarz) Lösungsmittelkonzentrationen. Abkürzungen für Lösungsmittelnamen: Etb, Ethylbenzol; Tol, Toluol; pCr, p-Cresol; Pol, Phenol; Mix, Gemisch aus Etb, Tol, pCr und Pol. Konzentrationsangabe [mM] bezieht sich auf die Konzentration im wässrigen Medium. (B) Modell einer RND-Efflux-Pumpe, die an der Lösungsmittel-Toleranz von *E. coli* (Komponenten in Schwarz) beteiligt ist. Ein ähnlicher Mechanismus wird für ein Lösungsmittel-spezifisches Efflux-System von Stamm EbN1 (Komponenten in Grau) angenommen, hier exemplarisch für *p*-Ethylphenol angegeben (verändert nach Yu et al. 2003 und Piddock 2006).

Nitrit-Reduktase durch aromatische Verbindungen hindeuten. In Succinat-verwertenden, Lösungsmittel-exponierten Zellen wurde NirS lediglich im Fall des Lösungsmittel-Gemisches signifikant hochreguliert (3,0-fach). Allerdings wurde die periplasmische NO-Reduktase (NosZ), die den vorletzten Schritt der Denitrifikation katalysiert, 5,1-, 5,2- bzw. 2,6-fach hoch reguliert (Schock mit Ethylbenzol, Toluol bzw. dem Lösungsmittel-Gemisch). Insgesamt lässt dieses regulatorische Muster vermuten, dass hohe Lösungsmittelkonzentrationen die Funktion periplasmatischer Proteine der Denitrifikation beeinträchtigen. So könnten diese Verbindungen den Elektronen-Fluss zu oder innerhalb NirS behindern, z.B. durch Inhibierung der Dimerisierung von NirS oder seiner Interaktion mit Cytochrom *c*. Entsprechend konnte in *Pseudomonas nautica* ein beeinträchtigter Elektronen-Fluss als Folge einer NirS Inaktivierung durch NO-Bindung festgestellt werden (Lopes et al. 2001). Eine verstärkte NirS-Bildung in Stamm EbN1 könnte daher einen Mechanismus darstellen, eine Lösungsmittel-bedingte Inaktivierung von NirS zu kompensieren.

3.3 Identifizierung eines potentiellen Efflux-Systems für Alkylbenzole und (Alkyl)Phenole

Bakterien haben im Laufe der Evolution verschiedene Mechanismen zur Detoxifizierung giftiger Verbindungen entwickelt. Einen dieser Mechanismen stellen die so genannten multidrug resistance (MDR) Efflux-Systeme dar. Sie katalysieren den aktiven Ausstoß vieler strukturell und funktionell verwandter Verbindungen aus dem bakteriellen Cytoplasma ins externe Medium (Ramos et al. 2002). Es wird angenommen, dass die physikalischen Eigenschaften der zu exportierenden Verbindungen (z.B. Ladung oder Hydrophobizität), van der Waals Interaktionen mit den aktiven Zentren und der Effektor-Bindestelle sowie die Flexibilität dieser Interaktionsstellen die Spezifität dieser Efflux-Systeme bestimmen (Neyfakh 2001). Vier Haupt-Familien von MDR Transportern wurden in Bakterien identifiziert: (i) major facilitator superfamily (MFS), (ii) small multidrug resistance elements, (iii) ATP-binding cassette Pumpen und (iv) Pumpen der resistance-nodulation-cell division (RND) Familie (Ramos et al. 2002). Alle bisher bekannten Efflux-Pumpen Gram-negativer Bakterien gehören zur RND Familie (Tabelle 1).

Tabelle 1: RND-Efflux-Pumpen zum aktiven Export von Lösungsmitteln. Die Größen der jeweiligen Proteine in Aminosäuren sind in Klammern angegeben (Ramos et al. 2002).

Efflux-Pumpe	Akzessorisches Protein	Kanal durch äußere Membran	Bakterium
AcrB (1049)	AcrA (397)	TolC (495)	*E. coli*
AcrF (1034)	AcrE (385)	TolC (495)	*E. coli*
MexB (1046)	MexA (383)	OprM (485)	*P. aeruginosa*
TtgB (1050)	TtgA (384)	TtgC (484)	*P. putida*
TtgE (1048)	TtgD (382)	TtgF (480)	*P. putida*
TtgH (1049)	TtgG (391)	TtgI (470)	*P. putida*
SrpB (1049)	SrpA (382)	SrpC (470)	*P. putida*
ArpB (1050)	ArpA (371)	ArpC (484)	*P. putida*
EbA327 (799)	EbA332 (454)	EbA335 (561)	"*A. aromaticum*" EbN1
EbA1928 (799)	EbA1932 (454)	EbA1936 (561)	"*A. aromaticum*" EbN1
EbA5763 (807)	EbA5766 (450)	EbA5768 (561)	"*A. aromaticum*" EbN1

Die RND Pumpen Gram-negativer Bakterien exportieren toxische Substanzen sowohl über die Cytoplasma- als auch die äußere Membran in einem einzigen, Energie-abhängigen Schritt. Diese Efflux-Systeme bestehen aus drei Komponenten: (i) einer Cytoplasmamembran durchspannenden Efflux-Pumpe, (ii) einem Kanal durch die äußere Membran und (iii) einem periplasmatischen akzessorischen Protein, welches die Interaktion der beiden anderen Proteine vermittelt (Abb. 11B). Die Beteiligung von Efflux-Systemen der RND-Familie an der Lösungsmittel-Toleranz (z.B. gegenüber *n*-Hexan oder Toluol) ist für mehrere Gram-negative Bakterien bekannt, vor allem *E. coli* und *Pseudomonas* spp. (Ramos et al. 2002; Yu et al. 2003). Das AcrAB-TolC Efflux-System aus *E. coli* und *Pseudomonas* spp. ist gut verstanden. Es besteht aus der membranständigen Efflux-Pumpe (AcrB), dem Kanal durch die äußere Membran (TolC) und einem weiteren Protein (AcrA), welches die AcrB-TolC Interaktion vermittelt (Abb. 11B; Ramos et al. 2002; Piddock 2006). Es wird angenommen, dass die membranständige Efflux-Pumpe AcrB das Substrat entweder aus dem Cytoplasma oder direkt aus der inneren Membran aufnimmt. Die Exkretion durch den Kanal TolC in der äußeren Membran wird dabei durch das Protonenpotential (Proton motive force) getrieben (Piddock 2006).

Während des anaeroben Wachstums von Stamm EbN1 mit *p*-Ethylphenol und *p*-Hydroxyacetophenon wurden Proteine unbekannter Funktion spezifisch gebildet (EbA329, EbA332 und EbA335 mit bis zu 259-fach erhöhter Abundanz), die Teile eines neuartigen Lösungsmittel-spezifischen Efflux-Systems darstellen könnten. Die korrespondierenden Gene sind zusammen mit einem weiteren hypothetischen Protein (EbA326) und einem möglichen Transport-Protein (EbA327) in einer Operon-ähnlichen Struktur 2,5 kb upstream der Gene des anaeroben *p*-Ethylphenol Abbauwegs kodiert (Abb. 10). EbA326 weist Domänen eines cytoplasmatischen universellen Stressproteins (UpsA) von *E. coli* auf (Mulder et al. 2007). In *E. coli* wird die *upsA* Expression verstärkt, wenn die Zellen längere Zeit Stress-Faktoren (z.B. Hitzeschock oder H_2O_2) ausgesetzt sind (Nyström und Neidhardt 1994). Dies unterstützt eine Lösungsmittelstress bezogene Funktion von EbA326 in Stamm EbN1. Die Aminosäuresequenz von EbA327 weist zwei Transmembran- und eine Sensor-Domäne auf, wie sie charakteristisch für die Efflux-Pumpe AcrB der RND-Familie von *E. coli* sind (Mulder et al. 2007).

Interessanter Weise besitzen die Sequenzen der hypothetischen Proteine EbA332 und EbA335 Signalsequenzen für eine periplasmatische Lokalisierung (Emanuelsson et al. 2007). Obwohl die Aminosäuresequenzen beider Proteine keine signifikante Ähnlichkeit zu AcrA bzw. TolC aufweisen, sind Sie jedoch ähnlich hinsichtlich der Molekulargrösse (Tabelle 1).

Darüber hinaus deutet die Vorhersage der Sekundärstruktur von EbA335 auf die Bildung zahlreicher Betafaltblätter und "Turn"-Regionen hin, wie sie charakteristisch für Porine der äußeren Membran sind (Delcour 2002). Die spezifische Bildung dieser Proteine in Gegenwart aromatischer Lösungsmittel sowie die chromosomale Co-Lokalisierung mit einem Stress Protein sind erste Hinweise dafür, dass EbA327, EbA332 und EbA335 ein bisher uncharakterisiertes, Lösungsmittel-spezifisches Efflux-System darstellen könnten, welches analog zum AcrAB-TolC System von *E. coli* und *Pseudomonas* spp. ist.

Bemerkenswerter Weise enthält das Genom von Stamm EbN1 zwei weitere Gen-Cluster, die eine sehr hohe Aminosäuresequenz Identität mit EbA326-335 aufweisen (76-97 %) und exakt die selbe Gen-Anordnung aufweisen (Abb. 6 und S3 in Publikation 3; Rabus et al. 2005). Darüber hinaus liegen diese beiden Gen-Cluster, ebenso wie *ebA326-335*, in direkter chromosomaler Nachbarschaft von Genen anaerober Aromaten-Abbauwege. Das erste Cluster (*ebdA1926-1936*) ist neben den Genen des anaeroben Toluol-Abbaus lokalisiert, während das zweite Cluster (*ebA5762-5768*) in der Nähe einer paralogen Phenylphosphat Synthase des anaeroben Phenol-Abbaus liegt (Rabus et al. 2005). Die korrespondierenden Genprodukte aller drei Gen-Cluster werden spezifisch gebildet, wenn das Substrat der in Nachbarschaft kodierten Abbauwege zugegen ist, d.h. *p*-Ethylphenol/*p*-Hydroxyacetophenon, Toluol und Phenol/*p*-Cresol. Die Bildung von EbA5762-5768 wurde auch beobachtet, wenn Succinat-verwertende Zellen plötzlich mit phenolischen Verbindungen geschockt wurden. Diese analogen regulatorischen Muster unterstützen somit die vermutete Lösungmittel-bezogene Funktion dieser Proteine und lassen auf ein fein abgestimmtes und Substrat-spezifisches System der Lösungsmittel-Toleranz in Stamm EbN1 vermuten.

An dieser Stelle sollte angemerkt werden, dass sehr ähnliche Gen-Cluster in den Genomen des Pflanzen-assoziierten *Azoarcus* sp. BH72 (42-80 % Aminosäuresequenz Identität; Krause et al. 2006), des anaeroben Aromaten-Abbauers *T. aromatica* K172 (80-88 % Aminosäuresequenz Identität; Leuthner et al. 1998) und des aeroben Aromaten-Abbauers *Pseudomonas putida* KT2440 (42-64 % Aminosäuresequenz Identität; Nelson et al. 2003) vorhanden sind. Im Falle von *Azoarcus* sp. BH72 und *T. aromatica* K172 sind diese Gene ebenfalls in der Nähe von Genen für katabole Wege aromatischer Verbindungen (Phenol bzw. Toluol) lokalisiert, was eine Lösungsmittel-Stress bezogene Funktion dieses Gen-Clusters zusätzlich unterstützt.

4. Genetische Manipulierbarkeit von Stamm EbN1

Auf Basis des vollständig sequenzierten Genoms von "*A. aromaticum*" EbN1 wurden die physiologischen Funktionen vieler Gene mittels vergleichender Genom-Analyse vorhergesagt (Rabus et al. 2005). Wie in allen bisher untersuchten Genomen wurden aber auch viele hypothetische Proteine gefunden, für die keine verlässliche funktionelle Zuweisung *in silico* gemacht werden konnte. Eine der post-genomischen Herausforderungen besteht nun darin die Genom-basierten Daten mit Hilfe klassischer Methoden (z.B. Physiologie, Biochemie oder molekulare Genetik) in den physiologischen Gesamtzusammenhang zu stellen. Die Bildung von Proteinen unbekannter Funktion unter distinkten Wachstumsbedingungen erlaubt eine erste funktionelle Einordnung. Für eine abschließende Funktionsbeschreibung ist jedoch ein genetisch-biochemischer Ansatz notwendig.

Stamm EbN1 hat aufgrund der umfangreichen genomisch/proteomischen Datenlage das Potential ein wichtiger Modellorganismus für die Forschung mit der "*Aromatoleum*"/*Azoarcus*/*Thauera*-Gruppe zu werden. Um diesem Potential gerecht zu werden, wurde im Rahmen der vorliegenden Arbeit ein genetisches System für Stamm EbN1 entwickelt. Die genetische Zugänglichkeit der anaeroben Aromaten-Abbauer *A. evansii* (Gescher et al. 2002; Rost et al. 2002), *Azoarcus* sp. Stamm T (Achong et al. 2001) und *T. aromatica* Stamm T1 (Coschigano 2002) wurde bereits beschrieben.

4.1 Etablierung von Techniken zur Kultivierung auf festem Medium und Generierung einer Streptomycin-resistenten Mutante (Stamm EbN1-SR7)

Die Grundvoraussetzung für molekulargenetische Experimente ist die Möglichkeit genetisch modifizierte Einzelkolonien auf festem Medium zu isolieren. Zu Beginn der vorliegenden Arbeit konnte Stamm EbN1 nur nach langer Inkubation (>14 Tage) zu geringem Wachstum (d.h. sehr kleine Koloniegröße) auf verfestigtem Mineralmedium gebracht werden. Stamm EbN1 ist nicht in der Lage, auf Standard Festmedien (z.B. LB- oder BHI-Agar) zu wachsen (sowohl aerob als auch anaerob). Ebenso kann das feste Medium, welches für den phylogenetisch verwandten *Azoarcus* sp. BH72 beschrieben wurde (Reinhold-Hurek et al. 1993a), nicht zum Wachstum für Stamm EbN1 genutzt werden.

Im Rahmen dieser Arbeit war es möglich eine Methode zur schnellen und effizienten Kultivierung von Stamm EbN1 auf festem Mineralmedium zu etablieren. Optimales Wachstum wurde mit einem Phosphat-gepufferten Mineralmedium und Inkubation unter einer N_2-Atmosphäre (im Anaerobentopf) erzielt. Das Festmedium enthielt, im Vergleich zum

flüssigen Medium, 4- bis 5-fach erhöhte Nitrat- und Ammoniumkonzentrationen (Endkonzentration je 30 mM), um eine Limitierung der reduzierten Stickstoffquelle sowie des Elektronenakzeptors (bei anaeroben Wachstum) zu vermeiden. Als gut verwertbare organische Substrate wurden Acetat (5 mM), Pyruvat (5 mM) und Benzoat (4 mM) angeboten. Mit diesem neu entwickelten Medium konnten bereits nach 5-7 Tagen Bakterien-Kolonien mit einem Durchmesser von 1-2 mm erzielt werden. Gleichartige Kolonien bildeten sich unter oxischen Bedingungen erst nach 9-10 Tagen. Um die Plattierungseffizienz zu bestimmen, wurde eine flüssig Verdünnungsreihe (ausgehend von $1{,}1 \times 10^9$ Zellen/ml) auf dem neu entwickelten Festmedium ausplattiert. Die Bildung von $1{,}5\ (\pm 0{,}2) \times 10^8$ CFU/ml belegte eine relativ hohe Plattierungseffizienz.

Das Wachstum von Stamm EbN1 wurde von einer Vielzahl Antibiotika sowohl in flüssigem wie festem Medium inhibiert, z.B. Ampicillin, Gentamycin, Kanamycin oder Streptomycin. Dies ermöglicht den Einsatz einer großen Bandbreite von Antibiotika-Resistenz-Markern zur Selektion auf genetische Varianten.

Um genetisch modifizierter Zellen von Stamm EbN1 gegenüber Donor-Stämmen (z.B. *E. coli*) selektieren zu können, wurde eine Streptomycin-resistente Mutante erzeugt (Stamm EbN1-SR7). In der Gegenwart von Streptomycin zeigte Stamm EbN1-SR7 im Vergleich zum Wildtyp etwas geringere Wachstumsraten und maximale optische Dichten. Die Verdopplungszeiten während des anaeroben Wachstums mit Benzoat waren jedoch sehr ähnlich (3,3 h für Stamm EbN-SR7 bzw. 3,0 h für den Wildtyp). Umfangreiche Wachstumstests zeigten, dass Stamm EbN1-SR7 dieselben Substrate wie der Wildtyp verwerten kann (Tabelle 1 in Publikation 4). Ebenso konnten in einer proteomischen Vergleichsanalyse (Silber-gefärbte 2DE Gele; pH-Bereich 3-10) nur weniger als 10 unterschiedliche Proteinspots (aus >1000 im Gel getrennten) nachgewiesen werden. Diese sind sehr wahrscheinlich der Streptomycin-Resistenz zuzuordnen. Insgesamt konnte gezeigt werden, dass Stamm EbN1-SR7 sich in seinen zentralen physiologischen Eigenschaften offenbar nicht vom Wildtyp unterscheidet und somit gut für genetische Experimente geeignet ist.

4.2 Etablierung von Techniken zum Transfer extrachromosomaler DNA mittels Konjugation für Stamm EbN1-SR7

Während der Konjugation können durch direkten Zellkontakt Plasmide oder auch chromosomale DNA übertragen werden. Da es sich um einen replikativen Vorgang handelt,

besitzen beide Zelltypen (Donor und Rezipient) nach Abschluss der Konjugation eine Kopie der übertragenen DNA. Essentiell für diesen Vorgang sind die Gene der *tra*-Funktion und der *oriT*. Letzterer stellt den Replikationsstartpunkt der zu übertragenden DNA-Kopie dar. Die "normale" Replikation startet hingegen am *oriV*. Die Gene der *tra*-Region lassen sich in 2 Gruppen unterteilen: Erstens, Gene, die zur Synthese spezieller Oberflächenstrukturen (dem Pilus) notwendig sind und zweitens, die *mob*-Region, welche die *Dtr*-Funktionen (DNA transfer and replication) zur Mobilisierung des Plasmids enthält. Plasmide, denen die *tra*-Region teilweise oder vollständig fehlt, können nur durch Zellen übertragen werden, die über die fehlenden Funktionen auf einem weiteren (Helfer-) Plasmid oder im Genom verfügen. Das in dieser Arbeit etablierte genetische System für Stamm EbN1 ist in Abb. 12 zusammenfassend dargestellt.

Die Konstruktion von Plasmidvektoren in *E. coli* erfordert Protokolle zum späteren Transfer des Vektor-Konstrukts in Stamm EbN1-SR7. Der Transfer nicht selbstübertragbarer Sicherheitsvektoren wurde mit *E. coli* S17-1 und dem Broad-Hoast-Range Plasmid pBBR1-MCS4, welches eine Ampicillin-Resistenz trägt, etabliert. Das Chromosom von *E. coli* S17-1 enthält die für den Transfer erforderlichen *tra*-Gene und ermöglicht so den Transfer des Plasmids auf Stamm EbN1-SR7. Verschiedene Konjugationsbedingungen mit unterschiedlichen Donor:Rezipient Verhältnissen und verschiedenen "Paarungs-Zeiten" (mating times) wurden getestet. Die besten Ergebnisse ergab ein Donor:Rezipient-Verhältnis von 1:3 und einer "Paarung" über Nacht. Unter diesen Bedingungen konnte eine Transformations-Effizienz von $1{,}3 \times 10^{-5}$ erzielt werden. Ähnliche Transformations-Effizienzen beim Plasmid-Transfer unter Nutzung des Donor-Stamms *E. coli* S17-1 wurden für die Proteobakterien *Allochromatium vinosum* ($3{,}6 \times 10^{-4}$) und *Bordetella henselae* (2×10^{-5}) beschrieben (Pattaragulwanit und Dahl 1995; Dehio und Meyer 1997).

4.3 Unmarkierte Gen-Deletion mittels homologer Rekombination am Beispiel der γ-Untereinheit einer paralogen Ethylbenzol Dehydrogenase (*ebdC2*)

Das Chromosom von Stamm EbN1 kodiert das erste Enzym des anaeroben Ethylbenzol-Abbaus, die Ethylbenzol Dehydrogenase (*ebdABCD*), in einer Operon-ähnlichen Struktur bei ~1,4 Mb (Rabus et al. 2002). Interessanter Weise enthält das Chromosom darüber hinaus noch Gene einer paralogen Ethylbenzol Dehydrogenase (*ebdABCD2*), welche an einer anderen Stelle des Chromosoms (bei ~3,4 Mb) lokalisiert sind (Rabus et al. 2005). Während die EbdABCD Proteine spezifisch während des anaeroben Wachstums mit Ethylbenzol

gebildet werden, trifft dies nicht auf die paralogen EbdABCD2-Proteine zu (Kühner et al. 2005). In dieser Studie konnte erstmals ihre Bildung während des anaeroben Wachstums mit *p*-Cresol beobachtet werden, obwohl sie sehr wahrscheinlich nicht am *p*-Cresol Abbauweg beteiligt sind. Die physiologische Funktion der paralogen Ethylbenzol Dehydrogenase (EbdABCD2) ist bisher unklar.

Die unmarkierte Deletion der γ-Untereinheit der paralogen Ethylbenzol Dehydrogenase (*ebdC2*) mittels homologer Rekombination in Stamm EbN1-SR7 sollte die Funktionalität des genetischen Systems belegen. Darüber hinaus sollte auf diese Weise nach ersten Hinweisen auf eine potentielle Funktion gesucht werden. Der gewählte Suizid-Vektor pK19mobsacB trägt eine Kanamycin Resistenz (Schäfer et al. 1994) und ist nicht in der Lage, in Stamm EbN1-SR7 selbständig zu replizieren. Im Gegensatz zu Broad-Host-Range Plasmiden, können derartige Suizid-Vektoren (oder "Narrow-Host-Range" Vektoren) nur in den Rezipienten-Zellen verbleiben, wenn sie in dessen Genom integrieren. Anhand des im Vektor kodierten Markers (hier eine Kanamycin-Resistenz) kann auf Zellen mit integriertem Vektor selektiert werden. Daher sind Suizid-Vektoren für die gerichtete Mutagenese durch homologe Rekombination geeignet. Um ein Gen zu deletieren oder mittels Insertions-Mutagenese zu inaktivieren, ist ein so genanntes Doppel-Crossover notwendig. Hierzu müssen zunächst die flankierenden Bereiche des zu inaktivierenden Gens in den Suizid-Vektor kloniert werden. Nach dessen Integration in das Genom (Single-Crossover) muss auf ein Doppel-Crossover selektiert werden, bei dem das generierte Konstrukt das Wildtypallel ersetzt. Um ein solches Screening zu vereinfachen, trägt der gewählte Suizid-Vektor das *sacB*-Gen, welches die Levansucrase kodiert. In Gegenwart von Saccharose bildet dieses Enzym das Polymer Levan, welches toxisch auf die meisten Bakterien wirkt. Durch den Einsatz Saccharose-haltigen Mediums, können somit Zellen selektiert werden, die nach Verlust des Vektors entweder den gewünschten Genotyp (d.h. die Deletion) aufweisen oder bei denen der Wildtyp-Genotyp wieder hergestellt wurde.

Um eine Integration in das Chromosom von Stamm EbN1-SR7 zu ermöglichen, wurden die Kopien der *ebdC2*-flankierenden Gene *ebdB2* und *ebdD2* in den pK19mobsacB Vektor kloniert. Das in *E. coli* S17-1 generierte Konstrukt enthielt anstatt eines kompletten *ebdC2*-Gens lediglich das Start- und Stop-Codon von *ebdC2*, welche durch eine XbaI-Schnittstelle getrennt waren (Abb. 12 sowie Abb. 3 in Publikation 4). Nach dem konjugativen Transfer des Konstruktes (Plasmid pK19ebdB2D2) in Stamm EbN1-SR7 konnten Kanamycin-resistente Klone erhalten werden, die das Konstrukt ins Chromosom integriert hatten (Single-Crossover). Eine anschließende Kultivierung in Kanamycin-freiem Medium

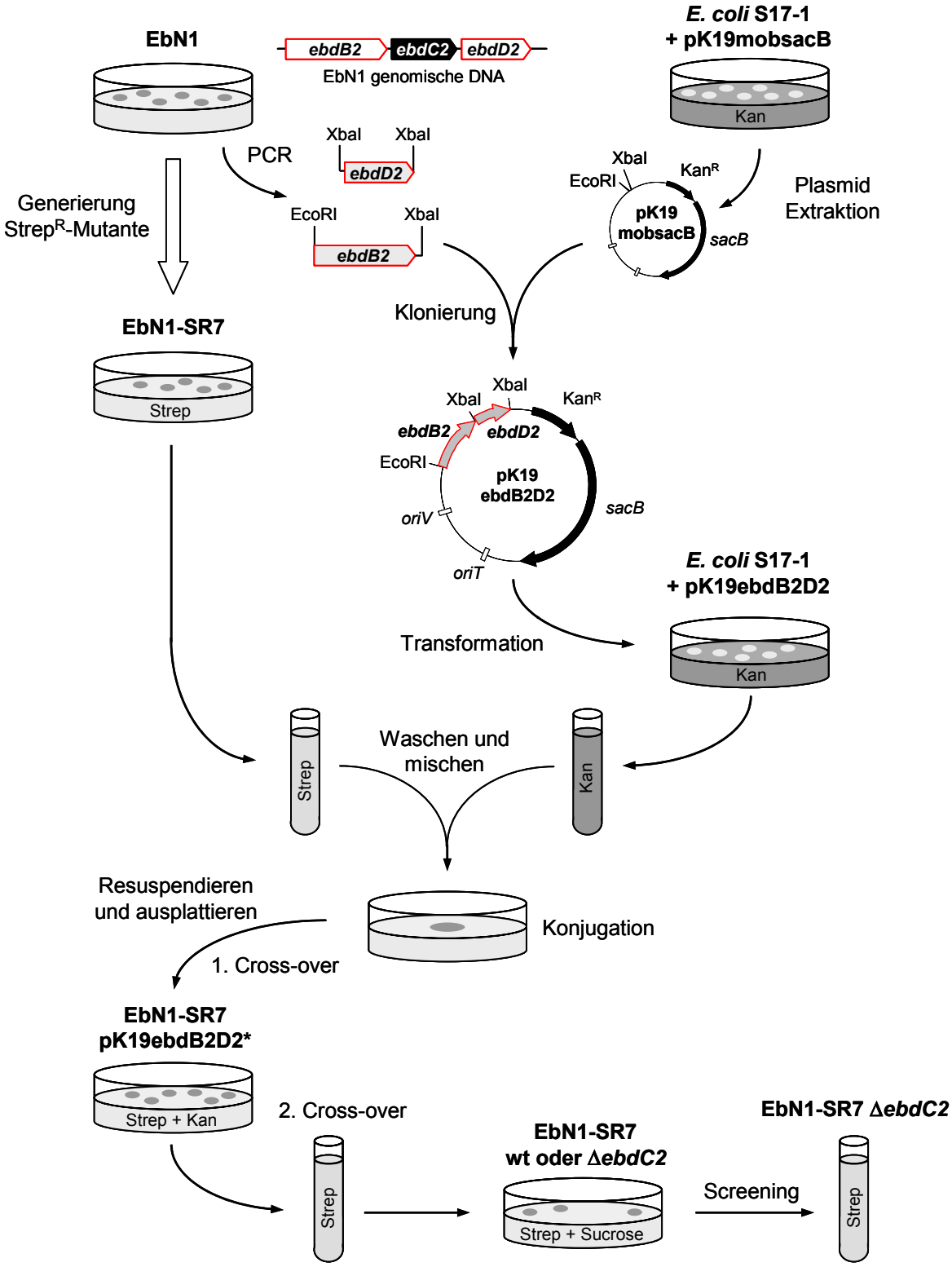


Abb. 12: Funktionsweise des genetischen Systems für Stamm EbN1. Besondere Bestandteile des Mineralmediums (Hellgrau) oder des Vollmediums (Dunkelgrau) sind angegeben. Die *ebdC2* flankierenden Gene *ebdB2* und *ebdD2* wurden mittels PCR amplifiziert, dabei mit Restriktions-Schnittstellen versehen, und in den pK19mobsacB Vektor kloniert. Dieser wurde mittels Konjugation in Stamm EbN1-SR7 übertragen und anschließend auf den Δ*ebdC2* Genotyp selektiert. Kan, Kanamycin; Strep, Streptomycin. (*) Integriert in das Chromosom von Stamm EbN1-SR7.

erlaubte genetische Rekombination (zweites Crossover) ohne Selektionsdruck. Das Screening von Kolonien, die auf Saccharose-haltigem Medium gewachsen waren zeigte, dass das zweite Crossover ein seltenes Ereignis war. Weniger als 4 % der untersuchten Kolonien wiesen eine Deletion des *ebdC2*-Gens auf. Die Deletion des Gens in der generierten Mutante Stamm EbN1-SR7 Δ*ebdC2* konnte mittels PCR und Sequenzierung der PCR-Produkte zweifelsfrei nachgewiesen werden (Abb. 3 in Publikation 4).

Expressionsanalysen von *ebdB2*, *ebdC2* und *ebdD2* in Wildtyp-Zellen von Stamm EbN1 zeigten, dass die Gene der paralogen Ethylbenzol Dehydrogenase während des anaeroben Wachstums mit *p*-Cresol abgelesen werden. In der EbN1-SR7 Δ*ebdC2* Mutante hingegen, werden nur Transkripte von *ebdB2* und *ebdD2* gebildet, was die korrekte (in frame) *ebdC2*-Deletion bestätigt. Die Transkription von *ebdD2* zeigt, dass die generierte Deletion keinen schwerwiegenden Einfluss auf die Transkription von Genen hat, die downstream des deletierten *ebdC2* liegen und wahrscheinlich in einem polycistronischen Transkript abgelesen werden. Ein neuer Phänotyp wäre somit auf die Deletion von *ebdC2* zurückzuführen und nicht auf eine deutlich veränderte Expression anderer Gene.

Für die phenotypische Charakterisierung war eine Komplementation der EbN1-SR7 Δ*ebdC2* Mutante notwendig. Bei der Komplementation wird das inaktivierte oder deletierte Gen in seiner "Wildtyp-Form" wieder in die Mutante eingebracht, um den ursprünglichen Phänotyp wiederherzustellen. In dieser Arbeit wurde eine Komplementation *in trans* durchgeführt, d.h. das deletierte Gen (*ebdC2*) wurde mit Hilfe eines Broad-Host-Range Vektors (hier das Plasmid pBBR1-MCS4 *ebdC2*) in die Zellen eingebracht und exprimiert. Die Expression ist konstitutiv, d.h. unabhängig von den Wachstumsbedingungen. Während die Bildung von EbdC2 im Wildtyp bisher lediglich während des anaeroben Wachstums mit *p*-Cresol beobachtet wurde, wird das heterolog exprimierte EbdC2 unter allen Bedingungen gebildet. Entsprechend war es möglich, in der komplementierten EbN1-SR7 Δ*ebdC2* Mutante auch während des anaeroben Wachstums mit Benzoat ein *ebdC2*-Transkript nachzuweisen. Die anderen Gene des *ebd2*-Clusters wurden, genau wie im Wildtyp, unter diesen Bedingungen nicht exprimiert wurden.

4.4 Potentielle physiologische Funktion der paralogen Ethylbenzol Dehydrogenase

Interessanter Weise hatte die Deletion der γ-Untereinheit der paralogen Ethylbenzol Dehydrogenase keinen nachweisbaren Phänotyp zur Folge. Die generierte Mutante war in der Lage, mit allen bekannten Wachstumssubstraten des Wildtyps zu wachsen (siehe Tabelle 1 in Publikation 4; Rabus und Widdel 1995). Dabei war das Wachstumsverhalten der Mutante nicht signifikant anders als das von Stamm EbN1-SR7. So war beispielsweise die Verdopplungszeit während des anaeroben Wachstums mit Ethylbenzol fast identisch: 5,18 h für Stamm EbN1-SR7 und 5,10 h für Stamm EbN1-SR7 Δ*ebdC2*. Ebenso konnte für die komplementierte Mutante kein distinkter Phänotyp festgestellt werden.

Die hohe Aminosäuresequenz Identität der Ethylbenzol Dehydrogenase und seines Paralogs (52-83 %; Rabus 2005b) ließ vermuten, dass ein potentielles Substrat von EbdABC2 chemisch dem Ethylbenzol ähnlich sein müsste. Tatsächlich konnte in dieser Arbeit *p*-Ethylphenol als neues anaerobes Wachstumssubstrat festgestellt werden. Da die Δ*ebdC2* Mutante sowohl mit Ethylbenzol als auch mit *p*-Ethylphenol wuchs, wurden umfangreiche Wachstumstests mit verschiedenen, strukturell dem Ethylbenzol ähnlichen aromatischen Verbindungen wurden durchgeführt. Allerdings unterstütze keine der folgenden Verbindungen das anaerobe Wachstum von Stamm EbN1: Ethyltoluole, Ethylanilin, Styrol, Xylole, Fluor-Toluole und Propylphenole sowie das heterocyclische Ethylpyridin und die Cyclopentadiene Ethylfuran und Ethylthiophen (siehe Tabelle 1 in Publikation 4). Die physiologische Funktion des paralogen Enzyms bleibt somit weiterhin unklar.

5. Ausblick

In der vorliegenden Arbeit konnte die differentielle Regulation von Abbauwegen aromatischer und aliphatischer Verbindungen unter oxischen und anoxischen Bedingungen umfassend auf physiologisch/proteomischer Ebene nachgewiesen werden. Dabei wurden lösliche Subproteome von Zellen untersucht, die an das Wachstum mit einzelnen Substraten angepasst waren. In weiterführenden Studien wäre es von Interesse zu untersuchen, welchen Effekt die gleichzeitige Präsenz mehrer Substrate auf die Ausprägung der jeweiligen Substrat-spezifischen Abbauleistungen hat.

Die umfassenden proteomischen Untersuchungen weisen darauf hin, dass in Stamm EbN1 übergeordnete Regulationssysteme existieren, die die Genexpression in Abhängigkeit

der Redox-Bedingungen kontrollieren. Im Genom von Stamm EbN1 wurden respiratorische Komplexe mit hoher und niedriger Sauerstoff-Affinität annotiert, was auf eine Unterscheidung zwischen aeroben und microaerophilen Bedingungen hinweisen könnte. Es wäre daher von Interesse zu untersuchen, ob es eine derartige Unterscheidung gibt, und wenn ja in welchem Ausmaß. Eine Vorraussetzung hierzu wäre allerdings zunächst die Kultivierung im Chemostat. In diesem Zusammenhang wäre anzumerken, dass eine Analyse des Membran-Proteoms weitere Aufschlüsse über die Regulation von Proteinen der Atmungskette oder auch spezifischen Transport-Proteinen ermöglichen würde. Mittlerweile ist es möglich auch Proteine mit mehreren Transmembrandomänen in einer besonderen Variante der zwei-dimensionalen Gelelektrophorese aufzutrennen (Braun et al. 2007).

Im Rahmen dieser Arbeit konnte *p*-Ethylphenol als anaerobes Wachstumssubstrat von Stamm EbN1 identifiziert und der Abbauweg sowie beteiligte Enzyme vorgeschlagen werden. Es wird angenommen, dass die Biotin-abhängige Carboxylase (XccAB) die Carboxylierung des Intermediates *p*-Hydroxyacetophenon katalysiert. Dieses Enzym weist allerdings keine signifikante Ähnlichkeit zur Acetophenon Carboxylase des anaeroben Ethylbenzol Abbaus auf und verfügt wahrscheinlich über einen distinkten, Biotin-abhängigen, katalytischen Mechanismus. Ebenso ist die vorgeschlagene Thiolase, welche die thiolytische Spaltung des Carboxylierungsprodukts katalysiert, nicht ähnlich zum analogen Enzym des anaeroben Ethylbenzol Abbauwegs, weshalb ein distinktes Carboxylierungsprodukt erwartet werden kann. Daher wäre es von besonderem Interesse das Carboxylierungsprodukt von *p*-Hydroxyacetophenon zu identifizieren.

In dieser Arbeit konnte die Bildung vieler Proteine unbekannter Funktion erstmals nachgewiesen werden, in einigen Fällen mit hoher Substrat-Spezifität und mit hoher Abundanz. Obwohl diese Proteine auf diese Weise mit einem bestimmten Wachstumszustand in Verbindung gebracht werden konnten, ist ihre genaue Funktion weiterhin unklar. Eine wichtige Frage zukünftiger Forschung sollte daher die funktionelle Untersuchung dieser Proteine darstellen. Das in dieser Arbeit entwickelte genetische System ermöglicht eine gerichtete Mutagenese der jeweiligen kodierenden Gene. Sollten diese Mutanten einen interpretierbaren Phänotyp aufweisen, könnte das betreffende Protein in Stamm EbN1 überexprimiert, gereinigt und biochemisch untersucht werden. Die hohe Lösungsmittel-Toleranz von Stamm EbN1 zusammen mit der Möglichkeit der heterologen Genexpression könnte darüber hinaus genutzt werden, um Proteine von verwandten Organismen (z.B. *Thauera* spp.) zu untersuchen, falls dies mit anderen Wirtstämmen (z.B. *E. coli*) Schwierigkeiten hervorrufen sollte.

C Literatur

Abril MA, Michan C, Timmis KN, Ramos JL (1989) Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J Bacteriol 171:6782-6790

Achong GR, Rodriguez AM, Spormann AM (2001) Benzylsuccinate synthase of *Azoarcus* sp strain T: Cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization. J Bacteriol 183:6763-6770

Aeckersberg F, Rainey FA, Widdel F (1998) Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. Arch Microbiol 170:361-369

Anders HJ, Kaetzke A, Kampfer P, Ludwig W, Fuchs G (1995) Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thauera,* as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the beta subclass of the Proteobacteria. Int J Syst Bacteriol 45:327-333

Ball HA, Johnson HA, Reinhard M, Spormann AM (1996) Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J Bacteriol 178:5755-5761

Bauer CE, Elsen S, Bird TH (1999) Mechanisms for redox control of gene expression. Annu Rev Microbiol 53:495-523

Beller HR (2002) Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring *in situ* BTEX biodegradaton. Environ Sci Technol 36:2724-2728

Beller HR, Spormann AM (1997) Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. Appl Environ Microbiol 63:3729-3731

Beller HR, Spormann AM, Sharma PK, Cole JR, Reinhard M (1996) Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl Environ Microbiol 62:1188-1196

Bérne F, Cordonnier J (1995) Industrial water treatment: Refining, petrochemicals and gas processing techniques. Gulf Publishing Company, Houston, TX

Biegert T, Altenschmidt U, Eckerskorn C, Fuchs G (1993) Enzymes of anaerobic metabolism of phenolic compounds. 4-Hydroxybenzoate-CoA ligase from a denitrifying *Pseudomonas* species. Eur J Biochem 213:555-561

Biegert T, Fuchs G, Heider J (1996) Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur J Biochem 238:661-668

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519-546

Boll M (2005a) Dearomatizing benzene ring reductases. J Mol Microbiol Biotechnol 10:132-142

Boll M (2005b) Key enzymes in the anaerobic aromatic metabolism catalysing Birch-like reductions. Biochim Biophys Acta 1707:34-50

Boll M, Fuchs G, Heider J (2002) Anaerobic oxidation of aromatic compounds and hydrocarbons. Curr Opin Chem Biol 6:604-611

Bossert ID, Whited G, Gibson DT, Young LY (1989) Anaerobic oxidation of *p*-cresol mediated by a partially purified methylhydroxylase from a denitrifying bacterium. J Bacteriol 171:2956-2962

Boyd DR, Sharma ND, Allen CC (2001) Aromatic dioxygenases: molecular biocatalysis and applications. Curr Opin Biotechnol 12:564-573

Bragg JR, Prince RC, Wilkinson JB, Atlas RM (1992) Bioremediation for shoreline cleanup following the 1989 Alaskan oil spill. Exxon Co.

Braun RJ, Kinkl N, Beer M, Ueffing M (2007) Two-dimensional electrophoresis of membrane proteins. Anal Bioanal Chem 389:1033-1045

Brautbar N, Williams J, 2nd (2002) Industrial solvents and liver toxicity: risk assessment, risk factors and mechanisms. Int J Hyg Environ Health 205:479-491

Breinig S, Schiltz E, Fuchs G (2000) Genes involved in anaerobic metabolism of phenol in the bacterium *Thauera aromatica.* J Bacteriol 182:5849-5863

Breitmaier E, Jung G (1994) Organische Chemie I, 3rd edn. Georg Thieme Verlag, Stuttgart

Cases I, de Lorenzo V (2005) Promoters in the environment: transcriptional regulation in its natural context. Nat Rev Microbiol 3:105-118

Champion KM, Zengler K, Rabus R (1999) Anaerobic degradation of ethylbenzene and toluene in denitrifying strain EbN1 proceeds via independent substrate-induced pathways. J Mol Microbiol Biotechnol 1:157-164

Coschigano PW (2002) Construction and characterization of insertion/deletion mutations of the *tutF*, *tutD*, and *tutG* genes of *Thauera aromatica* strain T1. FEMS Microbiol Lett 217:37-42

Coschigano PW, Wehrman TS, Young LY (1998) Identification and analysis of genes involved in anaerobic toluene metabolism by strain T1 - putative role of a glycine free radical. Appl Environ Microbiol 64:1650-1656

Coschigano PW, Young LY (1997) Identification and sequence analysis of two regulatory genes involved in anaerobic toluene metabolism by strain T1. Appl Environ Microbiol 63:652-660

Cronan JE, Waldrop GL (2002) Multi-subunit acetyl-CoA carboxylases. Prog Lipid Res 41:407-435

Cunningham JA, Hopkins GD, Lebron CA, Reinhard M (2000) Enhanced anaerobic bioremediation of groundwater contaminated by fuel hydrocarbons at Seal Beach, California. Biodegradation 11:159-170

Darby JM, Taylor DG, Hopper DJ (1987) Hydroquinone as the ring-fission substrate in the catabolism of 4-ethylphenol and 4-hydroxyacetophenone by *Pseudomonas putida* JD1. J Gen Microbiol 133:2137-2146

de Hoog CL, Mann M (2004) Proteomics. Annu Rev Genomics Hum Genet 5:267-293

de Leeuw JW, Largenau C (1993) A review of macromolecular organic compounds that comprise living organisms and their role in kerogen, coal, and petroleum formation. In: M.H. E, S.A. M (eds) Organic geochemistry - principles and applications. Plenum Publishing Corporation, New York, pp 23-65

Dehio C, Meyer M (1997) Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. J Bacteriol 179:538-540

Delcour AH (2002) Structure and function of pore-forming beta-barrels from bacteria. J Mol Microbiol Biotechnol 4:1-10

Dolfing J, Zeyer J, Binder-Eicher P, Schwarzenbach RP (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch Microbiol 154:336-341

Duldhardt I, Nijenhuis I, Schauer F, Heipieper HJ (2007) Anaerobically grown *Thauera aromatica*, *Desulfococcus multivorans*, *Geobacter sulfurreducens* are more sensitive towards organic solvents than aerobic bacteria. Appl Microbiol Biotechnol 77:705-711

Ehrenreich P, Behrends A, Harder J, Widdel F (2000) Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. Arch Microbiol 173:58-64

Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nat 2:953-971

Evans PJ, Ling W, Goldschmidt B, Ritter ER, Young LY (1992) Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl Environ Microbiol 58:496-501

Evans PJ, Mang DT, Kim KS, Young LY (1991) Anaerobic degradation of toluene by a denitrifying bacterium. Appl Environ Microbiol 57:1139-1145

Ficker M, Krastel K, Orlicky S, Edwards E (1999) Molecular characterization of a toluene-degrading methanogenic consortium. Appl Environ Microbiol 65:5576-5585

Fischer-Romero C, Tindall BJ, Juttner F (1996) *Tolumonas auensis* gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freshwater lake. Int J Syst Bacteriol 46:183-188

Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM, et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science 269:496-512

Foss S, Harder J (1998) *Thauera linaloolentis* sp. nov. and *Thauera terpenica* sp. nov., isolated on oxygen-containing monoterpenes (linalool, menthol, and eucalyptol) and nitrate. Syst Appl Microbiol 21:365-373

Fries MR, Zhou J, Chee-Sanford J, Tiedje JM (1994) Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl Environ Microbiol 60:2802-2810

Gade D, Gobom J, Rabus R (2005a) Proteomic analysis of carbohydrate catabolism and regulation in the marine bacterium *Rhodopirellula baltica*. Proteomics 5:3672-3683

Gade D, Stührmann T, Reinhardt R, Rabus R (2005b) Growth phase dependent regulation of protein composition in *Rhodopirellula baltica*. Environ Microbiol 7:1074-1084

Gade D, Thiermann J, Markowsky D, Rabus R (2003) Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium *Pirellula* sp. strain 1. J Mol Microbiol Biotechnol 5:240-251

Galushko A, Minz D, Schink B, Widdel F (1999) Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. Environ Microbiol 1:415-420

Gescher J, Zaar A, Mohamed M, Schagger H, Fuchs G (2002) Genes coding for a new pathway of aerobic benzoate metabolism in *Azoarcus evansii*. J Bacteriol 184:6301-6315

Gibson J, Harwood CS (2002) Metabolic diversity in aromatic compound utilization by anaerobic microbes. Annu Rev Microbiol 56:345-369

Gödeke S, Richnow HH, Weiss H, Fischer A, Vogt C, Borsdorf H, Schirmer M (2006) Multi tracer test for the implementation of enhanced in-situ bioremediation at a BTEX-contaminated megasite. J Contam Hydrol 87:211-236

Görg A, Weiss W, Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics 4:3665-3685

Gralewicz S (1999) Organic solvents and time-dependent sensitization. Int J Occup Med Environ Health 12:371-381

Grbic-Galic D, Vogel TM (1987) Transformation of toluene and benzene by mixed methanogenic cultures. Appl Environ Microbiol 53:254-260

Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol 4:117

Gulensoy N, Alvarez PJ (1999) Diversity and correlation of specific aromatic hydrocarbon biodegradation capabilities. Biodegradation 10:331-340

Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19:1720-1730

Hagedoorn PL, Chen T, Schroder I, Piersma SR, de Vries S, Hagen WR (2005) Purification and characterization of the tungsten enzyme aldehyde:ferredoxin oxidoreductase from the hyperthermophilic denitrifier *Pyrobaculum aerophilum*. J Biol Inorg Chem 10:259-269

Hammel KE (1995) Organopollutant degradation by ligninolytic fungi. In: Young LY, Cerniglia CE (eds) Microbial transformation and degradation of toxic organic chemicals. John Wiley & Sons Inc., New York, pp 331-346

Harder J, Foss S (1999) Anaerobic formation of the aromatic hydrocarbon *p*-cymene from monoterpenes by methanogenic enrichment cultures. Geomicrobiology Journal 16:295 - 305

Harms G, Zengler K, Rabus R, Aeckersberg F, Minz D, Rossello-Mora R, Widdel F (1999) Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl Environ Microbiol 65:999-1004

Harwood CS, Parales RE (1996) The beta-ketoadipate pathway and the biology of self-identity. Annu Rev Microbiol 50:553-590

Heider J (2007) Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. Curr Opin Chem Biol 11:188-194

Heider J, Boll M, Breese K, Breinig S, Ebenau-Jehle C, Feil U, Gad'on N, Laempe D, Leuthner B, Mohamed ME, Schneider S, Burchhardt G, Fuchs G (1998) Differential

induction of enzymes involved in anaerobic metabolism of aromatic compounds in the denitrifying bacterium *Thauera aromatica*. Arch Microbiol 170:120-131

Heider J, Ma K, Adams MW (1995) Purification, characterization, and metabolic function of tungsten-containing aldehyde ferredoxin oxidoreductase from the hyperthermophilic and proteolytic archaeon *Thermococcus* strain ES-1. J Bacteriol 177:4757-4764

Heipieper HJ, Neumann G, Cornelissen S, Meinhardt F (2007) Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl Microbiol Biotechnol 74:961-973

Hermann T (2003) Industrial production of amino acids by coryneform bacteria. J Biotechnol 104:155-172

Hermuth K, Leuthner B, Heider J (2002) Operon structure and expression of the genes for benzylsuccinate synthase in *Thauera aromatica* strain K172. Arch Microbiol 177:132-138

Hess A, Zarda B, Hahn D, Häner A, Stax D, Höhener P, Zeyer J (1997) *In situ* analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl Environ Microbiol 63:2136-2141

Himmelreich R, Hilbert H, Plagens H, Pirkl E, Li BC, Herrmann R (1996) Complete sequence analysis of the genome of the bacterium *Mycoplasma pneumoniae*. Nucleic Acids Res 24:4420-4449

Hongsthong A, Sirijuntarut M, Prommeenate P, Thammathorn S, Bunnag B, Cheevadhanarak S, Tanticharoen M (2007) Revealing differentially expressed proteins in two morphological forms of *Spirulina platensis* by proteomic analysis. Mol Biotechnol 36:123-130

Hopper DJ (1976) The hydroxylation of *p*-cresol and its conversion to *p*-hydroxybenzaldehyde in *Pseudomonas putida*. Biochem Biophys Res Commun 69:462-468

Hopper DJ, Bossert ID, Rhodes-Roberts ME (1991) *p*-Cresol methylhydroxylase from a denitrifying bacterium involved in anaerobic degradation of *p*-cresol. J Bacteriol 173:1298-1301

Hopper DJ, Taylor DG (1977) The purification and properties of *p*-cresol-(acceptor) oxidoreductase (hydroxylating), a flavocytochrome from *Pseudomonas putida*. Biochem J 167:155-162

Isken S, de Bont JA (1998) Bacteria tolerant to organic solvents. Extremophiles 2:229-238

Johnson HA, Pelletier DA, Spormann AM (2001) Isolation and characterization of anaerobic ethylbenzene dehydrogenase, a novel Mo-Fe-S enzyme. J Bacteriol 183:4536-4542

Kauppi B, Lee K, Carredano E, Parales RE, Gibson DT, Eklund H, Ramaswamy S (1998) Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. Structure 6:571-586

Kim YH, Cho K, Yun SH, Kim JY, Kwon KH, Yoo JS, Kim SI (2006) Analysis of aromatic catabolic pathways in *Pseudomonas putida* KT 2440 using a combined proteomic approach: 2-DE/MS and cleavable isotope-coded affinity tag analysis. Proteomics 6:1301-1318

Kloer DP, Hagel C, Heider J, Schulz GE (2006) Crystal structure of ethylbenzene dehydrogenase from *Aromatoleum aromaticum*. Structure 14:1377-1388

Kniemeyer O, Fischer T, Wilkes H, Glockner FO, Widdel F (2003) Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl Environ Microbiol 69:760-768

Kniemeyer O, Heider J (2001a) Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. J Biol Chem 276:21381-21386

Kniemeyer O, Heider J (2001b) (*S*)-1-Phenylethanol dehydrogenase of *Azoarcus* sp. strain EbN1, an enzyme of anaerobic ethylbenzene catabolism. Arch Microbiol 176:129-135

Kniemeyer O, Musat F, Sievert SM, Knittel K, Wilkes H, Blumenberg M, Michaelis W, Classen A, Bolm C, Joye SB, Widdel F (2007) Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-reducing bacteria. Nature 449:898-901

Krause A, Ramakumar A, Bartels D, Battistoni F, Bekel T, Boch J, Bohm M, Friedrich F, Hurek T, Krause L, Linke B, McHardy AC, Sarkar A, Schneiker S, Syed AA, Thauer R, Vorholter F-J, Weidner S, Puhler A, Reinhold-Hurek B, Kaiser O, Goesmann A (2006) Complete genome of the mutualistic, N_2-fixing grass endophyte *Azoarcus* sp. strain BH72. Nat biotechnol 24:1385-1391

Krieger CJ, Roseboom W, Albracht SP, Spormann AM (2001) A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus* sp. strain T. J Biol Chem 276:12924-12927

Kube M, Heider J, Amann J, Hufnagel P, Kühner S, Beck A, Reinhardt R, Rabus R (2004) Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1. Arch Microbiol 181:182-194

Kuhn EP, Colberg PJ, Schnoor JL, Wanner O, Zehnder AJP, Schwarzenbach RP (1985) Microbial transformations of substituted benzenes during infiltration of river water to groundwater: laboratory column studies. Environ Sci Technol 19:961-968

Kühner S, Wöhlbrand L, Fritz I, Wruck W, Hultschig C, Hufnagel P, Kube M, Reinhardt R, Rabus R (2005) Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1. J Bacteriol 187:1493-1503

Lack A, Fuchs G (1992) Carboxylation of phenylphosphate by phenol carboxylase, an enzyme system of anaerobic phenol metabolism. J Bacteriol 174:3629-3636

Lack A, Fuchs G (1994) Evidence that phenol phosphorylation to phenylphosphate is the first step in anaerobic phenol metabolism in a denitrifying *Pseudomonas* sp. Arch Microbiol 161:132-139

Laempe D, Jahn M, Breese K, Schagger H, Fuchs G (2001) Anaerobic metabolism of 3-hydroxybenzoate by the denitrifying bacterium *Thauera aromatica*. J Bacteriol 183:968-979

Langenhoff AAM, Nijenhuis I, Tan NCG, Briglia M, Zehnder AJB, Schraa G (1997) Characterisation of a manganese-reducing, toluene-degrading enrichment culture. FEMS Microbiol Ecol 24:113-125

Lau PC, Wang Y, Patel A, Labbe D, Bergeron H, Brousseau R, Konishi Y, Rawlings M (1997) A bacterial basic region leucine zipper histidine kinase regulating toluene degradation. Proc Natl Acad Sci U S A 94:1453-1458

Leuthner B, Heider J (1998) A two-component system involved in regulation of anaerobic toluene metabolism in *Thauera aromatica*. FEMS Microbiol Lett 166:35-41

Leuthner B, Heider J (2000) Anaerobic toluene catabolism of *Thauera aromatica*: the *bbs* operon codes for enzymes of beta oxidation of the intermediate benzylsuccinate. J Bacteriol 182:272-277

Leuthner B, Leutwein C, Schulz H, Horth P, Haehnel W, Schiltz E, Schagger H, Heider J (1998) Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol 28:615-628

Leutwein C, Heider J (2001) Succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase: an enzyme of the anaerobic toluene catabolic pathway in denitrifying bacteria. J Bacteriol 183:4288-4295

Leutwein C, Heider J (2002) (*R*)-Benzylsuccinyl-CoA dehydrogenase of *Thauera aromatica,* an enzyme of the anaerobic toluene catabolic pathway. Arch Microbiol 178:517-524

Lochmeyer C, Koch J, Fuchs G (1992) Anaerobic degradation of 2-aminobenzoic acid (anthranilic acid) via benzoyl-coenzyme A (CoA) and cyclohex-1-enecarboxyl-CoA in a denitrifying bacterium. J Bacteriol 174:3621-3628

Lopes H, Besson S, Moura I, Moura JJ (2001) Kinetics of inter- and intramolecular electron transfer of *Pseudomonas nautica* cytochrome *cd1* nitrite reductase: regulation of the NO-bound end product. J Biol Inorg Chem 6:55-62

Lovley DR (1997) Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. J Ind Microbiol Biot 18:75-81

Lovley DR, Baedecker MJ, Lonergan DJ, Cozzarelli IM, Phillips EJP, Siegel DI (1989) Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature 339:297-300

Lovley DR, Lonergan DJ (1990) Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl Environ Microbiol 56:1858-1864

Macy JM, Rech S, Auling G, Dorsch M, Stackebrandt E, Sly LI (1993) *Thauera selenatis* gen. nov., sp. nov., a member of the beta subclass of Proteobacteria with a novel type of anaerobic respiration. Int J Syst Bacteriol 43:135-142

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen Z, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer ML, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu P, Begley RF, Rothberg JM (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376-380

McIntire W, Hopper DJ, Singer TP (1985) *p*-Cresol methylhydroxylase. Assay and general properties. Biochem J 228:325-335

McKelvie JR, Lindstrom JE, Beller HR, Richmond SA, Sherwood Lollar B (2005) Analysis of anaerobic BTX biodegradation in a subarctic aquifer using isotopes and benzylsuccinates. J Contam Hydrol 81:167-186

Mechichi T, Stackebrandt E, Gad'on N, Fuchs G (2002) Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and

description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov. Arch Microbiol 178:26-35

Morett E, Segovia L (1993) The sigma54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J Bacteriol 175:6067-6074

Mosquéda G, Ramos-Gonzalez MI, Ramos JL (1999) Toluene metabolism by the solvent-tolerant *Pseudomonas putida* DOT-T1 strain, and its role in solvent impermeabilization. Gene 232:69-76

Mulder NJ, Apweiler R, Attwood TK, Bairoch A, Bateman A, Binns D, Bork P, Buillard V, Cerutti L, Copley R, Courcelle E, Das U, Daugherty L, Dibley M, Finn R, Fleischmann W, Gough J, Haft D, Hulo N, Hunter S, Kahn D, Kanapin A, Kejariwal A, Labarga A, Langendijk-Genevaux PS, Lonsdale D, Lopez R, Letunic I, Madera M, Maslen J, McAnulla C, McDowall J, Mistry J, Mitchell A, Nikolskaya AN, Orchard S, Orengo C, Petryszak R, Selengut JD, Sigrist CJA, Thomas PD, Valentin F, Wilson D, Wu CH, Yeats C (2007) New developments in the InterPro database. Nucleic Acids Res 35:D224-D228

Nair B (2001) Final report on the safety assessment of benzyl alcohol, benzoic acid, and sodium benzoate. Int J Toxicol 20 Suppl 3:23-50

Nealson KH (1997) Sediment bacteria: who's there, what are they doing, and what's new? Annu Rev Earth Planet Sci 25:403-434

Nelson KE, Weinel C, Paulsen IT, Dodson RJ, Hilbert H, Martins dos Santos VAP, Fouts DE, Gill SR, Pop M, Holmes M, Brinkac L, Beanan M, DeBoy RT, Daugherty S, Kolonay J, Madupu R, Nelson W, White O, Peterson J, Khouri H, Hance I, Lee PC, Holtzapple E, Scanlan D, Tran K, Fraser CM, et al. (2003) Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol 5:630

Neyfakh AA (2001) The ostensible paradox of multidrug recognition. J Mol Microbiol Biotechnol 3:151-154

Nyström T, Neidhardt FC (1994) Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest. Mol Microbiol 11:537-544

Pattaragulwanit K, Dahl C (1995) Developement of a genetic system for a purple sulfur bacterium: conjugative plasmid transfer in *Chromatium vinosum*. Arch Microbiol 164:217-222

Pelz O, Chatzinitas A, Andersen N, Bernasconi SM, Hesse C, Abraham WR, Zeyer J (2001) Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations. Arch Microbiol 175:270-281

Peters F, Rother M, Boll M (2004) Selenocysteine-containing proteins in anaerobic benzoate metabolism of *Desulfococcus multivorans*. J Bacteriol 186:2156-2163

Piddock LJV (2006) Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol 4:629-636

Prince RC (2002) Petroleum and other hydrocarbons, degradation of. In: Bitton G (ed) Encyclopedia of environmental microbiology. John Wiley, New York, pp 2402-2416

Rabus R (2005a) Biodegradation of hydrocarbons under anoxic conditions. In: Ollivier B, Magot M (eds) Petroleum Microbiology. ASM Press, Washington D.C.

Rabus R (2005b) Functional genomics of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Appl Microbiol Biotechnol 68:580-587

Rabus R, Heider J (1998) Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. Arch Microbiol 170:377-384

Rabus R, Kube M, Beck A, Widdel F, Reinhardt R (2002) Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EbN1. Arch Microbiol 178:506-516

Rabus R, Kube M, Heider J, Beck A, Heitmann K, Widdel F, Reinhardt R (2005) The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Arch Microbiol 183:27-36

Rabus R, Nordhaus R, Ludwig W, Widdel F (1993) Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl Environ Microbiol 59:1444-1451

Rabus R, Widdel F (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol 163:96-103

Rabus R, Widdel F (1996) Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Appl Environ Microbiol 62:1238-1241

Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, Rojas A, Teran W, Segura A (2002) Mechanisms of solvent tolerance in gram-negative bacteria. Annu Rev Microbiol 56:743-768

Ramos JL, Marques S, Timmis KN (1997) Transcriptional control of the *Pseudomonas* TOL plasmid catabolic operons is achieved through an interplay of host factors and plasmid-encoded regulators. Annu Rev Microbiol 51:341-373

Rana SV, Verma Y (2005) Biochemical toxicity of benzene. J Environ Biol 26:157-168

Rauh D, Graentzdoerffer A, Granderath K, Andreesen JR, Pich A (2004) Tungsten-containing aldehyde oxidoreductase of *Eubacterium acidaminophilum*. Eur J Biochem 271:212-219

Reeve CD, Carver MA, Hopper DJ (1990) Stereochemical aspects of the oxidation of 4-ethylphenol by the bacterial enzyme 4-ethylphenol methylenehydroxylase. Biochem J 269:815-819.

Reinhold-Hurek B, Hurek T (2000) Reassessment of the taxonomic structure of the diazotrophic genus *Azoarcus sensu lato* and description of three new genera and new species, *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus fungiphilus* gen. nov., sp. nov. Int J Syst Evol Microbiol 50:649-659

Reinhold-Hurek B, Hurek T, Claeyssens M, van Montagu M (1993a) Cloning, expression in *Escherichia coli*, and characterization of cellulytic enzymes of *Azoarcus* sp., a root-invading diazotroph. J Bacteriol 175:7056-7065

Reinhold-Hurek B, Hurek T, Gillis C, Hoste B, Van Canneyt C, Kersters K, De Ley J (1993b) *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int J Syst Bacteriol 43:574-584

Rosenberg E (2006) Hydrocarbon-oxidizing bacteria. In: The prokaryotes, 3rd edn. Springer Verlag, New York

Rost R, Haas S, Hammer E, Herrmann H, Burchhardt G (2002) Molecular analysis of aerobic phenylacetate degradation in *Azoarcus evansii*. Mol Genet Genomics 267:656-663

Roy R, Adams MW (2002) Tungsten-dependent aldehyde oxidoreductase: a new family of enzymes containing the pterin cofactor. Met Ions Biol Syst 39:673-697

Rudolphi A, Tschech A, Fuchs G (1991) Anaerobic degradation of cresols by denitrifying bacteria. Arch Microbiol 155:238-248

Rueter P, Rabus R, Wilkes H, Aeckersberg F, Rainey FA, Jannasch HW, Widdel F (1994) Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature 372:455-458

Sawers RG, Zehelein E, Bock A (1988) Two-dimensional gel electrophoretic analysis of *Escherichia coli* proteins: influence of various anaerobic growth conditions and the *fnr* gene product on cellular protein composition. Arch Microbiol 149:240-244

Schäfer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and

pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69-73

Schnell S, Bak F, Pfennig N (1989) Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. Arch Microbiol 152:556-563

Schocher RJ, Seyfried B, Vazquez F, Zeyer J (1991) Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch Microbiol 157:7-12

Schühle K, Fuchs G (2004) Phenylphosphate carboxylase: a new C-C lyase involved in anaerobic phenol metabolism in *Thauera aromatica*. J Bacteriol 186:4556-4567

Schühle K, Gescher J, Feil U, Paul M, Jahn M, Schagger H, Fuchs G (2003) Benzoate-coenzyme A ligase from *Thauera aromatica*: an enzyme acting in anaerobic and aerobic pathways. J Bacteriol 185:4920-4929

Segura A, Rojas A, Hurtado A, Huertas MJ, Ramos JL (2003) Comparative genomic analysis of solvent extrusion pumps in *Pseudomonas strains* exhibiting different degrees of solvent tolerance. Extremophiles 7:371-376

Shingler V (2003) Integrated regulation in response to aromatic compounds: from signal sensing to attractive behaviour. Environ Microbiol 5:1226-1241

Shinoda Y, Akagi J, Uchihashi Y, Hiraishi A, Yukawa H, Yurimoto H, Sakai Y, Kato N (2005) Anaerobic degradation of aromatic compounds by *Magnetospirillum* strains: isolation and degradation genes. Biosci Biotechnol Biochem 69:1483-1491

Shinoda Y, Sakai Y, Uenishi H, Uchihashi Y, Hiraishi A, Yukawa H, Yurimoto H, Kato N (2004) Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. Appl Environ Microbiol 70:1385-1392

Sikkema J, de Bont JA, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201-222

Slabas AR, Suzuki I, Murata N, Simon WJ, Hall JJ (2006) Proteomic analysis of the heat shock response in *Synechocystis* PCC6803 and a thermally tolerant knockout strain lacking the histidine kinase 34 gene. Proteomics 6:845-864

Song B, Haggblom MM, Zhou J, Tiedje JM, Palleroni NJ (1999) Taxonomic characterization of denitrifying bacteria that degrade aromatic compounds and description of *Azoarcus toluvorans* sp. nov. and *Azoarcus toluclasticus* sp. nov. Int J Syst Bacteriol 49:1129-1140

Song B, Palleroni NJ, Kerkhof LJ, Haggblom MM (2001) Characterization of halobenzoate-degrading, denitrifying *Azoarcus* and *Thauera* isolates and description of *Thauera chlorobenzoica* sp. nov. Int J Syst Evol Microbiol 51:589-602

Song B, Ward BB (2005) Genetic diversity of benzoyl coenzyme A reductase genes detected in denitrifying isolates and estuarine sediment communities. Appl Environ Microbiol 71:2036-2045

Spormann AM, Widdel F (2000) Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation 11:85-105

Springer N, Ludwig W, Philipp B, Schink B (1998) *Azoarcus anaerobius* sp. nov., a resorcinol-degrading, strictly anaerobic, denitrifying bacterium. Int J Syst Bacteriol 48 Pt 3:953-956

Szaleniec M, Hagel C, Menke M, Nowak P, Witko M, Heider J (2007) Kinetics and mechanism of oxygen-independent hydrocarbon hydroxylation by ethylbenzene dehydrogenase. Biochemistry 46:7637-7646

Tanner A, Hopper DJ (2000) Conversion of 4-hydroxyacetophenone into 4-phenyl acetate by a flavin adenine dinucleotide-containing Baeyer-Villiger-type monooxygenase. J Bacteriol 182:6565-6569

Tissot BP, Welte DH (1984) Petroleum formation and occurence, 2nd edn. Springer-Verlag, Berlin

Treutter D (2005) Significance of flavonoids in plant resistance and enhancement of their biosynthesis. Plant Biol 7:581-591

Tropel D, van der Meer JR (2004) Bacterial transcriptional regulators for degradation pathways of aromatic compounds. Microbiol Mol Biol Rev 68:474-500

Ünlü M, Morgan ME, Minden JS (1997) Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. Electrophoresis 18:2071-2077

Velculescu VE, Zhang L, Zhou W, Vogelstein J, Basrai MA, Bassett DE, Jr., Hieter P, Vogelstein B, Kinzler KW (1997) Characterization of the yeast transcriptome. Cell 88:243-251

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66-74

Wallin E, von Heijne G (1998) Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. Protein Sci 7:1029-1038

Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I (1995) Progress with gene-product mapping of the Mollicutes: *Mycoplasma genitalium*. Electrophoresis 16:1090-1094

Widdel F, Boetius A, Rabus R (2006) Anaerobic biodegradation of hydrocarbons including methane. In: Prokaryotes, 3rd edn. Springer Verlag, New York, pp 1028-1049

Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 12:259-276

Winderl C, Schaefer S, Lueders T (2007) Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker. Environ Microbiol 9:1035-1046

Wischgoll S, Heintz D, Peters F, Erxleben A, Sarnighausen E, Reski R, Van Dorsselaer A, Boll M (2005) Gene clusters involved in anaerobic benzoate degradation of *Geobacter metallireducens*. Mol Microbiol 58:1238-1252

Wöhlbrand L, Kallerhoff B, Lange D, Hufnagel P, Thiermann J, Reinhardt R, Rabus R (2007) Functional proteomic view of metabolic regulation in "*Aromatoleum aromaticum*" strain EbN1. Proteomics 7:2222-2239.

Yu EW, Aires JR, Nikaido H (2003) AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J Bacteriol 185:5657-5664

Zengler K (1999) Mikrobielle Diversität und neuartige Fähigkeiten beim anaeroben Abbau von Kohlenwasserstoffen. Dissertation, Universität Bremen, Bremen

Zengler K, Heider J, Rossello-Mora R, Widdel F (1999) Phototrophic utilization of toluene under anoxic conditions by a new strain of *Blastochloris sulfoviridis.* Arch Microbiol 172:204-212

Zeyer J, Kuhn EP, Schwarzenbach RP (1986) Rapid microbial mineralization of toluene and 1,3-dimethylbenzene in the absence of molecular oxygen. Appl Environ Microbiol 52:944-947

Zhou J, Fries MR, Chee-Sanford JC, Tiedje JM (1995) Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth of toluene and description of *Azoarcus tolulyticus* sp. nov. Int J Syst Bacteriol 45:500-506

Zieske LR (2006) A perspective on the use of iTRAQ reagent technology for protein complex and profiling studies. J Exp Bot 57:1501-1508

Teil II: Publikationen

A Publikationsliste mit Erläuterungen

Die Dissertation beruht zum großen Teil auf den folgenden fünf Publikationen. Die angefügten Erläuterungen zeigen meinen Beitrag an der jeweiligen Arbeit auf.

1. Functional proteomic view of metabolic regulation in "*Aromatoleum aromaticum*" strain EbN1

Lars Wöhlbrand, Birte Kallerhoff, Daniela Lange, Peter Hufnagel, Jürgen Thiermann, Richard Reinhardt und Ralf Rabus

Proteomics (2007) **7** (13): 2222-39

Mitwirkung an der Vesuchsplanung, Durchführung der zweidimensionalen Gelelektrophorese (2D DIGE) und Auswertung der differentiellen Proteomdaten. Erstellung des Manuskripts sowie der umfangreichen weiterführenden Information (Supplementary Material) in Zusammenarbeit mit Ralf Rabus.

2. Solvent stress response of the denitrifying bacterium "*Aromatoleum aromaticum*" strain EbN1.

Kathleen Trautwein, Simon Kühner, Lars Wöhlbrand, Thomas Halder, Kenny Kuchta, Alexander Steinbüchel und Ralf Rabus

Applied and Environmental Microbiology (2008) **74** (8): 2267-2274

Mitwirkung an der Versuchsplanung der Schock-Experimente mit Phenol, p-*Cresol und einem Lösungmittelgemisch. Durchführung der physiologischen Experimente mit Phenol,* p-*Cresol und dem Lösungsmittelgemisch sowie der chromatographischen Analysen (nur Phenol und* p-*Cresol) zusammen mit Jasmin Palfi und Kathleen Trautwein. Redaktionelle Mitarbeit am Manuskript mit Kathleen Trautwein und Ralf Rabus.*

3. Anaerobic degradation of *p*-ethylphenol by "*Aromatoleum aromaticum*" strain EbN1: pathway, involved proteins and regulation.

Lars Wöhlbrand, Heinz Wilkes, Thomas Halder und Ralf Rabus

Zur Veröffentlichung im Journal of Bacteriology eingereicht (März 2008)

Entwicklung des Konzepts in Zusammenarbeit mit Ralf Rabus, Durchführung der physiologischen Experimente und der Metabolitextraktion sowie der zweidimensionalen Gelelektrophorese und der real-time RT-PCR. Erstellung des Manuskripts zusammen mit Ralf Rabus.

4. Development of a genetic system for denitrifying "*Aromatoleum aromaticum*" Strain EbN1.

Lars Wöhlbrand und Ralf Rabus

Zur Veröffentlichung im Journal of Molecular Microbiology and Biotechnology eingereicht (März 2008)

Entwicklung des Konzepts in Zusammenarbeit mit Ralf Rabus und Durchführung aller Experimente. Erstellung des Manuskripts in Zusammenarbeit mit Ralf Rabus.

5. *Aromatoleum* gen. nov., a novel genus accommodating the phylogenetic lineage including *Azoarcus evansii* and related species, and proposal of *A. aromaticum* sp. nov., *A. petroleum* sp. nov., *A. bremensis* sp. nov., *A. toluolicum* sp. nov., and *A. diolicum* sp. nov.

Ralf Rabus, Lars Wöhlbrand, Daniela Lange, Markus Meyer, Barbara Reinhold-Hurek, Friedrich Widdel und Peter Kämpfer

Manuskript in Vorbereitung

Bestimmung der Substratspektren zusammen mit Daniela Lange und Untersuchungen zur Fähigkeit der Stickstofffixierung auf physiologischer und molekularer Ebene. Redaktionelle Mitarbeit am Manuskript.

B Publikationen

1

Functional proteomic view of metabolic regulation in *"Aromatoleum aromaticum"* strain EbN1

Lars Wöhlbrand[1], Birte Kallerhoff[1], Daniela Lange[1], Peter Hufnagel[2], Jürgen Thiermann[3], Richard Reinhardt[4] and Ralf Rabus[1,5]*

Proteomics (2007) **7**: 2222-2239

[1]Max-Planck-Institute for Marine Microbiology, Celsiusstrasse 1, 28359 Bremen, Germany

[2]Bruker Daltonik GmbH, Fahrenheitstrasse 4, 28359 Bremen, Germany

[3]GE Healthcare Europe GmbH, Oskar-Schlemmer-Str. 11, 80807 Munich, Germany

[4]Max-Planck-Institute for Molecular Genetics, Ihnestrasse 73, 14195 Berlin, Germany

[5]Institute for Chemistry and Biology of the Marine Environment (ICBM), University Oldenburg, Carl-von-Ossietzky. Str. 9-11, 26111 Oldenburg, Germany

* Corresponding author

2222 DOI 10.1002/pmic.200600987 *Proteomics* 2007, *7*, 2222–2239

RESEARCH ARTICLE

Functional proteomic view of metabolic regulation in "*Aromatoleum aromaticum*" strain EbN1

Lars Wöhlbrand[1], Birte Kallerhoff[1], Daniela Lange[1], Peter Hufnagel[2], Jürgen Thiermann[3], Richard Reinhardt[4] and Ralf Rabus[1, 5]

[1] Max-Planck-Institute for Marine Microbiology, Bremen, Germany
[2] Bruker Daltonik GmbH, Bremen, Germany
[3] GE Healthcare Europe GmbH, Munich, Germany
[4] Max-Planck-Institute for Molecular Genetics, Berlin, Germany
[5] Institute for Chemistry and Biology of the Marine Environment (ICBM), University Oldenburg, Oldenburg, Germany

The denitrifying "*Aromatoleum aromaticum*" strain EbN1 utilizes a wide range of aromatic and nonaromatic compounds under anoxic and oxic conditions. The recently determined genome revealed corresponding degradation pathways and predicted a fine-tuned regulatory network. In this study, differential proteomics (2-D DIGE and MS) was used to define degradation pathway-specific subproteomes and to determine their growth condition dependent regulation. Differential protein profiles were determined for cultures adapted to growth under 22 different substrate and redox conditions. In total, 354 different proteins were identified, 199 of which displayed significantly changed abundances. These regulated proteins mainly represented enzymes of the different degradation pathways, and revealed different degrees of growth condition specific regulation. In case of three substrate conditions (*e.g.* phenylalanine, anoxic), proteins previously predicted to be involved in their degradation were apparently not involved (*e.g.* Pdh, phenylacetaldehyde dehydrogenase). Instead, previously not considered proteins were specifically increased in abundance (*e.g.* EbA5005, predicted aldehyde:ferredoxin oxidoreductase), shedding new light on the respective pathways. Moreover, strong evidence was obtained for thus far unpredicted degradation pathways of three hitherto unknown substrates (*e.g.* *o*-aminobenzoate, anoxic). Comparing all identified regulated and nonregulated proteins provided first insights into regulatory hierarchies of special degradation pathways *versus* general metabolism in strain EbN1.

Received: December 8, 2006
Revised: February 15, 2007
Accepted: March 13, 2007

Keywords:
Anaerobic biodegradation / Aromatic compounds / Fluorescence two-dimensional difference gel electrophoresis / Functional proteomics / Mass spectrometry

1 Introduction

Aromatic compounds are widely distributed and abundant in nature. They are main constituents of crude oil (alkylbenzenes), important products of higher plants (*e.g.* phenolic compounds as building blocks of lignin), and present in the proteins of every living organism. Fuel-derived alkylbenzenes and phenolic compounds are produced in large quan-

Correspondence: Professor Dr. Ralf Rabus, Institute for Chemistry and Biology of the Marine Environment, University Oldenburg, Carl-von-Ossietzky Str. 9-11, D-26111 Oldenburg, Germany
E-mail: rabus@icbm.de
Fax: +49-441-798-3404

Abbreviations: AOR, aldehyde:ferredoxin oxidoreductase; **cCBB,** colloidal CBB; **PCA,** principle component analysis

tities [1] and used as solvents and start compounds for synthesis in chemical industry. Due to production, transport, or storage accidents, these toxic compounds are increasingly released to the environment (EPA, US Environmental Protection Agency, http://www.epa.gov/safewater/mcl.html 2002), often accumulating in anoxic zones of soils and sediments. Therefore, microbial degradation of aromatic compounds, in particular under anoxic conditions, is of increasing interest.

The chemical stability of aromatic compounds necessitates special biochemical reactions for their biodegradation. Aerobic degradation is a long known and well-understood process. Here, highly reactive oxygen species are used as cosubstrates in oxygenase catalyzed reactions for initial hydroxylation steps and subsequent ring cleavage [2, 3]. In contrast, anaerobic degradation is a more recently discovered process performed by a variety of newly isolated bacteria. Due to the absence of molecular oxygen many novel reactions are involved, which are fundamentally different from their aerobic counterparts. In many cases, the aromatic compounds are channelled *via* specific reaction sequences to the common intermediate benzoyl–CoA. Further degradation of the latter involves reductive dearomatization, hydrolytic ring cleavage, and β-oxidation-like reactions [4, 5]. Interestingly, these new bacteria also harbour unusual pathways and novel types of oxygenases for the aerobic degradation of aromatic compounds [6, 7].

The majority of newly discovered anaerobic aromatic compound degrading bacteria are denitrifiers belonging to the *Azoarcus/Thauera* cluster within the Betaproteobacteria [8–10]. This group will be proposed as the new genus "*Aromatoleum*" (Rabus, unpublished data). "*Aromatoleum aromaticum*" strain EbN1 is a metabolically versatile representative of this group [11]. Besides toluene and ethylbenzene, strain EbN1 anaerobically degrades a wide variety of polar aromatic compounds (including phenol and *p*-cresol), aliphatic carboxylic acids, ketones (including acetone) and alcohols. In addition, strain EbN1 is also able to degrade several aromatic compounds aerobically (Fig. 1).

The complete genome sequence of strain EbN1 was the first to be determined for a member of the "*Aromatoleum*"/*Azoarcus/Thauera* cluster and an anaerobic hydrocarbon degrader [12]. The genome revealed the presence of gene clusters for more than ten anaerobic and four aerobic aromatic compound degradation pathways. The absence of these pathways in the aerobic aromatic compound degrader *Pseudomonas putida* [13] and aerobic alkane degrader *Alcanivorax borkumensis* [14] underline the metabolic distinctiveness of strain EbN1. The only other genome sequenced bacterium also capable of anaerobic aromatic compound degradation is the phototrophic *Rhodopseudomonas palustris* [15]. In addition, the genome of strain EbN1 revealed the presence of multiple respiratory complexes and a large number of regulatory proteins. Thus, strain EbN1 is assumed to be well equipped to adapt to fluctuating availability of organic substrates and electron acceptors in the environment.

Regulation of individual aerobic or anaerobic pathways for aromatic compound degradation has already been studied with different organisms, *e.g. P. putida* [16], *Thauera aromatica* [17], *R. palustris* [18], and strain EbN1 [19]. The availability of the genome of strain EbN1 [12] together with 2-D DIGE-enabled quantitative protein profiling [19] and mass spectrometric protein identification [20] now allows for the first time global investigation of the entirety of anaerobic and aerobic degradation pathways. Thus, the present study pursued the following lines of research: (i) To what extent do the proteomic data corroborate the earlier genome-based pathway predictions? (ii) Do we find new protein components for individual pathways due to substrate-specific coregulation? (iii) How specifically are the individual pathways induced by their respective substrates? (iv) To what degree do individual substrates act as gratuitous inducer for other pathways? (v) Do different levels of regulatory hierarchy exist? (vi) Can regulated subproteomes be differentiated from a common constitutive subproteome?

2 Materials and methods

2.1 Medium and cultivation

The denitrifying strain EbN1 was cultivated under nitrate-reducing conditions as previously described [11]. Cultivation was performed in 500 mL flat glass bottles, containing 400 mL of medium. Acetate (5 mM), acetone (2 mM), *o*-aminobenzoate (4 mM), benzaldehyde (2 mM), benzoate (4 mM), benzyl alcohol (2 mM), 2-butanol (5 mM), 2-butanone (2 mM), *p*-cresol (2 mM), *m*-hydroxybenzoate (4 mM), *p*-hydroxybenzoate (4 mM), phenol (0.5 mM), phenylacetate (4 mM), phenylalanine (2 mM), 2-propanol (5 mM), and propionate (5 mM) were added from sterile-stock solutions.

Aerobic cultivation was performed in 500 mL glass bottles filled with 100 mL ascorbate- and nitrate-free phosphate buffered medium [11]. The gas headspace (400 mL) contained sterile air. The amount of oxygen in the headspace was sufficient for complete oxidation of the organic substrates. Acetate (5 mM), benzoate (4 mM), gentisate (2 mM), *m*-hydroxybenzoate (4 mM), phenylacetate (1 mM), and phenylalanine (1 mM) were added in the same way as under anoxic conditions.

Prior to mass cultivation, both aerobic and anaerobic cultures were adapted for at least five passages to the respective substrate. A new passage for a growth condition was achieved by transferring 5% v/v of a freshly grown culture to new substrate containing medium. Transfer of culture liquid was performed with N_2-flushed sterile syringes in case of anaerobic cultivation.

2.2 Mass cultivation

Mass cultivation was performed to supply sufficient cell material for proteomic analysis. Anaerobic mass cultivation was carried out as described before [21]. Aerobic mass cul-

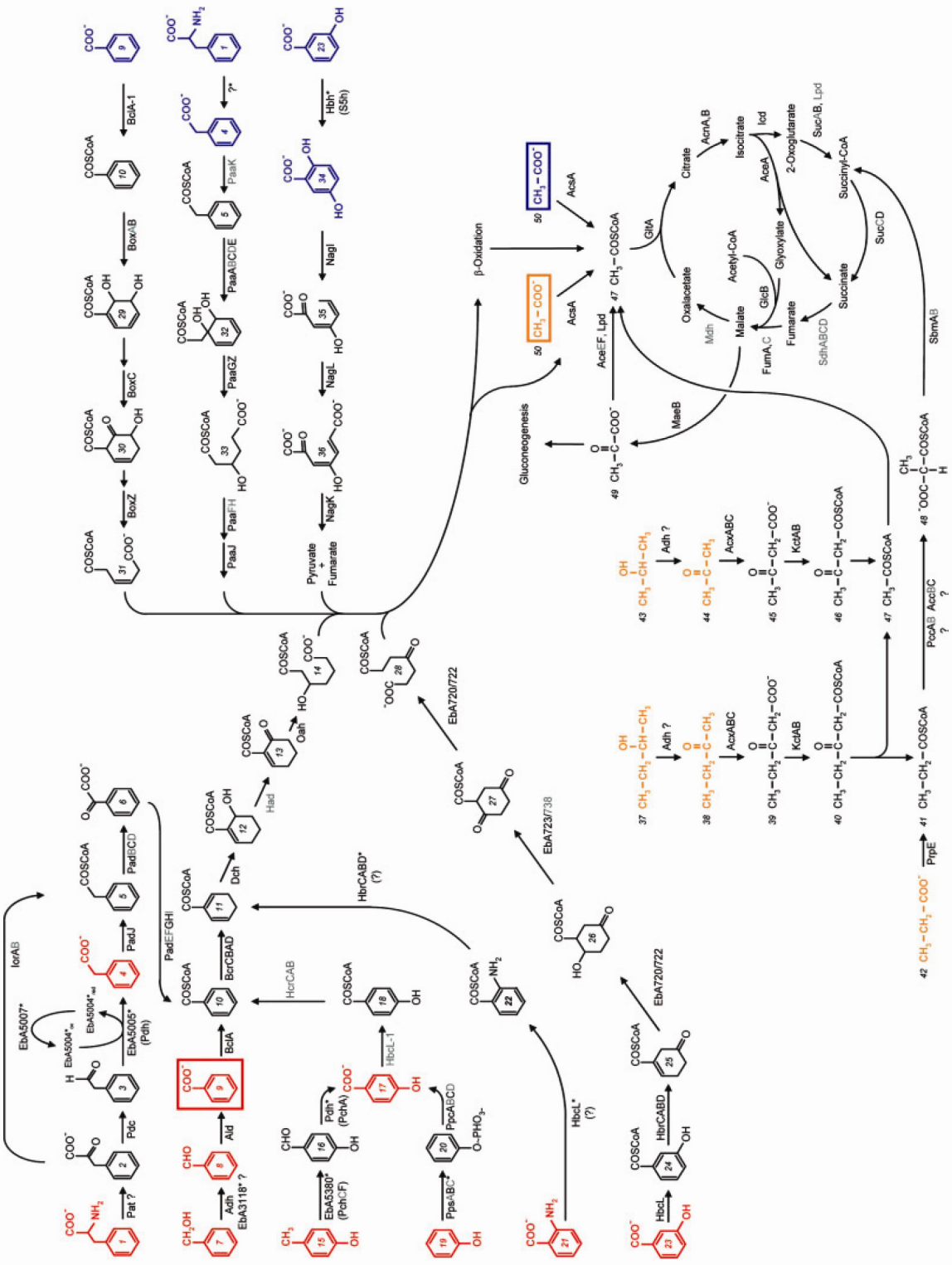


Figure 1. Main degradation pathways of "*Aromatoleum aromaticum*" strain EbN1 investigated for their substrate-specific regulation. Color-coding reflects main physiological groups for 2-D DIGE analysis: red, anaerobic degradation of aromatic compounds; orange, anaerobic degradation of aliphatic compounds; blue, aerobic degradation of aromatic compounds. Boxed compounds represent the reference states and the other colored compounds the analyzed test states. Compound names: (1) phenylalanine, (2) phenylpyruvate, (3) phenylacetaldehyde, (4) phenylacetate, (5) phenylacetyl–CoA, (6) phenylglyoxylate, (7) benzyl alcohol, (8) benzaldehyde, (9) benzoate, (10) benzoyl–CoA, (11) cyclohex-1,5-diene-1-carbonyl–CoA, (12) 6-hydroxycyclohex-1-ene-1-carbonyl–CoA, (13) 6-oxocyclohex-1-ene-1-carbonyl–CoA, (14) 3-hydroxypimelyl–CoA, (15) *p*-cresol, (16) *p*-hydroxybenzaldehyde, (17) *p*-hydroxybenzoate, (18) *p*-hydroxybenzoyl–CoA, (19) phenol, (20) phenylphosphate, (21) *o*-aminobenzoate, (22) *o*-aminobenzoyl–CoA, (23) *m*-hydroxybenzoate, (24) *m*-hydroxybenzoyl–CoA, (25) 2-ene-5-oxo-cyclohex-1-carbonyl–CoA, (26) 2-hydroxy-5-oxo-cyclohex-1-carbonyl–CoA, (27) 2,5-dioxo-cyclohex-1-carbonyl–CoA, (28) 4-oxopimelyl–CoA, (29) 2,3-dihydroxy-4,6-diene-cyclohexylcarbonyl–CoA, (30) 2-oxo-3-hydroxy-5-ene-cyclohexylcarbonyl–CoA, (31) β-ene-adipyl–CoA, (32) *cis*-dihydrodiol derivative of phenylacetyl–CoA, (33) β-hydroxy-adipyl–CoA, (34) gentisate, (35) maleylpyruvate, (36) fumarylpyruvate, (37) 2-butanol, (38) 2-butanone, (39) 3-oxo-valerate, (40) 3-oxo-valeryl–CoA, (41) propionyl–CoA, (42) propionate, (43) 2-propanol, (44) acetone, (45) 3-oxo-butyrate, (46) 3-oxo-butyryl–CoA, (47) acetyl–CoA, (48) methylmalonyl–CoA, (49) pyruvate, (50) acetate. Enzyme names (in alphabetical order): Color coding: black, identified; grey, not identified. Enzyme names in brackets were predicted *in silico*. Enzyme names marked with (*) were assigned in this study. A function in the respective pathway of enzymes marked with (?) could neither be supported nor disproved by this study. (AceA) isocitrate lyase, (AceEF, Lpd) pyruvate dehydrogenase multienzyme complex, (AcnA, AcnB) aconitase isoenzymes, (AcsA) acetyl–CoA synthetase, (AcxABC) acetone carboxylase, (Adh) alcohol dehydrogenase, (Ald) aldehyde dehydrogenase, (BclA) benzoate–CoA ligase, (BclA-1) "aerobic" benzoate–CoA ligase, (BcrCBAD) benzoyl–CoA reductase, (BoxAB) benzoyl–CoA oxidation complex, (BoxC) enoyl–CoA hydratase/isomerase, (BoxZ) aldehyde dehydrogenase, (Dch) cyclohex-1,5-diene-1-carbonyl–CoA hydratase, (EbA720/722) putative 3-oxo-5-ene-cyclohexylcarbonyl–CoA hydratase and 3,5-dioxo-cyclohexylcarbonyl–CoA hydrolase, (EbA723/738) putative 3-oxo-5-hydroxy-cyclohexylcarbonyl–CoA dehydrogenase, (EbA3118) Zn-containing alcohol dehydrogenase, (EbA5004) iron–sulfur cluster-binding protein, similar to 4Fe–4S ferredoxin, (EbA5005) predicted AOR, (EbA5007) ferredoxin:NADH oxidoreductase, (EbA5380) putative *p*-cresol methylhydroxylase, (FumA, FumC) fumarase isoenzymes, (GlcB) malate synthase, (GltA) citrate synthase, (Had) 6-hydroxycyclohex-1-ene-1-carbonyl–CoA dehydrogenase, (HbcL) *m*-hydroxybenzoate–CoA ligase, (HbcL-1) *p*-hydroxybenzoate–CoA ligase, (Hbh) hydroxybenzoate hydroxylase, (HbrCBAD) *m*-hydroxybenzoyl–CoA reductase, (HcrCAB) *p*-hydroxybenzoyl–CoA reductase, (Icd) isocitrate dehydrogenase, (IorAB) indolepyruvate:ferredoxin oxidoreductase, (KctAB) succinyl–CoA:3-ketoacid–CoA ligase, (MaeB) malic enzyme, (Mdh) malate dehydrogenase, (NagI) gentisate-1,2-dioxygenase, (NagK) fumarylpyruvate hydrolase, (NagL) maleylpyruvate isomerase, (Oah) 6-oxocyclohex-1-ene-1-carbonyl–CoA hydrolase, (PaaABCDE) phenylacetyl–CoA (di)oxygenase, (PaaGZ) ring opening enzyme, (PaaG) enoyl–CoA hydratase/isomerase, (PaaZ) aldehyde dehydrogenase, (PaaFHJ) involved in β-oxidation *via* β-ketoadipyl–CoA, (PaaK) phenylacetate–CoA ligase, (PadBCD) phenylacetyl–CoA:acceptor oxidoreductase, (PadEFGHI) phenylglyoxylate:acceptor oxidoreductase, (PadJ) phenylacetate–CoA ligase, (Pat) phenylalanine aminotransferase, (PccAB, AccBC) propionyl–CoA carboxylase, (PchA) *p*-hydroxybenzaldehyde dehydrogenase, (PchCF) *p*-cresol methylhydroxylase, (Pdc) phenylpyruvate decarboxylase, (Pdh) phenylacetaldehyde dehydrogenase or putative *p*-hydroxybenzaldehyde dehydrogenase, (PpcABCD) phenylphosphate carboxylase, (PpsABC) phenylphosphate synthetase, (PrpE) propionate–CoA ligase, (SbmAB) methylmalonyl–CoA mutase, (SdhABCD) succinate dehydrogenase, (SucAB, Lpd) 2-oxoglutarate dehydrogenase complex, (SucCD) succinyl–CoA synthetase. Modified from Rabus [60].

tures were performed in 5 L glass bottles containing 1 L phosphate buffered medium (see above). Cells were harvested during the midlinear growth phase as described [11]. Cell pellets were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

2.3 2-DE

Cell disruption with the PlusOne Sample grinding kit (GE Healthcare, Munich, Germany) as well as preparation of protein extracts was performed as described before [22]. Protein concentration was determined according to the method described by Bradford [23].

IEF was carried out as described [19, 22] using the IPGphor (GE Healthcare) and commercial 24-cm IPG strips with linear pH gradients of 4–7 (GE Healthcare). The EttanDalt II system (GE Healthcare) was used for separation according to molecular mass in 12.5% acrylamide or duracryl (in case of silver staining; Genomic solutions, Ann Arbor, Mi, USA) gels. Low fluorescence glass plates (GE Healthcare) were used for 2-D DIGE. The quality of the sample preparation method was verified by silver-stained gels according to the method described by Heukeshoven and Dernick [24]. Independent samples were used for silver staining, colloidal CBB (cCBB) staining and 2-D DIGE.

2.4 2-D DIGE

2-D DIGE (Ettan DIGE Technology) was essentially carried out as described by Gade *et al.* [22]. A total of 200 pmol of CyDye DIGE Fluors (GE Healthcare) were used to label 50 μg of the protein sample. Labeling with different fluorescence dyes prior to electrophoresis allows coseparation of up to three samples in a single gel. An individual experiment in the present study contained (*per* gel) reference state, test state, and internal standard. To achieve statistical confidence, five parallel gels were run *per* experiment.

Due to the broad range of test states, the project was divided into three main physiological groups: (i) anaerobic growth with aromatic compounds, (ii) aerobic growth with aromatic compounds, and (iii) anaerobic growth with alcohols, ketones, and carboxylic acids (Supporting Information 1: Fig. 1.1). For group (i), protein extracts from cells grown anaerobically with benzoate served as reference state since its activated form benzoyl–CoA is the central intermediate of anaerobic aromatic compound degradation. The reference state was labeled with Cy5. Protein extracts from cells grown anaerobically with *o*-aminobenzoate, benzyl alcohol, benzaldehyde, *p*-cresol, *m*-hydroxybenzoate, *p*-hydroxybenzoate, phenol, phenylacetate, or phenylalanine represented the test states and were each labeled with Cy3. All performed experiments of group (i) contained the same internal standard, which was composed of equal amounts of the reference state and all test states and labeled with Cy2.

In case of group (ii), protein extracts from aerobically acetate grown cells were used as reference state and were labeled with Cy5. This reference was chosen because acetyl–CoA represents a common intermediate of all tested compounds. Protein extracts from cells grown aerobically with benzoate, *m*-hydroxybenzoate, gentisate, phenylacetate, or phenylalanine served as test states and were each labeled with Cy3. The internal standard was composed of equal amounts of the reference state and all test states and labeled with Cy2.

Protein extracts from anaerobically acetate grown cells also represented the reference state of group (iii) and were labeled with Cy5. Except for propionate, acetyl–CoA is a central intermediate of aliphatic compound degradation. Protein extracts from cells grown anaerobically with acetone, 2-butanol, 2-butanone, 2-propanol, or propionate served as test states and were labeled with Cy3. Equal amounts of the reference and all test states were used as internal standard and labeled with Cy2.

2-D DIGE gels were scanned immediately after electrophoresis with a Typhoon 9400 scanner (GE Healthcare). Analysis of cropped images was performed with the DeCyder software (version 5.0, GE Healthcare). Parameters for codetection of spots were as follows: (i) detection of 3000 spots and (ii) exclusion of signals with a slope of >1, an area <200, a peak height of <190, and a volume of $<60\,000$. Statistical analysis was based on independent spot maps. Regulated protein spots (*i.e.* protein spots with significantly changed abundances) fulfilled the following criteria: an average ratio (fold change) of <-2.5 or >2.5, an ANOVA p value of <0.05, t-test value of $<10^{-4}$, and matched in at least 75% of all gels. It should be noted that the studied biological system determines the level of a reasonable threshold. In the present study, a stringent threshold ($\geq|2.5|$) was applied to achieve a high level of confidence and since mainly catabolic (abundant) proteins were targeted. According to the applied threshold, proteins with average ratios of $<|2.5|$ are classified as nonregulated or constitutive (*i.e.*, relative changes in abundance are regarded as not significant). This includes also those proteins with average ratios of 1 or −1, *i.e.*, with absolutely unchanged abundance.

The main physiological group (i) comprised 45 gels (representing 135 gel images) which contained on average 1345 detected protein spots. In case of group (ii), 25 gels (corresponding to 75 gel images) were analyzed containing on average 1548 detected protein spots. The main physiological group (iii) covered 25 gels (representing 75 gel images) containing on average 1047 detected protein spots.

Advanced data analysis was performed with the Extended Data Analysis (EDA) module (version 1.0) of DeCyder (version 6.5; GE Healthcare). Principle component analysis (PCA) was performed with the NIPALS algorithm [25]. The calculation is based on a minimal number of principle components required to describe 80–90% of dataset variability and the two principle components containing most information (*i.e.* most of the variability in the dataset) are displayed (Fig. 2).

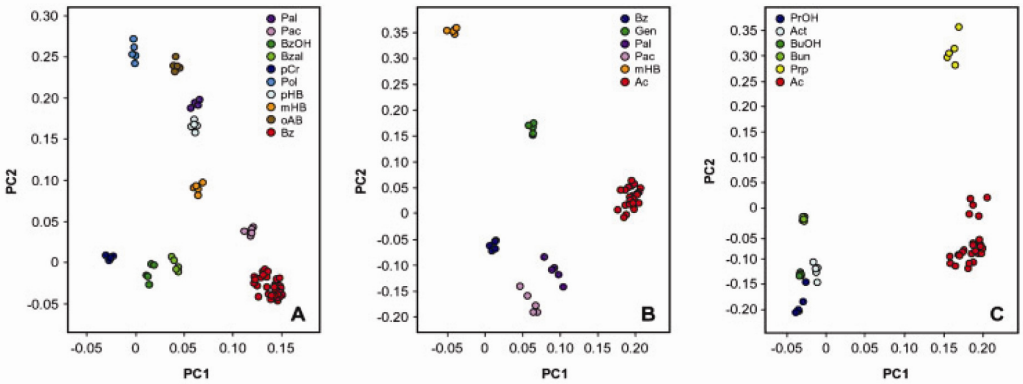


Figure 2. PCA of the spotmaps for each analyzed 2-D DIGE gel of all three main physiological groups. Relative values at the axis represent the variability of the respective principle component (PC1 and PC2). For each test state five parallel gels were analyzed. Correspondingly, 45 (group i) or 25 (groups ii and iii) reference gels were included in the analysis. The tight clusters reflect the low data variability between the corresponding spotmaps, *i.e.* high reproducibility within each tested state. Conversely, the individual states are clearly separated. The inserts contain the color coding of the analyzed states: Group (i), anaerobic growth with aromatic compounds (A); group (ii), aerobic growth with aromatic compounds (B); and group (iii), anaerobic growth with aliphatic alcohols, ketones, and carboxylic acids (C). Compound abbreviations: (Ac) acetate, (Act) acetone, (BuOH) 2-butanol, (Bun) 2-butanone, (Bz) benzoate, (Bzal) benzaldehyde, (BzOH) benzyl alcohol, (Gen) gentisate, (mHB) *m*-hydroxybenzoate, (oAB) *o*-aminobenzoate, (Pac) phenylacetate, (Pal) phenylalanine, (pCr) *p*-cresol, (pHB) *p*-hydroxybenzoate, (Pol) phenol, (PrOH) 2-propanol, (Prp) propionate. Reference states are indicated in red.

While the 2-D DIGE experiments confirmed and quantified the regulated proteins, initially defined by silver staining, they also allowed detection and identification of additional specifically regulated proteins.

In order to obtain sufficient amounts of protein for MS analysis, separate preparative gels were run with each of the 22 studied growth conditions. These gels were stained with cCBB according to the method described by Doherty *et al.* [26]. The high reproducibility of spot patterns (Supporting Information 1: Fig. 1.2.) allowed MS-based identification of proteins selected by the 2-D DIGE/DeCyder analysis from cCBB stained gels; only unambiguously matching protein spots were included in the present study.

2.5 Protein identification by MS

Selected regulated and constitutive protein spots were excised with an automatic excision workstation (PROTEINEER spII; Bruker Daltonik, Bremen, Germany) equipped with an on-board visual scanner for image acquisition. Sample preparation and tryptic digest of the excised proteins were performed as described [20]. MALDI–TOF–MS(/MS) analysis was done on an ultraflex or ultraflex II (Bruker Daltonik) as previously described [20]. Based on MS or MS/MS data, fingerprinting was done on a MASCOT server (Matrix Science, London, UK) and a ProFound server (Genomic Solutions, MS only) against a genomic database of strain EbN1, translated into amino acid sequences [20].

Identification was based on matching spots from at least two parallel gels with a MASCOT score of at least 95 or a ProFound score of 2.2. The average scores of the identified proteins of the present study were 201 (MASCOT) and 2.34 (ProFound). Three percent of the proteins were identified from two parallel gels, 14% from three gels, and 74% from more than three gels. The average sequence coverage was 50.8%. For details see ref. [20].

3 Results and discussion

Substrate-dependent regulation in "*A. aromaticum*" strain EbN1 was quantitatively investigated with the 2-D DIGE approach. Gel-based protein separation was preferred over LC-based peptide separation, since the former approach allows reducing analytical complexity and maintains the structural protein information; both aspects facilitate the efficiency of subsequent MS-based protein identification. To study substrate-dependent regulation, strain EbN1 was adapted to grow with 17 different aromatic and nonaromatic compounds under anoxic or oxic conditions, resulting in a total of 22 different growth conditions. All of these growth conditions were assigned to three main physiological groups: (i) anaerobic growth with aromatic compounds, (ii) aerobic growth with aromatic compounds, and (iii) anaerobic growth with aliphatic alcohols, ketones, and carboxylic acids (Fig. 1). In each of the three groups, the various catabolic routes converge at common intermediates: benzoyl–CoA in case of group (i) and acetyl–CoA in case of groups (ii) and (iii). The precursors of these two central intermediates (benzoate and acetate) were used as reference states to detect and identify proteins specifically regulated in response to the tested growth conditions. Notably, 8 out of the 17 tested growth substrates represent

intermediates in the catabolic pathways of other compounds, *e.g.* phenylacetate in the phenylalanine pathways (Fig. 1). Thus, the present study also allowed defining the subproteomes of connected pathway modules (*e.g.* from phenylalanine to phenylacetate *vs.* from phenylacetate to benzoyl–CoA during anaerobic degradation) and the extent of their substrate-dependent regulation. The entirety of the proteins with significantly changed abundances (fold change $\geq|2.5|$) provided a first global experimental perspective on the *in silico* predicted regulatory potential of strain EbN1. Conversely, identification of a large number of proteins not changing significantly in abundance (fold change $<|2.5|$) revealed the extent and nature of the constitutive subproteome. Extensive Supporting Information is provided, including biochemical background information for each analyzed pathway as well as annotated gel images and gene context for each analyzed substrate condition.

3.1 General properties of the analyzed dataset

The present study builds on a comprehensive, high quality dataset. The differential protein profiles determined by 2-D DIGE were essentially the same as revealed by independent silver- or cCBB-stained 2-DE gels (Supporting Information 1: Fig. 1.1 for general workflow and Fig. 1.2 for reproducibility of spot patterns). For the quantification of differential protein profiles by 2-D DIGE, a total of 285 digital gel images containing an average of 1047–1548 detected protein spots were analyzed. PCA demonstrated the high reliability of the generated spot maps in the case of all tested growth conditions within a 95% significance level (Fig. 2). Protein spots not meeting the 95% significance level according to PCA represent specifically and strongly regulated proteins, rather than analytical artifacts (Supporting Information 1: Fig. 1.3.). The threshold of significance for fold changes in protein abundance was set at the rather high value of $\geq|2.5|$, to limit the number of biological false positives. In total, more than 4500 2-DE-separated proteins spots were analyzed by high-through-put MS, resulting in the highly reliable identification of 354 different proteins. The identification was based on matching spots from at least two independent 2-DE gels and a high average MASCOT score of 201 (see Section 2).

3.2 Anaerobic growth with aromatic compounds

3.2.1 Phenylalanine and phenylacetate

All predicted proteins of the anaerobic phenylalanine degradation pathway, *i.e.* phenylalanine aminotransferase (Pat), phenylpyruvate decarboxylase (Pdc), and phenylacetaldehyde dehydrogenase (Pdh), were identified on the 2-DE gels of phenylalanine-adapted cells (Fig. 3). Remarkably, expression of *pat* is apparently not specifically upregulated during anaerobic growth with phenylalanine (Fig. 4). One may speculate that Pat is constitutively formed or that an alternative aminotransferase (16 predicted) catalyzes this initial reaction, but may have escaped our analytical window. In contrast, Pdc is 27.5-fold increased in abundance, supporting its proposed function in phenylalanine degradation. Pdh was again not increased in abundance during anaerobic growth with phenylalanine, but specifically with *p*-cresol (7.4-fold), implicating a dedicated function in *p*-cresol rather than phenylalanine degradation (see Section 3.2.3. "*p*-cresol").

The differential protein profiling suggested a predicted aldehyde:ferredoxin oxidoreductase (AOR) (EbA5005) as a possible substitute for Pdh. The encoding gene, *ebA5005*, is colocalized with *ebA5004* (iron–sulfur cluster-binding protein) and *ebA5007* (ferredoxin:NADH oxidoreductase) in an operon-like structure (Fig. 3). All three proteins were specifically increased in abundance during anaerobic growth with phenylalanine (9.7-, 49.7-, and 6.0-fold, respectively). Enzymes of the AOR family are widespread among prokaryotes and catalyze the oxidation of a broad range of both aliphatic and aromatic aldehydes to their corresponding carboxylic acid using ferredoxin as electron acceptor [27, 28]. Investigation of the substrate spectrum of purified AOR from *Thermococcus* strain ES-1 and *Eubacterium acidaminophilum*, indeed, exhibited a high activity with phenylacetaldehyde [29, 30]. EbA5005 may be supported by the two additional proteins. EbA5004 may function as electron acceptor for EbA5005, since it exhibits high similarities to ferredoxin from *Rhodoferax ferrireducens* T118 (69% identities, as determined by BLAST-analysis [31]) and is classified by InterPro as a 4Fe–4S ferredoxin [32]. Moreover, the ferredoxin:NADH oxidoreductase (EbA5007) could be involved in regeneration of the ferredoxin cofactor.

Subunits of all three proteins of the anaerobic phenylacetate degradation pathway (Fig. 1) were identified in this study. The phenylacetate–CoA ligase (PadJ), a subunit of the phenylacetyl–CoA:acceptor oxidoreductase (PadC) and two subunits of phenylglyoxylate:acceptor oxidoreductase (PadG and PadH) were most strongly increased in abundance (ranging from 5.5- to 29.5-fold) during anaerobic growth with phenylalanine and phenylacetate (Fig. 4), corroborating this pathway for the degradation of both compounds in strain EbN1.

An alternative pathway shortcutting the phenylalanine degradation from phenylpyruvate directly to phenylacetyl–CoA was previously speculated to involve indolepyruvate:ferredoxin oxidoreductase (IorAB) [12]. However, the present proteomic data do not support this possibility, since identified IorA was not differentially regulated (Fig. 4).

3.2.2 Benzyl alcohol and benzaldehyde

Analysis of the strain EbN1 genome suggested the presence of various dehydrogenases for aromatic alcohols and aldehydes possibly involved in anaerobic benzyl alcohol and benzaldehyde degradation [12]. The present proteomic study revealed a multifaceted regulatory pattern, which did not allow defining dedicated dehydrogenases for the two oxidation reactions. Notably, the genomically predicted dehydrogenases were also increased in abundance during anae-

robic growth with other aromatic compounds, *e.g. p*-cresol (Fig. 4; further information is provided in Supporting Information 3).

The protein spot revealing the largest increase in abundance during anaerobic growth with benzyl alcohol (144.5-fold) as well as benzaldehyde (66.2-fold) is EbA5637, a putative mandelate racemase [12]. In addition, products of genes forming an operon-like structure with *ebA5637* (an acyl–CoA transferase (EbA5640), an acyl–CoA dehydrogenase (EbA5641), the benzaldehyde dehydrogenase (Ald), and another acyl–CoA transferase (EbA5643)) also revealed strongly increased abundances during anaerobic growth with benzyl alcohol and benzaldehyde as well as with *p*-cresol (Fig. 4). However, strain EbN1 cannot utilize either of the two-mandelate isomers (data not shown), agreeing with the absence of mandelate and benzylformate dehydrogenase encoding genes in the genome sequence of strain EbN1. In known mandelate utilizing bacteria, such as *Acinetobacter calcoaceticus*, the respective catabolic operon is not induced by benzaldehyde [33, 34], suggesting that the observed regulation in strain EbN1 might be nonspecific and a relict, originating from gene recruitment or genome evolution (further information is provided in Supporting Information 3).

3.2.3 *p*-Cresol

One subunit of the first enzyme of anaerobic *p*-cresol degradation, *p*-cresol methylhydroxylase (PchF) as well as the second enzyme, *p*-hydroxybenzaldehyde dehydrogenase (PchA), could be identified. However, both did not display significant increases in abundance during anaerobic growth with *p*-cresol, but surprisingly with benzaldehyde and benzyl alcohol (PchA: 7.8- and 5.3-fold, respectively, Fig. 4).

The largest increase in abundance in *p*-cresol grown cells (352.2-fold) was observed for a predicted FAD-dependent oxidase (EbA5380), which displays high similarities to *p*-cresol methylhydroxylases of *P. putida* and *Nitrosomonas eutropha* (60% identities and 78% positives each). Notably, the *ebA5380* gene of strain EbN1 forms an operon-like structure with a predicted phenylacetaldehyde dehydrogenase (*pdh/ebA5381*) and a putative regulatory protein (*ebA5384*). The product of *ebA5381* is specifically formed (7.4-fold increase) during anaerobic growth with *p*-cresol, and its previous functional assignment as Pdh in the phenylalanine degradation pathway could not be supported by the present study (see Section 3.2.1. "phenylalanine and phenylacetate"). The described regulatory patterns suggest that strain EbN1 uses EbA5381 to oxidize the aldehyde group of *p*-hydroxybenzaldehyde rather than that of phenylacetaldehyde. Thus, the products of *ebA5380* (putative *p*-cresol methylhydroxylase) and *ebA5381* (putative *p*-hydroxybenzaldehyde dehydrogenase) are suggested to be involved in anaerobic *p*-cresol degradation, rather than PchCF and PchA.

p-Cresol may also function as gratuitous inducer for other pathways of anaerobic aromatic compound degradation. Firstly, several proteins involved in anaerobic toluene degradation (BssE, BbsA, BbsH, EbA1929, EbA1932, and EbA1936) were observed to be increased in abundance (6.8- to 72.7-fold) in *p*-cresol grown cells. While previous studies indicated a rather strict specificity of the predicted TdiS sensor for toluene (not responsive to ethylbenzene or acetophenone [19]), the present results suggest that TdiS may also recognize the structurally highly similar *p*-cresol. Secondly, during anaerobic growth with *p*-cresol also two proteins (Ped2 and EbA5797) encoded in a paralogous *ebd* operon exhibited an increased abundance (235.4- and 12.1-fold, respectively). The gene products of this operon are highly similar to the enzymes of the "upper" pathway of ethylbenzene degradation to acetophenone (*e.g.* 77.8% identity in case of the catalytic EbdA subunit of ethylbenzene dehydrogenase). However, ethylbenzene and toluene do not induce this paralogous operon. Thus, the unexpected *p*-cresol dependent induction may provide a first hint onto the nature of the true, so far unknown, substrate for these paralogous enzymes, *e.g.* the structurally similar *p*-ethylphenol. This hypothesis is currently tested in our laboratory.

3.2.4 Phenol

Subunits of both enzymes of the anaerobic phenol degradation pathway, *i.e.* PpsB (EbA3134) of phenylphosphate synthase and PpcAC of phenylphosphate carboxylase, whose genes are encoded in an operon-like structure, were identified on the 2-DE gels. In phenol grown cells of strain EbN1, all three proteins revealed specifically increased abundances (*e.g.* 120.3-fold for PpcC). Correspondingly, enzymatic and proteomic analysis of *T. aromatica* also revealed strict phenol-specific induction of both enzymes [35, 36].

In contrast, the abundance of a paralogous PpsB protein (EbA5783), encoded by a paralogous *ppsB* gene at a different chromosomal position, was increased in abundance during growth with *p*-cresol (19.3-fold) and slightly with phenylacetate (2.8-fold), but its abundance was not significantly changed with phenol. Thus, an essential role of paralogous PpsB in anaerobic phenol degradation appears unlikely. In addition, the respective paralogous *pps* gene cluster does not contain a PpsC encoding gene, which was demonstrated before to markedly increase (four-fold) the *in vitro* activity of phenylphosphate synthase (purified PpsAB) in *T. aromatica* [37]. In strain EbN1, the second *pps*-operon is flanked by genes encoding transposases of the IS4 family. Indeed, the actual position of *ppsC* is occupied by a transposase (*tnp46*) *in trans*. This might be a hint for genome evolution, reshuffling of genes within the genome, or assimilation of new genes by lateral gene transfer. Thus, one may speculate that the paralogous *pps* gene cluster is involved in a hitherto unknown degradation pathway.

In addition to the Ppc proteins, several heat-shock proteins (EbA2730, EbB88, and EbA4332) and proteins related to oxidative stress (superoxide dismutase (SodB) and glutathion peroxidase (BtuE)) or DNA protection (Dps) revealed

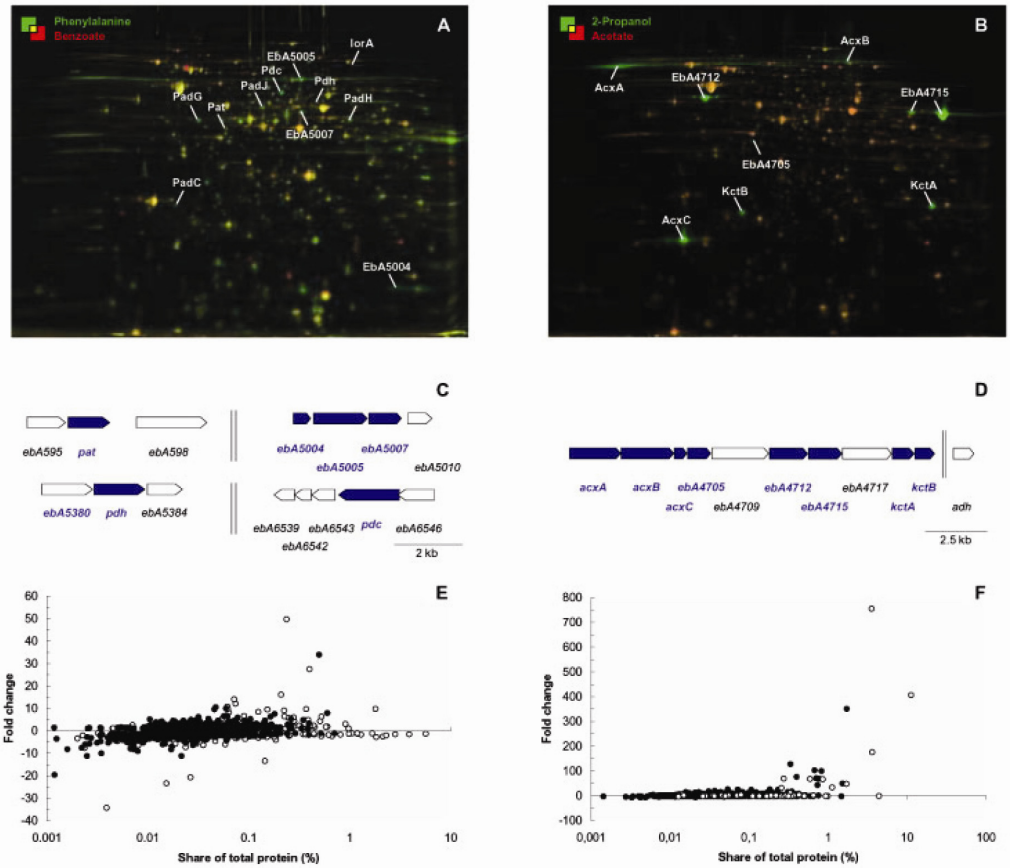
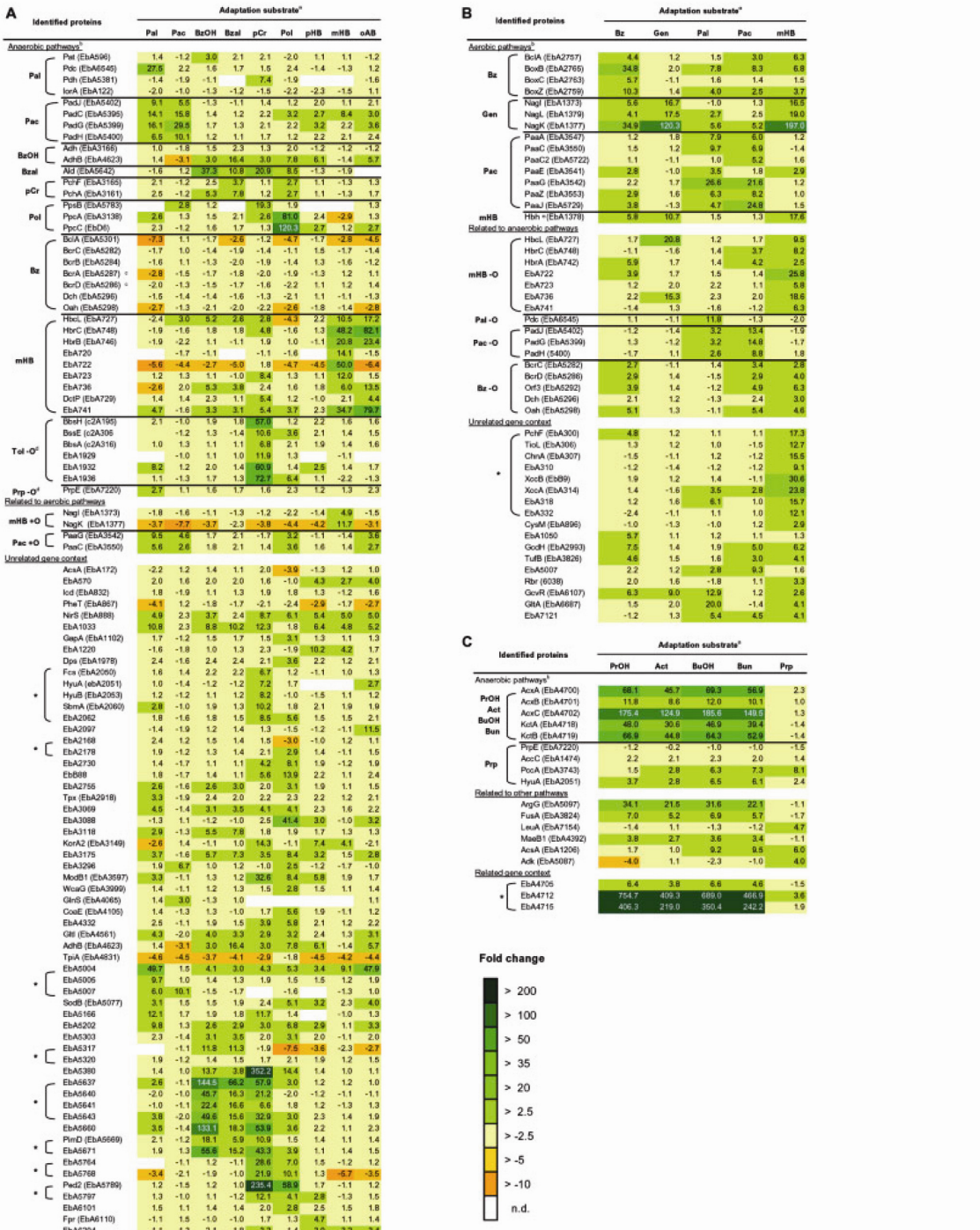


Figure 3. Differential protein profiles (2-D DIGE) representing anaerobic growth of strain EbN1 with phenylalanine (A) and 2-propanol (B). Scale models of genes predicted [12] to be involved in anaerobic degradation of phenylalanine (C) and 2-propanol (D). Genes encoding identified proteins are highlighted in blue. Fold changes in protein abundance and their relative share of total 2-DE separated protein in phenylalanine (E) and 2-propanol (F) grown cells. Each dot represents a spot on the 2-DE gel, except for repeatedly identified proteins (values summed up). (○) Identified proteins, (●) not identified proteins.

Figure 4. Fold changes in abundance of identified proteins during growth of strain EbN1 with different aromatic compounds under anoxic (A) or oxic (B) conditions as well as with aliphatic alcohols, ketones, or carboxylic acids under anoxic conditions (C). Color code indicates increased (fold change >2.5, green), decreased (fold change <−2.5, red), or unchanged (fold change <2.5 and >−2.5, yellow) abundances (white, not determined). The more intense the color, the stronger the fold change (see colored scale bar). (a) Cells were adapted over at least five passages to the respective growth condition. Fold changes were determined on the basis of five parallel gels. For details see Section 2. Substrate abbreviations: (Ac) acetate, (Act) acetone, (BuOH) 2-butanol, (Bun) 2-butanone, (Bz) benzoate, (Bzal) benzaldehyde, (BzOH) benzyl alcohol, (Gen) gentisate, (mHB) *m*-hydroxybenzoate, (oAB) *o*-aminobenzoate, (Pac) phenylacetate, (Pal) phenylalanine, (pCr) *p*-cresol, (pHB) *p*-hydroxybenzoate, (Pol) phenol, (PrOH) 2-propanol, (Prp) propionate. (b) Proteins are listed in order of catalytic activity in the respective pathway. Protein names and ORF numbers (*e.g.* EbA720) are as described in ref. [12]. Substrate abbreviations are as described above. Tol, toluene. (c) The paralogs of BcrA and HbrA as well as BcrD and HbrD share 100% sequence identity and are therefore not distinguishable by PMF. (d) Anaerobic growth with toluene or propionate were not included in this group of the DIGE analysis. (e) Hbh was originally named S5h. (*) Proteins with unrelated gene context, organized in operon-like structures, are marked by parenthesis.

Proteomics 2007, *7*, 2222–2239



increased abundances in phenol grown cells. The majority of these stress proteins were also increased in the presence of the solvent *p*-cresol, though to a lesser extent. Furthermore, a hypothetical protein EbA3088 was highly upregulated (41.4-fold), which was predicted to play a role in the cell envelope. These results are consistent with the toxic nature of organic solvents that forces the cell to pursue complex survival strategies [38].

3.2.5 *p*-Hydroxybenzoate

While *p*-hydroxybenzoate is a true substrate for anaerobic growth of strain EbN1, it also represents the first common intermediate of anaerobic *p*-cresol and phenol degradation (see Fig. 1). While genes of the anaerobic *p*-hydroxybenzoate pathway (encoding *p*-hydroxybenzoate–CoA-ligase (*hbcL-1*), and *p*-hydroxybenzoyl–CoA reductase (*hcrCAB*)) are present on the chromosome of strain EbN1 [12], none of their products could be identified in the present study.

3.2.6 *m*-Hydroxybenzoate

In case of anaerobic *m*-hydroxybenzoate degradation, the specific CoA-ligase (HbcL), the paralogous *m*-hydroxybenzoyl–CoA reductase (HbrCABD) as well as the hydrolases and dehydrogenases (EbA720, EbA722, EbA723, EbA736, and EbA738; Fig. 1) of the subsequent metabolism of the alicyclic intermediate were identified. All of them revealed a large increase in abundance during anaerobic growth with *m*-hydroxybenzoate, supporting the assumed pathway. The regulation was highly substrate-specific, with the exception of *o*-aminobenzoate (see below).

3.2.7 *o-Aminobenzoate*

Anthranilic acid (*o*-aminobenzoate) is an intermediate of tryptophan synthesis as well as degradation. In *T. aromatica* a single benzoyl–CoA ligase is used for the activation of benzoate as well as *o*-aminobenzoate, but not for monohydroxybenzoate analog (*e.g. m*-hydroxybenzoate) or phenylacetate [39]. Activation of the latter compounds is achieved by other specific CoA-ligases [40–42]. Interestingly, benzoate–CoA ligase (BclA) is 4.5-fold decreased in abundance in *o*-aminobenzoate grown cells of strain EbN1, while the CoA-ligase predicted to be specific for *m*-hydroxybenzoate (HbcL) revealed a marked increase in abundance (17.2-fold).

At present, the postulated aminobenzoate reductase and its coding gene(s) have not been discovered in *T. aromatica* or strain EbN1. However, we identified a putative ferredoxin (EbA5004) which was strongly increased in abundance (47.9-fold) in *o*-aminobenzoate adapted cells of strain EbN1. One may speculate that EbA5004 may function as electron donor for reductive deamination or dearomatization.

In addition to HbcL also HbrB and HbrC (subunits of hydroxybenzoyl–CoA reductase) are specifically increased in abundance. Thus, dearomatization of *o*-aminobenzoate is most likely performed by the same enzyme proposed for the *m*-hydroxybenzoate pathway. Since the proteins involved in ring cleavage and further oxidation of the *m*-hydroxybenzoate pathway (EbA720, EbA722, and EbA723) are not induced by *o*-aminobenzoate, subsequent degradation might proceed *via* the anaerobic benzoate pathway to 3-hydroxypimelyl–CoA involving Dch, Had, and Oah as described for *T. aromatica*.

It should be noted that three more proteins of the *hbr*-operon, EbA736, EbA741, and DctP, are also upregulated in the presence of *o*-aminobenzoate, even though their role in the degradation pathway is presently unknown.

3.3 Aerobic growth with aromatic compounds

3.3.1 Benzoate

Four of five predicted gene products of a new aerobic benzoate pathway [43, 44], namely benzoate–CoA ligase (BclA-1), one subunit of benzoyl–CoA oxygenase (BoxB), enoyl–CoA-hydratase/isomerase (BoxC), and the aldehyde dehydrogenase involved in ring opening (BoxZ), could be identified. All four proteins were strongly increased in abundance (4.4- to 34.8-fold; Fig. 4) during aerobic growth with benzoate.

A certain degree of regulatory unspecificity was observed in two directions. Firstly, the identified proteins of the aerobic benzoate pathway were not only formed during aerobic growth with benzoate, but also with phenylalanine, phenylacetate, or *m*-hydroxybenzoate, even though to a lesser extent. The implicated gratuitous induction of the aerobic benzoate pathway by such compounds is supported by reduced BoxA activity in phenylacetate and *m*-hydroxybenzoate grown cells of *A. evansii* [44] and proteomic detection of benzoate dioxygenase and subsequent enzymes in *p*-hydroxybenzoate grown cells of *P. putida* KT 2440 [45].

Secondly, aerobic growth with benzoate did not only lead to the formation of the enzymes of the respective pathway, but also to a lower level upregulation of enzymes from the aerobic gentisate and the anaerobic benzoate pathways. Interestingly, the fumarylpyruvate hydrolase NagK (aerobic gentisate pathway) was next to BoxB the most strongly increased protein (34.9-fold; for comparison: 120.3-fold with gentisate) during aerobic growth with benzoate. Considering that both NagK and salicylate 5-hydroxylase (S5h; suggested to be renamed as hydroxybenzoate hydroxylase (Hbh); see below) were also increased in abundance during anaerobic growth with benzoate (acetate grown cells used as reference state, data not shown), the latter obviously functions as gratuitous inducer of the aerobic gentisate pathway. From the anaerobic benzoate pathway, the abundance of subunits of the benzoyl–CoA reductase (BcrCD) and proteins of the subsequent β-oxidation steps (Oah and Orf3) were slightly increased (Fig. 4). Conversely, proteins of the aerobic benzoate pathway were also slightly increased in abundance dur-

ing anaerobic growth with benzoate (acetate grown cells used as reference state; data not shown). Thus, redox conditions apparently do not control the protein constituents of aerobic and anaerobic degradation pathways for benzoate with absolute stringency.

3.3.2 *m*-Hydroxybenzoate and gentisate

While *m*-hydroxybenzoate degradation in *Pseudomonas*, *Bacillus*, and *Klebsiella* species does not require initial activation to *m*-hydroxybenzoyl-CoA [46–48], this has been reported for *T. aromatica* [49]. In both cases, a hydroxylation then yields gentisate. Since orthologous genes for a respective CoA-ligase and hydroxylase were previously not identified in the genome of strain EbN1, the pathway for aerobic *m*-hydroxybenzoate degradation in this strain could not be predicted *in silico*.

Gentisate 1,2-dioxygenase (NagI), maleylpyruvate isomerase (NagL), and fumarylpyruvate hydroxylase (NagK), predicted to be involved in aerobic gentisate degradation were identified in this study. Furthermore, a predicted salicylate 5-hydroxylyse (S5h), encoded in an operon-like structure with *nagI*, *nagL*, and *nagK*, was identified. All four proteins were found to be specifically and strongly formed in cells growing aerobically with gentisate or *m*-hydroxybenzoate. Overall, a markedly larger increase in abundance was observed in *m*-hydroxybenzoate *versus* gentisate grown cells: NagK (197.0- *vs.* 120.3-fold) and S5h (17.6- *vs.* 10.7-fold). This agrees with previous studies showing higher rates for *m*-hydroxybenzoate and gentisate consumption in *m*-hydroxybenzoate *versus* gentisate grown cells of *Pseudomonas* spp. [47].

In particular, *m*-hydroxybenzoate dependent formation of S5h implicated that this protein represents an *m*-hydroxybenzoate hydroxylase rather than the previously predicted salicylate 5-hydroxylase; S5h should therefore be renamed as hydroxybenzoate hydroxylase (Hbh). This assumption is corroborated by two further lines of evidence. Firstly, strain EbN1 cannot grow with salicylate. Secondly, orthologous gene clusters related to gentisate catabolism from *Polaromonas naphthalenivorans* CJ2 and *P. alcaligenes* NCIMB 9867 also contain genes similar to *hbh*, namely *xlnD* and *orf2*, respectively [50, 51]. Furthermore, XlnD was characterized as 3-hydroxybenzoate 6-hydroxylase catalyzing the NADH-dependent conversion of *m*-hydroxybenzoate to gentisate [52]. Hbh of strain EbN1 also displays high sequence similarities to 3-hydroxybenzoate 6-hydroxylases of *P. entomophila* L48 (61% identity) and *K. pneumoniae* (62% identity).

Interestingly, seven out of eight identified proteins of the anaerobic *m*-hydroxybenzoate pathway were also increased in abundance during aerobic growth with *m*-hydroxybenzoate, even though mostly to a lesser extent. Remarkably, HbcL and EbA736 were also strongly increased in cells grown aerobically with gentisate (20.8- and 15.3-fold, respectively), but none of the other proteins of the anaerobic *m*-hydroxybenzoate pathway. Conversely, NagI and NagK from the aerobic gentisate pathway also displayed an increased abundance under anoxic conditions (data not shown). As in case of the benzoate pathways, these results indicate a relaxed stringency of redox control of anaerobic *versus* aerobic degradation pathways.

m-Hydroxybenzoate may also function as gratuitous inducer for another possible operon, the products of which have diverse predicted functions (*e.g.* a subunit of *p*-cresol methylhydroxylase (PchF), a thiolase (TioL), or a carboxylase (XccAB) were identified) and are strongly increased in abundance (9.1- to 30.6-fold, see Fig. 4). However, one should consider that the observed induction of this operon may be erroneous, since transposases flanking the operon [12] could indicate remnants of genome evolution or reshuffling of the genome.

3.3.3 Phenylalanine and phenylacetate

Aerobic phenylalanine degradation in strain EbN1 is assumed to be channeled into the aerobic phenylacetate pathway (Fig. 1) for several reasons. (i) Abundant formation of the predicted proteins for aerobic phenylacetate degradation (Paa) in aerobically phenylalanine grown cells of strain EbN1 indicates intermediate formation of phenylacetyl–CoA, which is also known to induce *paa* gene expression in *E. coli* and *P. putida* U [53]. (ii) Substrate-specific enzyme activities and *paa* gene expression in *A. evansii* also support this pathway [7]. (iii) A catabolic route *via* tyrosine connecting to the homogentisate pathway as described for *P. putida* [54] seems unlikely, since genes encoding a phenylalanine hydroxylase (*phhA*; tyrosine forming) and *p*-hydroxyphenylpyruvate dioxygenase (*hpd*) could not be detected in the genome of strain EbN1 [12]. Furthermore, the only present gene from the tyrosine pathway, *tyrB*, is not upregulated in phenylalanine or phenylacetate grown cells of strain EbN1 (Fig. 5). (iv) Tyrosine does not support growth of strain EbN1 (data not shown).

In this study, three subunits of the ring hydroxylating enzyme (phenylacetyl–CoA oxygenase (PaaACE)) and the enzymes responsible for ring opening (enoyl–CoA hydratase/isomerase (PaaG) and aldehyde dehydrogenase (PaaZ)) of the aerobic phenylacetate degradation pathway were identified. The respective genes are encoded in a gene cluster (at approx. 2.1 Mb) bearing most of the genes of aerobic phenylacetate degradation, except for enoyl–CoA hydratase (*paaF*) and 3-ketoadipyl–CoA thiolase (*paaJ*). Another subunit of the ring hydroxylating enzyme (PaaC2) and PaaJ, encoded in a second gene cluster (at approx. 3.4 Mb), could also be identified.

All of these proteins were specifically formed during aerobic growth with phenylacetate and phenylalanine. Except for PaaJ and PaaE, the increase in abundance is slightly larger with phenylalanine than with phenylacetate. This is consistent with previous observations from adaptation studies with *A. evansii* [7].

Since Paa protein abundance was only increased in phenylalanine and phenylacetate grown cells, a strict regulatory circuit can be envisioned, with the predicted regulator PaaR displaying high specificity for the possible

A

Identified proteins[b]	Adaptation substrate[a]								
	Pal	Pac	BzOH	Bzal	pCr	Pol	pHB	mHB	oAB
DNA replication									
DnaN (EbA2847)	1.1	-1.1	-1.1	-1.1	-1.2	1.1	1.3	-1.3	1.0
Transcription									
RpoA (EbA3853)	-1.4	-1.2	-1.3	-1.3	-1.4	-1.1	-1.1	-1.3	-1.2
NusG (EbA3811)	1.0	1.2	-1.0	-1.0	1.3	2.1	1.6	1.2	1.1
GreA (EbA4816)	-1.6	-1.3	1.0	1.1	-1.6	1.1	-1.3	-1.1	-1.5
RseB (EbA5465)	1.5	-1.2	1.6	1.2	1.4	1.3	1.4	1.1	1.5
SpoOJ (EbA2907)	-1.0	-1.1	-1.1	-1.0	-1.4	-2.3	-1.1	1.2	1.1
SspA (EbA1195)	-1.0	1.1	1.0	1.0	1.0	1.4	1.1	-1.1	1.0
TldD (EbA969)	1.2	-1.0	1.2	1.2	-1.1	-1.0	-1.0	-1.2	1.2
Translation									
GatA (EbA3030)	-1.1	-1.2	-1.2	-1.1	-1.4	-1.0	-1.3	-1.2	-1.2
GatB (EbA3029)	-1.1	-1.2	1.2	1.2	1.0	1.1	1.0	-1.2	1.2
AspS (EbA4339)	-1.8	1.3	-1.5	-1.4	-1.7	-2.1	-2.0	-1.4	-1.5
TyrS (EbA931)	1.1	-1.0	1.2	1.1	1.4	-1.1	1.0	1.3	2.4
ProS (EbA950)	-2.0	1.3	-1.3	-1.3	-1.1	-2.6	-1.3	-1.1	-1.4
Translation factors									
EbA825	1.3	-1.6	1.2	1.2	1.1	-1.5	-1.1	-1.3	1.2
TufB (EbA3826)	-1.1	-1.0	-1.2	-1.2	-1.3	1.3	-1.1	-1.3	-1.3
PrfB (EbA5809)	-1.4	1.2	-1.5	-1.3	-1.6	-1.5	-1.0	-1.2	-1.3
Tsf (EbA5987)	-1.8	-1.1	-1.4	-1.3	-1.7	-1.5	-1.3	-1.4	-1.4
Efp (EbA2973)	-1.9	-1.0	-1.4	-1.5	-1.6	-1.8	1.0	1.1	1.2
Ribosomal proteins									
RpsA (EbA903)	-1.7	1.1	-1.5	-1.3	-1.8	-2.2	-1.6	-1.5	-1.5
RplY (EbA1409)	-1.5	1.1	-1.2	-1.3	-1.3	1.1	1.3	-1.1	-1.3
Other translational functions									
CafA (EbA3964)	1.4	-1.8	1.6	1.4	1.6	-1.2	-1.9	-1.7	-1.1
Heat shock proteins and chaperons									
GroEL (EbA1185)	-1.2	1.1	1.1	-1.2	1.3	-1.1	-1.2	-1.1	1.2
GroES (EbB35)	-1.1	1.0	1.1	-1.3	1.6	2.1	1.4	1.1	1.5
DnaK (EbA4794)	-2.3	1.2	-1.5	-1.7	1.0	-2.0	-2.2	-1.6	-1.5
HptG (4865)	-1.8	1.5	-1.2	-1.3	1.3	-1.7	-1.6	-1.2	-1.3
ClpB (EbA6667)	1.5	1.0	1.4	1.2	1.9	1.2	-1.4	-1.1	1.6
Pcm (EbA1645)	-1.1	1.3	1.2		1.1	1.3	1.7	1.1	
PpiB (EbA4788)	1.8	-1.4	1.7	1.5	1.7	1.7	1.7	1.1	1.8
PpiD (EbA6685)	1.2	-1.0	1.2	1.3	1.3	1.1	-1.1	1.1	1.2
Tig (EbA4969)	-1.4	1.1	-1.2	-1.0	-1.5	-1.7	-1.1	-1.1	-1.2
Cell envelope biosynthesis									
DdlB (EbA1442)	1.3	1.2	1.5	1.2	1.2	-1.6	1.5	1.4	1.3
OmpC (EbA4244)	-1.2	1.5	-1.3	-1.3	1.3	-1.3	1.9	-1.0	-1.2
Cell division									
MreB (EbA3033)	-1.2	1.1	-1.3	-1.3	-1.3	1.0	-1.3	-1.0	-1.3
Mrp (EbA4409)	1.2	-1.1	1.2	1.3	-1.1	-1.9	-1.2	-1.1	1.0
MinD (EbA6838)	1.5	1.0	1.4	1.3	1.5	1.6	1.5	1.3	1.6
Chemotaxis, motility and secretion									
SecB (EbA1056)	1.2	1.2	1.4	1.6	1.3	-1.0	1.4	1.3	1.1
PilM (EbA2266)	1.1	-1.2	1.2	1.2	1.3	1.5	1.4	1.7	1.9
Anaerobic benzyl alcohol degradation									
Adh (EbA3166)	1.0	-1.8	1.5	2.3	1.3	1.9	-1.2	-1.2	-1.2
Anaerobic benzoate degradation									
BcrB (EbA5284)	-1.6	1.1	-1.3	-2.0	-1.9	-1.4	1.3	-1.6	-1.2
BcrC (EbA5282)	-1.7	1.0	-1.4	-1.9	-1.4	-1.1	1.5	-1.7	-1.4
BcrD (EbA5286)	-2.0	-1.3	-1.5	-1.7	-1.6	-2.2	1.1	1.2	1.4
BzdZ (EbA5300)	-1.5	-1.6	-1.2	-1.4	-1.1	-1.3	1.2	-1.2	-1.4
Dch (EbA5296)	-1.5	-1.4	-1.4	-1.6	-1.3	-2.1	1.1	-1.1	-1.3
Oah (EbA5298)	-2.1	1.0	-1.4	-1.4	-1.5	-2.0	-1.5	-1.1	-2.0
EbA5307	1.2	-1.6	1.2	-1.1	-1.1	1.3	1.4	1.0	1.0
Orf3 (EbA5292)	-1.5	-1.7	-1.5	-1.5	-1.5	-1.2	1.5	-1.1	-1.3
Aerobic benzoate degradation									
BoxC (EbA2763)	-1.5	-1.5	1.3	1.6	-1.3	-1.2	-1.3	-1.1	-1.6
Anaerobic propionate degradation									
AccC (EbA1474)	1.7	1.2	1.1	1.1	-1.1	1.9	1.2	-1.0	1.3
Glycolysis and gluconeogenesis									
GpmA (EbA1052)	-1.2	1.4	1.2	1.1	1.4	-1.0	1.5	1.3	1.2
Pgk (EbA1103)	-1.1	-1.3	-1.2	-1.1	-1.6	-1.2	-1.1	1.1	1.2
FbaA (EbA1107)	1.9	1.3	1.5	1.2	1.2	1.4	1.1	1.2	1.6
Eno (EbA6162)	1.0	-1.2	1.1	1.2	1.2	-1.5	-1.0	-1.2	-1.4
Fbp (EbA1191)	1.1	-1.2	1.1	1.0	-1.1	-1.2	1.2	-1.1	-1.0
Glucose degradation									
EpsT (EbA6378)	1.2	1.1	1.2	1.2	-1.1	-1.4	-1.0	1.1	1.1
Pentose phosphate pathway									
Rpe (EbA4173)	1.3	1.5	1.2	1.3	-1.2	-2.0	1.2	1.3	1.1
TCA-cycle									
FumA (EbA173)	-1.2	1.3	-1.0	-1.1	-1.0	-1.8	-1.1	1.1	1.1
Icd (EbA829)	1.8	-1.9	1.1	1.3	1.9	1.8	1.3	-1.2	1.6
SucD (EbA1271)	2.4	1.1	1.3	1.2	1.4	2.3	1.5	1.0	1.5
GltA (EbA6687)	-1.1	-1.3	-1.3	-1.1	-2.1	-1.8	-1.9	-1.4	-1.3
SdhA (EbA6690)	1.5	1.1	1.2	-1.2	-1.1	1.2	-1.1	1.2	1.5
SdhB (EbA6689)	1.7	1.3	1.2	1.2	1.8	1.7	1.2	1.1	1.4
Respiratory energy generation									
AtpA (EbA3004)	-1.1	-1.1	-1.1	-1.1	-1.4	-1.3	-1.4	-1.6	-1.5
AtpD (EbA3007)	-1.1	1.3	-1.1	-1.1	-1.3	1.2	-1.1	-1.3	-1.2
NuoF (EbA4840)	-1.6	-1.0	-1.3	-1.1	-1.4	-1.9	-1.5	-1.2	-1.5
SdhA (EbA6690)	1.5	1.1	1.2	-1.2	-1.1	1.2	-1.1	1.2	1.5
SdhB (EbA6689)	1.7	1.3	1.2	1.2	1.8	1.7	1.2	1.1	1.4
Oxidative phosphorylation									
Ppa (EbA7053)	2.0	1.4	1.8	1.8	1.9	2.1	2.5	1.7	2.0
Acyl-CoA dehydrogenases									
EbA3640	1.2	-1.2	1.4	-1.0	-1.2	-1.4	-1.5	-1.8	-1.4
GcdH (EbA2993)	-1.5	-1.0	-1.6	-1.6	-2.2	-2.3	-1.7	-1.0	-1.7
Electron transfer flavoproteins									
EtfA (EbA6510)	1.0	-1.3	1.7	1.9	1.1	-1.1	1.3	-1.0	-1.1
Enoyl-CoA hydratases/isomerases									
	1.4	-1.2	1.3	1.4	1.0	-1.0	1.4	1.0	1.2
EbA2313	-1.1	1.0	-1.0	-1.1	-1.1	-1.3	1.0	1.1	1.1
EbA6516	2.4	-1.1	2.1	1.8	1.6	-1.5	1.5	1.6	1.9

Identified proteins[b]	Adaptation substrate[a]								
	Pal	Pac	BzOH	Bzal	pCr	Pol	pHB	mHB	oAB
Short chain alcohol dehydrogenases									
EbA2528	-1.0	-1.1	1.2	1.1	1.6	-1.1	1.1	1.6	1.1
HbdA (EbA4742)	-1.2	-1.2	-1.3	-1.3	-1.4	-1.7	-1.3	-1.6	-1.3
EbA5320	1.9	-1.2	1.4	1.5	1.7	2.1	1.9	1.2	1.5
Thiolases									
EbA5202	2.3	1.1	1.6	1.6	2.1	1.8	1.1	1.1	1.2
FeS cluster biosynthesis									
HscA (EbA6396)	-1.4	-1.3	-1.3	-1.3	-1.2	-1.9	-1.3	-1.2	1.1
Cytochrome c biosynthesis									
HemL (EbA1743)	1.3	1.1	1.4	1.3	1.5	1.1	1.1	1.1	1.3
HemE (EbA2822)	1.0	1.1	1.0	1.1	-1.2	-1.6	-1.1	-1.0	-1.1
Oxygen and NO detoxification									
KatA (EbA2102)	-1.1	1.0	2.2	2.1	2.2	1.2	1.7	2.3	2.1
Peroxidases									
AhpC (EbA4399)	1.1	-1.7	-1.2	-1.2	-1.4	-1.5	1.1	1.2	-1.0
ATP-binding proteins									
EbA1337	1.2	-1.3	1.3	1.2	1.5	1.4	1.1	-1.2	1.1
Proteases									
MucD (EbA2969)	1.0	-1.4	1.2	1.3	1.3	-1.1	-1.8	-2.4	-1.5
Protein kinases									
PrkA (EbA4966)	1.9	-1.9	1.4	1.7	1.5	-1.0	-1.1	-1.3	1.3
Amino acid synthesis									
MetK (EbA3942)	-1.1	1.0	-1.1	-1.1	-1.2	1.3	1.0	-1.3	-1.3
EbA5412	2.0	-1.2	2.0	1.4	1.9	-1.1	1.4	1.2	1.5
alpha-ketoglutarate family									
ArgB (EbA4330)	1.4	-1.2	1.5	1.3	1.4	1.3	1.3	1.1	1.4
ArgG (EbA5097)	-1.1	1.1	-1.1	-1.1	-1.4	-2.0	-1.3	-1.1	-1.1
GdhA (EbA5425)	-1.2	1.1	1.1	1.1	-1.2	-2.3	-1.3	-1.1	-1.1
CarA (EbA4813)	-1.4	1.2	1.3	1.1	-1.1	-1.1	-1.1	1.1	1.2
EbA2547	-1.0	-1.1	-1.1	-1.1	-1.4	-1.0	-1.4	-1.2	-1.3
aspartate family									
DapA (EbA774)	1.4	1.1	1.4	1.4	1.2	-1.0	1.5	1.4	1.4
LysA (EbA2271)	1.2	-1.1	1.1	-1.1	1.1	-1.3	1.3	1.1	-1.1
DapF (EbA3950)	1.7	-1.3	1.5	1.6	1.9	2.1	1.5	1.2	1.5
Asd (EbA4761)	1.1	1.3	1.2	1.2	-1.0	-2.0	1.0	1.2	1.1
DapB (EbA4812)	1.5	-1.2	1.5	1.3	1.3	1.1	1.4	1.1	1.5
ThrA (EbA4952)	-1.4	-1.2	-1.2	-1.3	-1.5	-1.9	-1.1	-1.2	-1.2
DapD (EbA6386)	1.1	1.0	1.3	1.2	1.4	-1.0	1.2	1.1	1.2
pyruvate family									
IlvE (EbA993)	-1.1	1.1	1.2	1.0	-1.1	-1.1	-1.1	-1.0	1.5
LeuC (EbA4757)	1.5	2.1	1.8	1.1	1.8	-1.3	1.5	1.6	1.7
LeuD (EbA4758)	1.8	1.7	1.6	1.2	1.5	1.2	1.9	1.7	2.0
LeuB (EbA4760)	1.1	1.1	1.2	-1.1	1.1	-1.7	1.1	1.1	1.2
IlvC (EbA7134)	-1.3	1.1	-1.1	1.0	-1.5	-1.5	-1.4	-1.2	-1.3
LeuA (EbA7154)	-1.4	1.2	1.0	-1.4	-1.2	-2.0	-1.2	-1.0	1.1
TyrB (EbA5437)	-1.2	-1.2	-1.0	-1.2	-1.3	-1.9	-1.5	-1.4	-1.3
triose family									
CysM (EbA896)	-1.6	-1.3	1.0	-1.1	-1.7	-2.4	-1.2	-1.2	1.1
SerC (EbA907)	1.6	-1.2	1.4	1.4	1.2	-1.1	1.3	1.0	1.3
CysH (EbA2623)	-1.3	-1.2	1.2	1.1	-1.1	-1.8	-1.1	-1.0	-1.1
CysD (EbA2625)	1.1	1.0	1.1	1.1	1.1	1.1	1.3	1.1	1.4
CysK (EbA6678)	1.7	-1.2	1.6	1.5	1.4	2.0	1.8	1.4	2.0
CysP (EbA6204)	1.6	-1.4	1.4	1.1	1.7	2.3	2.2	1.4	2.4
aromatic amino acid family									
TrpC (EbA4201)	1.4	1.1	1.4	1.3	1.4	-1.4	1.5	1.3	1.4
TnaA (EbA605)	-1.0	-1.6	-1.2	-1.2	1.0	-2.2	-1.8	-1.8	-1.5
histidine									
HisF (EbA1291)	1.4	1.1	1.3	1.2	1.4	1.2	1.6	1.4	1.5
Purine and pyrimidine synthesis									
PurH (EbA1153)	-1.5	-1.1	-1.0	-1.1	-1.4	-1.9	-1.7	-1.6	-1.6
PurD (EbA1155)	1.1	-1.2	1.0	1.1	-1.0	-1.4	-1.1	-1.2	-1.1
PurM (EbA7104)	-1.0	-1.0	1.0	1.1	-1.0	-1.2	-1.1	-1.0	1.3
Adk (EbA5087)	1.7	-1.2	1.5	1.5	1.3	1.5	1.5	1.1	1.3
TmB (EbA7042)	-2.1	-1.6	-1.1	1.1	-1.3	1.1	-1.1	1.2	1.5
Lipid synthesis									
FabD (EbA5456)	-1.2	-1.1	-1.1	-1.2	-1.4	-1.6	-1.2	-1.1	-1.2
FabF (EbA5459)	-1.1	1.1	-1.1	-1.2	-1.1	-1.3	1.2	-1.2	1.1
FabI (EbA6664)	1.2	1.0	1.4	1.2	1.1	1.0	1.2	1.2	1.3
Riboflavin synthesis									
RibAB (EbA3567)	1.1	-1.1	-1.0	1.0	2.0	1.9	1.3	1.4	1.6
RibE (EbA3569)	1.0	-1.2	1.2	1.0	-1.1	-2.2	1.2	-1.0	-1.1
Folate synthesis									
PabA (EbA4199)	1.4	-1.1	1.2	1.2	1.1	1.7	1.6	1.2	1.5
Panthothenic acid / CoA synthesis									
PanC (EbA7120)	1.6	1.2	1.4	1.1	1.7	1.6	1.9	1.4	2.1
Terpenoid synthesis									
IspA (EbA4440)	1.1	-1.0	1.4	1.3	1.0	1.4	1.4	1.3	2.0
IspH (EbA4444)	1.9	-1.1	1.6	1.6	1.3	-1.7	1.3	1.1	1.5
Polyhydroxybutyrate synthesis and degradation									
PhbC (EbA3628)	-1.1	1.1	-1.1	-1.1	-1.9	-1.0	-1.9		-1.2
PhbR (EbA4732)	-1.1	-1.1	1.0	1.1	1.2	-1.0	1.4	1.2	1.3
Biosynthesis of molybdenum cofactor									
EbA2717	1.3	-1.2	1.1	1.0	1.2	1.3	1.4	-1.0	1.1
Mog (EbA1027)	1.2	-1.3	1.2	1.0	1.6	1.5	1.3	1.0	1.4
Biosynthesis of cofactors and prosthetic groups									
FolD (EbA119)	1.3	-1.1	1.1	1.1	-1.3	-1.1	1.0	1.2	-1.0
Unknown function									
IorA (122)	-1.9	-1.0	-1.3	-1.2	-1.5	-2.2	-2.3	-1.5	1.1
Hypothetical proteins									
EbA8332	-1.5	1.2	-1.2	-1.2	1.0	-1.2	-1.4	-1.1	-1.1
Conserved hypothetical proteins									
EbA1403	1.1	1.1	1.2	1.2	1.5	1.2	1.2	1.3	1.3
EbA3532	-1.4	1.0	1.4	1.6	1.7	-1.2	2.4	2.0	1.5
EbA3951	1.2	-1.7	1.1	-1.1	1.3	1.7	1.4	-1.1	1.2
EbA5268	-2.2	-1.7	-1.5	-1.2	1.1	1.7	-1.4	-1.8	-1.6
EbA6073	1.5	-1.1	1.9	2.1	2.2	1.2	2.3	2.2	1.9
EbA6152	1.2	1.1	1.2	1.2	1.9	-1.6	1.6	1.7	1.9

B

Identified proteins[b]	Adaptation substrate[a]				
	Bz	Gen	Pal	Pac	mHB
Transcription					
Fur (EbB167)	-1.6	1.0	1.1	-1.1	1.1
Translation					
AspS (EbA4339)	-1.1	1.5	1.0	-1.1	1.1
Translation factors					
TufB (EbA3826)	-1.0	-1.1	-1.0	1.5	-2.0
Tsf (EbA5987)	-1.2	-1.1	-1.2	-1.0	-1.3
Heat shock proteins and chaperons					
GroEL (EbA1185)	1.1	-1.1	1.0	-1.1	1.0
GroES (EbB35)	-1.4	-1.2	-1.1	-1.3	-1.1
DnaK (EbA4794)	1.2	-1.1	-1.1	1.1	1.6
HptG (4865)	-1.1	-1.4	-1.3	1.0	1.0
ClpB (EbA6667)	-1.3	1.0	-1.1	-1.2	1.3
EbA1594	-1.03	1.33	-1.35	1.25	-1.36
Tig (EbA4969)	-1	-1.06	-1.1	1.18	-1.13
Cell envelope biosynthesis					
DdlB (EbA1442)	-1.3	1.1	1.1	-1.3	-1.3
Cell divison					
MinD (EbA6838)	-1.5	1.0	-1.1	-1.2	-1.6
Transporter					
EbA2168	-1.22	1.09	1.38	-1.32	1.17
Anaerobic benzoate degradation					
BzdZ (EbA5300)	1.41	-1.36	-1.08	1.3	1.63
Glycolysis and gluconeogenesis					
Pgk (EbA1103)	1.8	1.2	1.3	1.4	1.5
Glucose degradation					
EpsT (EbA6378)	-1.28	-1.05	1.02	-1.21	-1.41
TCA-cycle					
Icd (EbA829)	2.0	1.1	2.2	1.7	1.2
AcnB (EbA5265)	-1.2	-1.1	-1.4	-1.7	-1.7
Lpd (EbA136)	-1.09	2.29	1.21	-1.03	1.7
Respiratory energy generation					
AtpD (EbA3007)	-1.2	-1.6	-1.1	-1.2	-1.4
Electron transfer flavoproteins					
EtfA (EbA6510)	1.4	1.3	1.1	1.5	1.1
Enoyl-CoA hydratases/isomerases					
Ech (EbA1321)	-1.3	-1.2	1.2	-1.6	-2.2
FeS cluster biosynthesis					
HscA (EbA6396)	1.0	-1.2	1.2	-1.0	-1.2
Oxygen and NO detoxification					
SodB (EbA5077)	1.3	1.1	2.1	1.1	1.1
Gst (EbA5076)	-1.26	1.08	-1.19	1.92	-1.52
Peroxidases					
AhpC (EbA4399)	1.5	1.9	1.3	1.1	1.9
ATP-binding proteins					
EbA1337	-1.52	1.02	-1.02	-1.23	-1.24
Amino acid synthesis					
aspartate family					
DapD (EbA6386)	-1.6	-1.2	-1.3	-1.0	-1.5
pyruvate family					
LeuB (EbA4760)	-1.4	-1.3	-1.5	-1.4	-1.3
TyrB (EbA5437)	1.19	1.04	1.22	-1.05	1.16
Purine and pyrimidine synthesis					
PurM (EbA7104)	-1.2	-1.2	-1.0	-1.1	-1.5
PrtAA (EbA4497)	1.8	1.07	1.57	1.48	1.16
Lipid synthesis					
AcpD (EbA3879)	1.9	1.4	-2.4	2.0	2.0
Terpenoid synthesis					
IspB (EbA4512)	-1.2	-1.0	-1.1	-1.1	-1.9
Biosynthesis of molybdenum cofactor					
Mog (EbA1027)	1.22	1.1	1.36	1.27	1.97
Biosynthesis of cofactors and prosthetic groups					
FolD (EbA118)	-1.33	-1.09	-1.04	-1.08	-1.23
Hypothetical					
EbA3488	-1.44	1.04	1.01	-1.35	-1.3
Conserved hypothetical proteins					
EbA3951	-1.2	1	-1.03	-1.23	1.11
EbA6101	-1.43	-1.59	-1.72	-1.29	-1.68

C

Identified proteins[b]	Adaptation substrate[a]				
	PrOH	Act	BuOH	Bun	Prp
DNA replication					
DnaN (EbA2847)	-1.4	-1.3	-1.2	-1.3	-1.1
Transcription					
RpoA (EbA3853)	-1.8	-1.5	-1.6	-1.4	-1.1
Translation					
TyrS (EbA931)	-1.3	-1.5	-1.7	-1.5	-1.9
Translation factors					
Efp (EbA2973)	-1.5	-1.2	-1.2	-1.2	-1.0
Ribosomal proteins					
RplI (EbA4078)	-1.6	-1.2	-1.4	-1.3	-1.1
Heat shock proteins and chaperons					
GroEL (EbA1185)	-1.0	1.1	1.1	1.2	1.0
GroES (EbB35)	-1.1	1.1	-1.0	1.1	1.1
HptG (4865)	-1.3	1.2	-1.1	1.1	1.9
Outer membrane proteins					
EbA5996	1.35	1.39	1.45	1.48	-1.33
Anaerobic benzoate degradation					
BcrD (EbA5286)	-1.42	-1.11	1.05	1.35	-1.15
TCA-cycle					
SucB (EbA6684)	-1.3	-1.4	-1.7	-1.5	-1.9
AceF (EbA135)	1.1	1.5	1.9	1.9	2.5
Respiratory energy generation					
AtpD (EbA3007)	-1.4	-1.4	-1.5	-1.4	-1.5
Acyl-CoA dehydrogenases					
PrpE (7220)	-1.2	-1.8	-1.0	-1.0	-1.5
EbA3640	-1.2	-1.5	2.1	2.3	1.3
EbA6506	1.2	-1.1	1.5	1.3	2.2
Electron transfer flavoproteins					
EtfA (EbA6510)	1.4	1.4	1.6	1.5	2.1
Oxygen and NO detoxification					
SodB (EbA5077)	1.1	-1.2	-1.1	-1.2	-1.3
Peroxidases					
AhpC (EbA4399)	1.0	-1.4	-1.1	-1.1	-1.3
Amino acid synthesis					
EbA5412	1.31	-1.07	1.19	1.02	-1.13
pyruvate family					
TyrB (EbA5437)	-1.1	-1.03	-1.09	-1.06	1.05
Purine and pyrimidine synthesis					
Adk (EbA5087)	1.29	1.17	1.2	1.12	1.18
Hypothetical protein					
EbA2178	-1.02	-1.38	-1.18	-1.36	1.06
Conserved hypothetical protein					
EbA4217	-1.53	-1.12	-1.18	-1.19	1.13

Fold change

Figure 5. Identified proteins comprising the constitutive subproteome and their fold changes of protein abundance across the analyzed growth conditions: anaerobic growth (A) or aerobic growth (B) with aromatic compounds and anaerobic growth with aliphatic alcohols, ketones, and carboxylic acids (C). Changes in protein abundance are defined as nonsignificant in the present study, when the determined fold change is below the threshold of significance ($<|2.5|$). Color codes indicate different degrees of fold changes within the defined window of nonsignificance ($<|2.5|$). (a) Cells were adapted over at least five passages to the respective growth condition. Fold changes were determined on the basis of five parallel gels; benzoate- or acetate-grown cells were used as reference state. For details see Section 2. Substrate abbreviations: (Ac) acetate, (Act) acetone, (BuOH) 2-butanol, (Bun) 2-butanone, (Bz) benzoate, (Bzal) benzaldehyde, (BzOH) benzyl alcohol, (Gen) gentisate, (mHB) *m*-hydroxybenzoate, (oAB) *o*-aminobenzoate, (Pac) phenylacetate, (Pal) phenylalanine, (pCr) *p*-cresol, (pHB) *p*-hydroxybenzoate, (Pol) phenol, (PrOH) 2-propanol, (Prp) propionate. (b) Protein abbreviations are as described in ref. [12]. (c) The maximal fold change is 2.5.

inductor phenylacetyl–CoA. It should be noted, however, that Paa proteins were also slightly increased in cells grown anaerobically with phenylalanine or phenylacetate.

3.4 Anaerobic growth with aliphatic alcohols, ketones, and carboxylic acids

3.4.1 2-Propanol, 2-butanol, acetone, and 2-butanone

Surprisingly, no short-chain alcohol dehydrogenase possibly involved in the oxidation of the tested alcohols to the respective ketones, could be identified. One may speculate that the involved alcohol dehydrogenase is constitutively formed or escaped the applied analytical window.

In contrast, all subunits of acetone carboxylase (AcxABC) and succinyl–CoA:3-ketoacyl–CoA transferase (KctAB) were identified in the present proteomic study displaying highly increased abundances (ranging from 8.6- to 185.6-fold) during anaerobic growth with either of the two alcohols or ketones. No increase in abundance was observed, however, during anaerobic growth with propionate (Fig. 4). Substrate-dependent regulation is probably mediated by the regulator AcxR, which is encoded in a gene cluster together with *acxABC* and *kctAB*. Similar regulation of the acetone carboxylase has also been shown for *Xanthobacter autotrophicus*,

Rhodococcus rhodochrous B276, and *Rhodobacter capsulatus* strain B10 [55–58]. Analogous regulatory patterns of AcxABC in acetone and 2-butanone grown cells of strain EbN1 indicated that the same enzyme was used for the carboxylation of the two different ketones. This is supported by previous results with the purified enzymes of *R. rhodochrous* and *X. autotrophicus* [58].

The *acx* and *kct* genes of strain EbN1 frame genes for one acetyl–CoA synthase and four hypothetical proteins, together representing a large operon-like structure (Fig. 3). Three of the latter (EbA4705, EbA4712, and EbA4715) were highly increased in abundance in response to the alcohols and ketones. Most interestingly, EbA4712 displayed the most pronounced increase in abundance (409.3- to 754.7-fold) of the entire study. Furthermore, EbA4715 had an unusually high share of more than 11% from the total 2-DE-separated protein; for comparison, acetone carboxylase (AcxABC) had a maximal share of 6.2%. Considering that no *ebA4705*, *ebA4712*, or *ebA4715* homologs could be detected in the available genome sequences of *X. autotrophicus* Py2 and *Rhodobacter sphaeroides* (www.jgi.doe.gov), one may speculate that their products serve essential, hitherto unknown functions in anaerobic ketone degradation of strain EbN1.

3.4.2 Propionate

Propionyl–CoA ligase (PrpE), a subunit of propionyl–CoA carboxylase (PccA) as well as a subunit of biotin carboxylase (AccC), possibly delivering carboxylated biotin carrier protein, were identified. PccA was increased in abundance during growth with propionate (8.1-fold), 2-butanol (6.3-fold), and butanone (7.3-fold), but not with 2-propanol and acetone. This agreed with the methylmalonyl–CoA pathway not being required for the degradation of the latter two compounds. Surprisingly, PrpE and AccC were not differentially regulated, raising doubts about their role in the propionate degradation. Also no subunits of methylmalonyl–CoA mutase (SbmAB) were identified in this main physiological group (iii).

3.5 Regulated subproteomes

3.5.1 Extent of regulation

The high number of regulated proteins (199 identified) is contrasted by a suite of constitutive proteins (155 identified) displaying highly stable abundances (a general overview of all identified proteins with respect to observed maximal fold changes in abundance is presented in Supporting Information 1, Fig. 1.4.). The vast majority of regulated proteins displayed increases in abundance with fold changes ranging from 2.5 to 50. The highest numbers of proteins with increased abundance were observed during anaerobic growth with aromatic compounds. For example, >200 protein spots with changed abundance were observed during anaerobic growth with phenol (reference benzoate; this study). In fact, this number should be even higher, since additional >120 protein spots are specifically formed during anaerobic growth with benzoate (reference acetate; data not shown). In contrast, only >115 protein spots with changed abundance were detected during aerobic growth with *m*-hydroxybenzoate and 70 during anaerobic growth with 2-butanol. These higher numbers of proteins with changed abundance probably reflect the complex and diverse biochemical reactions and adaptations involved in anaerobic degradation of aromatic compounds.

The metabolic relevance of a regulated protein is not only reflected by the degree of its fold change, but also by its relative abundance (share from total 2-DE-separated protein), as exemplified in the following (see also Supporting Information 1: Table 1.1). During anaerobic growth with aromatic compounds on average >100 protein spots (out of ~1300) were changed in abundance, comprising ~20% of the total 2-DE-separated protein. During anaerobic growth with 2-propanol or 2-butanol only about 60 protein spots (out of ~1000) were found to be regulated. However, they comprised more than 40% of the total 2-DE-separated protein, emphasizing their prominent metabolic role and the relatively simple metabolic context.

In addition to proteins with assigned pathway functions, multiple other proteins were also specifically increased in abundance under each analyzed growth condition. These additional proteins displayed other predicted or unknown function (Fig. 6). This unexpected finding may indicate that some of the pathways involve more protein components than previously known (*e.g.* highly specific formation of EbA4705, EbA4712, and EbA4715 – all of unknown function – during anaerobic degradation of ketones), that a certain degree of unspecific regulation occurs or that the toxic properties of aromatic compounds (*e.g. p*-cresol and phenol) activate stress response mechanisms.

3.5.2 Specificity of regulation

The large number of regulatory proteins predicted from the genome of strain EbN1 suggested a fine-tuned regulatory network to adapt to changing growth conditions [12]. The present comprehensive study allows for the first time to assess this hypothesis on an experimental basis. In fact, various degrees of regulatory specificity were observed depending on individual substrate and redox conditions.

In case of anaerobic aromatic compound degradation, individual pathways generally displayed a rather strict regulation in response to the respective substrate. Furthermore, also modular pathways were strictly regulated, *e.g.* phenylacetate only induced the formation of proteins involved in its degradation (lower pathway), but not those converting phenylalanine to phenylacetate (upper pathway). Exceptions were proteins, which were previously not correlated to any of the pathways but were increased in abundance in response to several different aromatic compounds (*e.g.* EbA1033).

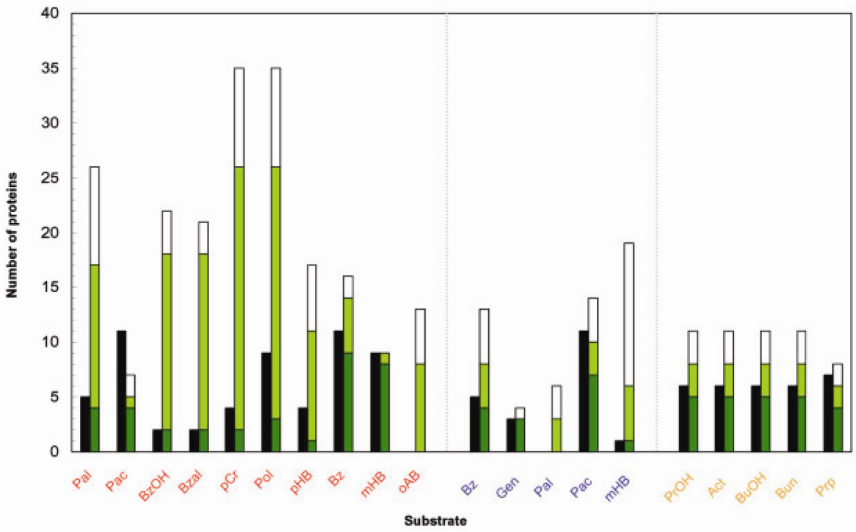


Figure 6. Number of proteins involved in the analyzed pathways predicted from genome analysis *versus* identified upregulated proteins as determined by proteomics. Abbreviations are as described in legend to Fig. 4. Red indicates anaerobic growth with aromatic compounds, blue aerobic growth with aromatic compounds, and orange anaerobic growth with aliphatic alcohols, ketones, and carboxylic acids. Proteins with assigned pathway functions, as previously predicted by genome analysis (black bars). Specifically upregulated and identified (this study) proteins with assigned functions in pathway (dark green bars), with other predicted functions (light green bars), or with unknown functions (open bars).

In case of anaerobic growth with aliphatic alcohols, ketones and carboxylic acids also a high degree of regulatory specificity was observed. For example, propionyl–CoA carboxylase of the propionate pathway was only formed during growth with propionate, 2-butanol and 2-butanone, agreeing with their degradation *via* the methylmalonyl–CoA pathway.

In case of aerobic aromatic compound degradation, the specificity of regulation was less pronounced than under anoxic conditions. While the proteins of a given degradation pathway generally displayed highest fold changes in the presence of the respective substrate, they were also formed in the presence of other substrates, indicating a certain degree of crossregulation on the substrate level (Fig. 4). For some aromatic compounds (*e.g.* benzoate), strain EbN1 possesses next to the aerobic also an anaerobic degradation pathway. Interestingly, these anaerobic pathways were also induced under oxic conditions, even though to a lesser extent. Supporting these proteomic findings, low-level expression of benzoyl–CoA reductase during aerobic growth with aromatic compounds was also observed in *T. aromatica* [36]. Conversely, low abundance of proteins involved in aerobic benzoate degradation was observed in cells of strain EbN1 grown anaerobically with benzoate (reference state: anaerobic growth with acetate; data not shown). These unexpected low-level protein formation may be due to relaxed regulatory specificities, but one may also speculate that it serves to maintain a basal level of enzymes, allowing immediate response to the emergence of the respective environmental conditions.

Overall, comparison of anoxic and oxic conditions revealed that also in strain EbN1 the latter have an overruling regulatory effect, even though not with absolute stringency. Considering the presence of several terminal oxidases with different O_2-affinities in strain EbN1, it will be interesting to study the fine-tuned response to varying O_2 partial pressures.

3.6 Constitutive subproteome

Overall, 92% of all detected protein spots were not significantly changed in abundance and therefore attributed to the constitutive subproteome (Supporting Information 1: Table 1.1.). Most of the identified nonregulated proteins possess functions of general cell metabolism (*e.g.* TCA-cycle or pyruvate metabolism), biosynthesis (*e.g.* amino acid or tRNA synthesis), or have cellular functions (energy conservation, detoxification, DNA/RNA metabolism, transcription or translation). Notably, the central metabolic pathways (*e.g.* glycolysis and TCA-cycle) could be almost completely reconstructed on the basis of identified proteins (Fig. 5 and Supporting Information 4 and 5). Interestingly, the constitutive proteins (155 identified) displayed a low average fold change in abundance of ~|1.3|-fold (SD ± 0.30; Fig. 5), being only slightly affected by changing growth conditions. For example, the fold changes of GroEL abundances ranged between 1.07 and 1.16 (SD_{max} ± 0.07) across all tested growth condi-

tions. Such marginally changed protein abundances were previously also observed for TCA-cycle enzymes of *Rhodopirellula baltica* [59].

Expanding this analysis to all detected, nonregulated protein spots (representing mostly unidentified proteins) also yielded highly similar fold changes. For example, in the group of anaerobic aromatic compound degradation, the average fold change only increased from 1.34 (SD ± 0.31; based on 146 identified protein spots) to 1.36 (SD ± 0.32; based on 693 identified as well as unidentified protein spots).

3.7 Hypothetical proteins

A total of 37 hypothetical or conserved hypothetical proteins were identified in the present study, demonstrating their principle formation for the first time. Eighteen of them were specifically increased in abundance under at least one of the analyzed growth conditions, the majority (12) being regulated during anaerobic growth with aromatic compounds (Figs. 4 and 6). Often, the coding genes are not located in close proximity to the designated pathway genes, impairing a possible functional assignment based on the observed coregulation. Prominent exceptions are the hypothetical proteins EbA4705, EbA4712, and EbA4715, which could be correlated to anaerobic ketone degradation (see above).

4 Concluding remarks

The comprehensiveness of the present study allows unprecedented insights into the metabolic regulation of a relevant environmental bacterium on the proteomic level. The majority of the predicted pathways (for 22 different growth conditions) could be confirmed by the differential proteomic data. In case of three pathways (anaerobic degradation of phenylalanine, benzyl alcohol, and *p*-cresol), the predicted proteins could not be confirmed. In fact, the differential protein profiles indicated the involvement of other thus far not considered proteins (*e.g. p*-cresol methylhydroxylase). In case of the previously unknown growth substrates *o*-aminobenzoate (anoxic), phenylalanine, and *m*-hydroxybenzoate (both oxic), respective degradation pathways could not be predicted by genome analysis. In all three cases, substrate-specific protein formation advanced our catabolic understanding (*e.g.* discovery of a putative *m*-hydroxybenzoate hydroxylase). In addition, the extent of regulated proteins demonstrates the high-regulatory flexibility and thereby environmental adaptability of strain EbN1, as previously predicted by genome analysis.

Overall, the present study demonstrates the benefit of physiological proteomics for the functional interpretation of genomic data, by actually verifying *in silico* predictions and providing unexpected functional perspectives based on coregulation. The latter generated multiple new hypotheses for future genetic and biochemical followup studies.

We are grateful to A. Beck for expert bioinformatics support, D. Gade for initial support with 2-DE, and F. Widdel for general support of proteomic studies in our group. This study was supported by the Deutsche Forschungsgemeinschaft, the Max-Planck-Society, Bruker Daltonics and GE Healthcare.

5 References

[1] Koch, R., Wagner, B. O., *Umweltchemikalien: Physikalisch-chemische Daten, Toxizitäten, Grenz- und Richtwerte, Umweltverhalten*, VCH Verlagsgesellschaft, Weinheim 1989.

[2] Gibson, D. T., Parales, R. E., *Curr. Opin. Biotechnol.* 2000, *11*, 236–243.

[3] Harwood, C. S., Parales, R. E., *Annu. Rev. Microbiol.* 1996, *50*, 553–590.

[4] Heider, J., Fuchs, G., *Eur. J. Biochem.* 1997, *243*, 577–596.

[5] Gibson, J., Harwood, C. S., *Annu. Rev. Microbiol.* 2002, *56*, 345–369.

[6] Zaar, A., Gescher, J., Eisenreich, W., Bacher, A., Fuchs, G., *Mol. Microbiol.* 2004, *54*, 223–238.

[7] Mohamed, M. E. S., Ismail, W., Heider, J., Fuchs, G., *Arch. Microbiol.* 2002, *178*, 180–192.

[8] Spormann, A. M., Widdel, F., *Biodegradation* 2000, *11*, 85–105.

[9] Heider, J., Spormann, A. M., Beller, H. R., Widdel, F., *FEMS Microbiol. Rev.* 1998, *22*, 459–473.

[10] Widdel, F., Rabus, R., *Curr. Opin. Biotechnol.* 2001, *12*, 259–276.

[11] Rabus, R., Widdel, F., *Arch. Microbiol.* 1995, *163*, 96–103.

[12] Rabus, R., Kube, M., Heider, J., Beck, A. *et al.*, *Arch. Microbiol.* 2005, *183*, 27–36.

[13] Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J. *et al.*, *Environ. Microbiol.* 2003, *5*, 630.

[14] Schneiker, S., dos Santos, V., Bartels, D., Bekel, T. *et al.*, *Nat. Biotechnol.* 2006, *24*, 997–1004.

[15] Larimer, F. W., Chain, P., Hauser, L., Lamerdin, J. *et al.*, *Nat. Biotechnol.* 2004, *22*, 55–61.

[16] Gallegos, M. T., Williams, P. A., Ramos, J. L., *J. Bacteriol.* 1997, *179*, 5024–5029.

[17] Leuthner, B., Heider, J., *FEMS Microbiol. Lett.* 1998, *166*, 35–41.

[18] Peres, C. M., Harwood, C. S., *J. Bacteriol.* 2006, *188*, 8662–8665.

[19] Kühner, S., Wöhlbrand, L., Fritz, I., Wruck, W. *et al.*, *J. Bacteriol.* 2005, *187*, 1493–1503.

[20] Hufnagel, P., Rabus, R., *J. Mol. Microbiol. Biotechnol.* 2006, *11*, 53–81.

[21] Champion, K. M., Zengler, K., Rabus, R., *J. Mol. Microbiol. Biotechnol.* 1999, *1*, 157–164.

[22] Gade, D., Thiermann, J., Markowsky, D., Rabus, R., *J. Mol. Microbiol. Biotechnol.* 2003, *5*, 240–251.

[23] Bradford, M. M., *Anal. Biochem.* 1976, *72*, 248–254.

[24] Heukeshoven, J., Dernick, R., *Electrophoresis* 1988, *9*, 28–32.

[25] Wold, H., in: P. R.Krishnaiah (Ed.), *Estimation of Principle Components and Related Models by Iterative Least Squares*

in Multivariate Analysis, Academic press, NY 1966, pp. 391–420.

[26] Doherty, N. S., Littman, B. H., Reilly, K., Swindell, A. C. *et al.*, *Electrophoresis* 1998, *19*, 355–363.

[27] Roy, R., Adams, M. W. W., *Molybdenum And Tungsten: Their Roles In Biological Processes* 2002, *39*, 673–697.

[28] Hagedoorn, P. L., Chen, T. H., Schroder, I., Piersma, S. R. *et al.*, *J. Biol. Inorg. Chem.* 2005, *10*, 259–269.

[29] Heider, J., Ma, K., Adams, M. W. W., *J. Bacteriol.* 1995, *177*, 4757–4764.

[30] Rauh, D., Graentzdoerffer, A., Granderath, K., Andreesen, J. R., Pich, A., *Eur. J. Biochem.* 2004, *271*, 212–219.

[31] Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H. *et al.*, *Nucleic Acids Res.* 1997, *25*, 3389–3402.

[32] Quevillon, E., Silventoinen, V., Pillai, S., Harte, N. *et al.*, *Nucleic Acids Res.* 2005, *33*, W116–W120.

[33] Livingstone, A., Fewson, C. A., *Biochem. J.* 1971, *121*, 8P–9P.

[34] Rosenberg, S. L., *J. Bacteriol.* 1971, *108*, 1257–1269.

[35] Breinig, S., Schiltz, E., Fuchs, G., *J. Bacteriol.* 2000, *182*, 5849–5863.

[36] Heider, J., Boll, M., Breese, K., Breinig, S. *et al.*, *Arch. Microbiol.* 1998, *170*, 120–131.

[37] Schmeling, S., Narmandakh, A., Schmitt, O., Gad'on, N. *et al.*, *J. Bacteriol.* 2004, *186*, 8044–8057.

[38] Isken, S., Debont, J. A. M., *Extremophiles* 1998, *2*, 229–238.

[39] Schühle, K., Gescher, J., Feil, U., Paul, M. *et al.*, *J. Bacteriol.* 2003, *185*, 4920–4929.

[40] Biegert, T., Altenschmidt, U., Eckerskorn, C., Fuchs, G., *Eur. J. Biochem.* 1993, *213*, 555–561.

[41] Laempe, D., Jahn, M., Breese, K., Schagger, H., Fuchs, G., *J. Bacteriol.* 2001, *183*, 968–979.

[42] Mohamed, M. E.-S., Fuchs, G., *Arch. Microbiol.* 1993, *159*, 554–562.

[43] Gescher, J., Ismail, W., Olgeschlager, E., Eisenreich, W. *et al.*, *J. Bacteriol.* 2006, *188*, 2919–2927.

[44] Gescher, J., Zaar, A., Mohamed, M., Schagger, H., Fuchs, G., *J. Bacteriol.* 2002, *184*, 6301–6315.

[45] Kim, Y. H., Cho, K., Yun, S. H., Kim, J. Y., *et al.*, *Proteomics* 2006, *6*, 1301–1318.

[46] Crawford, R. L., *Appl. Microbiol.* 1975, *30*, 439–444.

[47] Wheelis, M. L., Palleroni, N. J., Stanier, R. Y., *Arch. Mikrobiol.* 1967, *59*, 302–314.

[48] Suarez, M., Ferrer, E., Garridopertierra, A., Martin, M., *FEMS Microbiol. Lett.* 1995, *126*, 283–290.

[49] Altenschmidt, U., Oswald, B., Steiner, E., Herrmann, H., Fuchs, G., *J. Bacteriol.* 1993, *175*, 4851–4858.

[50] Yeo, C. C., Wong, M. V. M., Feng, Y. M., Song, K. P., Poh, C. L., *Gene* 2003, *312*, 239–248.

[51] Jeon, C. O., Park, M., Ro, H. S., Park, W., Madsen, E. L., *Appl. Environ. Microbiol.* 2006, *72*, 1086–1095.

[52] Gao, X. L., Tan, C. L., Yeo, C. C., Poh, C. L., *J. Bacteriol.* 2005, *187*, 7696–7702.

[53] Ferrandez, A., Garcia, J. L., Diaz, E., *J. Biol. Chem.* 2000, *275*, 12214–12222.

[54] Arias-Barrau, E., Olivera, E. R., Luengo, J. M., Fernandez, C. *et al.*, *J. Bacteriol.* 2004, *186*, 5062–5077.

[55] Sluis, M. K., Small, F. J., Allen, J. R., Ensign, S. A., *J. Bacteriol.* 1996, *178*, 4020–4026.

[56] Sluis, M. K., Larsen, R. A., Krum, J. G., Anderson, R. *et al.*, *J. Bacteriol.* 2002, *184*, 2969–2977.

[57] Boyd, J. M., Ellsworth, H., Ensign, S. A., *J. Biol. Chem.* 2004, *279*, 46644–46651.

[58] Clark, D. D., Ensign, S. A., *J. Bacteriol.* 1999, *181*, 2752–2758.

[59] Gade, D., Gobom, J., Rabus, R., *Proteomics* 2005, *5*, 3672–3683.

[60] Rabus, R., *Appl. Microbiol. Biotechnol.* 2005, *68*, 580–587.

2

Solvent Stress Response of the Denitrifying Bacterium "*Aromatoleum aromaticum*" Strain EbN1

Kathleen Trautwein[1,2†], Simon Kühner[2†], Lars Wöhlbrand[2], Thomas Halder[3], Kenny Kuchta[4], Alexander Steinbüchel[4], and Ralf Rabus[1,2*]

Applied and Environmental Microbiology (2008) **74** (8): 2267-2274

[1]Institute of Chemistry and Biology of the Marine Environment (ICBM), University Oldenburg, Carl-von-Ossietzky Str. 9-11, D-26111 Oldenburg, Germany

[2]Max Planck Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany

[3]TopLab GmbH, Fraunhoferstr. 18a, D-82152 Martinsried, Germany

[4]Institute for Molecular Microbiology and Biotechnology, Westfälische Wilhelms University Münster, Corrensstr. 3, D-48149 Münster, Germany

* Corresponding author

† These authors contributed equally to this work.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2008, p. 2267–2274 Vol. 74, No. 8
0099-2240/08/$08.00+0 doi:10.1128/AEM.02381-07

Solvent Stress Response of the Denitrifying Bacterium "*Aromatoleum aromaticum*" Strain EbN1▽†

Kathleen Trautwein,[1,2]‡ Simon Kühner,[2]‡ Lars Wöhlbrand,[2] Thomas Halder,[3] Kenny Kuchta,[4] Alexander Steinbüchel,[4] and Ralf Rabus[1,2]*

Institute of Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, Carl-von-Ossietzky Str. 9-11, D-26111 Oldenburg, Germany[1]; Max Planck Institute for Marine Microbiology, Celsiusstr. 1, D-28359 Bremen, Germany[2]; TopLab GmbH, Fraunhoferstr. 18a, D-82152 Martinsried, Germany[3]; and Institute for Molecular Microbiology and Biotechnology, Westfälische Wilhelms University of Münster, Corrensstr. 3, D-48149 Münster, Germany[4]

Received 23 October 2007/Accepted 28 January 2008

The denitrifying betaproteobacterium "*Aromatoleum aromaticum*" strain EbN1 degrades several aromatic compounds, including ethylbenzene, toluene, *p*-cresol, and phenol, under anoxic conditions. The hydrophobicity of these aromatic solvents determines their toxic properties. Here, we investigated the response of strain EbN1 to aromatic substrates at semi-inhibitory (about 50% growth inhibition) concentrations under two different conditions: first, during anaerobic growth with ethylbenzene (0.32 mM) or toluene (0.74 mM); and second, when anaerobic succinate-utilizing cultures were shocked with ethylbenzene (0.5 mM), toluene (1.2 mM), *p*-cresol (3.0 mM), and phenol (6.5 mM) as single stressors or as a mixture (total solvent concentration, 2.7 mM). Under all tested conditions impaired growth was paralleled by decelerated nitrate-nitrite consumption. Additionally, alkylbenzene-utilizing cultures accumulated poly(3-hydroxybutyrate) (PHB) up to 10% of the cell dry weight. These physiological responses were also reflected on the proteomic level (as determined by two-dimensional difference gel electrophoresis), e.g., up-regulation of PHB granule-associated phasins, cytochrome *cd*$_1$ nitrite reductase of denitrification, and several proteins involved in oxidative (e.g., SodB) and general (e.g., ClpB) stress responses.

BTEX (*b*enzene, *t*oluene, *e*thylbenzene, and *x*ylenes) and phenolic compounds are of environmental concern. They represent major constituents of crude oil (in the case of BTEX) (27) and are widely used as solvents and starting compounds in chemical synthesis. Due to accidental spillage during production, storage, and transport, these compounds are increasingly released into the natural environment (13). Contamination with BTEX or phenolic compounds in the vicinity of groundwater aquifers is of particular concern, since these compounds also exhibit considerable water solubility. For example, 1:20 mixtures of gasoline and water contain 3.2 mg liter^{-1} ethylbenzene, 70 mg liter^{-1} toluene, and altogether 130 mg liter^{-1} BTEX (10). The frequent occurrence of oxygen-limited or even anoxic conditions in such environments emphasizes the significance of anaerobic biodegradation. In past years many novel bacterial strains that are able to degrade aromatic compounds under anoxic conditions employing many novel and intriguing biochemical reactions have been isolated (18, 51).

The denitrifying bacterium strain EbN1 is able to degrade toluene and ethylbenzene under anoxic conditions and also directly from crude oil (36). This organism belongs to a novel cluster of anaerobic degraders within the *Betaproteobacteria*, the proposed new genus "*Aromatoleum*," which is distinct from the plant-associated *Azoarcus* sensu stricto species (R. Rabus, L. Wöhlbrand, D. Lange, B. Reinhold-Hurek, F. Widdel, and P. Kämpfer, unpublished). In addition, strain EbN1 also utilizes *p*-cresol, phenol, and some other polar aromatic compounds under anoxic conditions. Recently, the complete genome sequence of strain EbN1 was determined, allowing detailed reconstruction of its metabolic network and in-depth proteomic analysis of substrate-dependent regulation of individual degradation pathways (28, 35, 54). Strain EbN1 represents a promising model organism for in situ processes, since toluene-amended microcosms from oil-contaminated and pristine sites displayed rapid consumption of nitrate (31) and contained high numbers of bacteria related to the phylogenetic cluster harboring strain EbN1 (20, 31). Correspondingly, strain EbN1 removes toluene with high efficiency in laboratory two-dimensional aquifer microcosms which mimic in situ concentration gradients (2). Moreover, the detection of *bssA* expression (BssA is the catalytic subunit of toluene-activating benzylsuccinate synthase) in contaminated aquifers (3, 53) further emphasized the significance of anaerobic degradation for in situ bioremediation.

The toxicity of aromatic compounds generally correlates with their hydrophobicity, which is described by the logarithm of their partition coefficients in a mixture of *n*-octanol and water (log $P_{O/W}$). Compounds with a log $P_{O/W}$ between 1 and 4 are cytotoxic, since they preferentially dissolve in biological membranes (40). Consequently, membrane fluidity increases, which leads to a loss of ions, ATP, and other cellular metabolites. Furthermore, dissipation of the proton motive force and denaturation of mem-

* Corresponding author. Mailing address: Institute of Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, Carl-von-Ossietzky Str. 9-11, D-26111 Oldenburg, Germany. Phone: 49-441-798-3884. Fax: 49-441-798-3404. E-mail: rabus@icbm.de.

† Supplemental material for this article may be found at http://aem.asm.org/.

‡ These authors contributed equally to this work.

▽ Published ahead of print on 8 February 2008.

brane proteins (e.g., respiratory complexes or nutrient transporters) result in severe energetic problems in solvent-exposed cells (for an overview, see reference 41).

Despite the recent insights into the organisms, biochemistry, and genetics of anaerobic aromatic compound degradation, little is known about the strategies that the novel isolates use to cope with the toxicity of their growth substrates. Until now, bacterial solvent tolerance was studied mainly with aerobic, biotechnologically relevant microorganisms. The solvent stress response of these organisms involved mainly solvent efflux pumps, heat shock proteins, and modifications of the cytoplasmic membrane (19, 22). Here, we investigated for the first time the physiological and proteomic responses of the denitrifying bacterium "*Aromatoleum aromaticum*" strain EbN1 to semi-inhibitory concentrations of its aromatic growth substrates.

MATERIALS AND METHODS

Medium and cultivation. The denitrifying bacterium strain EbN1 was cultivated under nitrate-reducing conditions as previously described (36). Cultivation was carried out in 500-ml flat glass bottles containing 400 ml medium that were anoxically sealed with butyl rubber stoppers under an N_2-CO_2 (90/10%, vol/vol) atmosphere. The alkylbenzene substrates for anaerobic growth were provided as dilutions in 20 ml of deaerated 2,2,4,4,6,8,8-heptamethylnonan (HMN) as an inert carrier phase. Succinate was directly added to the medium from sterile stock solutions. The chemicals used were analytical grade.

Physiological experiments. The present study was based on two different lines of experiments comprising a total of seven different solvent stress conditions. (i) Strain EbN1 was adapted to "standard" substrate concentrations of 5% (vol/vol) ethylbenzene in HMN and 2% (vol/vol) toluene in HMN for at least five passages. These cultures served as inocula for subsequent cultivation at low, standard, and semi-inhibitory (about 50% growth inhibition) substrate concentrations (see Table S1 in the supplemental material). In the case of ethylbenzene these concentrations were 2, 5, and 8% (corresponding to determined equilibrium concentrations of 0.08, 0.21, and 0.32 mM, respectively, in the culture medium), and in the case of toluene they were 0.5, 2, and 5.5% (corresponding to 0.07, 0.24, and 0.74 mM, respectively). In this first line of experiments the alkylbenzenes served as growth substrates. (ii) Cultures of strain EbN1 adapted for at least five passages to anaerobic growth with 10 mM succinate were suddenly exposed (shocked) to ethylbenzene, toluene, *p*-cresol, or phenol as a single stressor and to a mixture of all four compounds during early linear growth with succinate. Application of the solvent mixture should have mimicked an in situ multiple-stress situation. In the second line of experiments succinate served as the growth substrate. The aromatic stressors were added directly to the aqueous medium, and the culture bottles were shaken vigorously to dissolve the hydrophobic compounds. The determined semi-inhibitory concentrations of the single solvents in the culture medium were 0.5 mM ethylbenzene, 1.2 mM toluene, 3.0 mM *p*-cresol, and 6.5 mM phenol (see Table S1 in the supplemental material). The solvent mixture had a semi-inhibitory effect at a total solvent concentration of 2.7 mM, corresponding to about one-quarter of the individual solvent concentrations mentioned above.

The same sampling procedure was used for all seven experiments. Samples were taken from the aqueous phase of the cultures using N_2-flushed, sterile syringes to determine the concentration(s) of the aromatic compound(s), the nitrate-nitrite concentrations, and the optical density. Carrier phase-containing culture bottles were inverted to avoid retrieving HMN together with the sample. Contact between the inert carrier phase and rubber stoppers was generally avoided. Each sample (2.5 ml) was divided into two subsamples (1 and 1.5 ml), which were processed as follows. The 1-ml subsamples containing alkylbenzenes were filtered (Spartan 13/0.2 RC; Schleicher & Schuell, Dassel, Germany) into 1 ml methanol (high-performance liquid chromatography [HPLC] grade) and stored in Teflon-sealed vials at 4°C. The 1-ml subsamples containing phenol or *p*-cresol were not diluted with methanol, but otherwise were treated in the same way. The second subsample (1.5 ml) was first used to record growth by measuring the optical density at 660 nm (UV-mini 1240; Shimadzu, Duisburg, Germany). Subsequently, cells were removed by filtration (Spartan 13/0.2 RC), and the filtrates were stored at 4°C for HPLC analysis of nitrate and nitrite. Parallel cultures (≥3) yielded very similar time courses of growth and concentrations of aromatic compounds, nitrate, and nitrite (data not shown). The concentrations of aromatic compounds in the aqueous medium remained essentially constant during the incubation time (data not shown). Controls lacking either the aromatic compounds or inoculum were treated in the same way.

Theoretical membrane concentrations of the aromatic solvents were calculated based on their log $P_{O/W}$ values (obtained from www.inchem.org) as described previously (40).

Mass cultivation. Mass cultivation was performed to obtain sufficient cell material for proteomic and polyhydroxyalkanoate (PHA) analysis. Cultures grown with alkylbenzenes and cultures grown with succinate were treated differently, as follows. To obtain compact cell pellets from alkylbenzene-utilizing cultures, a phosphate-buffered mineral medium supplemented with NaCl (1 g $liter^{-1}$) was used (46). Twelve parallel cultures for each of the three different concentrations of ethylbenzene and toluene were inoculated with correspondingly adapted precultures. All cultures were harvested at an optical density at 660 nm of around 0.2, corresponding to the half-maximal optical density during growth with 0.32 mM ethylbenzene. The latter culture yielded the lowest maximal optical density among the cultures containing the six alkylbenzene concentrations used. Six parallel succinate-utilizing cultures were harvested for each aromatic stressor and time point (45, 250, and 600 min after stressor addition). Unexposed succinate-utilizing cultures were harvested at the same time points. Harvesting and storage of cells were performed as described previously (8).

Chemical analysis. Concentrations of the alkylbenzenes, *p*-cresol, and phenol were determined with an HPLC system (Sykam, Fürstenfeldbruck, Germany) using a reversed-phase column as described previously (36). The eluent was composed of either 80% (vol/vol) acetonitrile (for analysis of alkylbenzenes) or 45% (vol/vol) acetonitrile (for analysis of *p*-cresol and phenol) and 0.75 mM phosphoric acid. In both cases the flow rate was 1 ml min^{-1}. The aromatic compounds were detected at 215 nm (alkylbenzenes), 254 nm (phenol), and 270 nm (*p*-cresol) and had retention times of 4.4 min (phenol), 4.5 min (toluene), 5.1 min (ethylbenzene), and 5.4 min (*p*-cresol).

Nitrate and nitrite samples were analyzed with an HPLC system (Sykam) as previously described (36).

2D DIGE. Cells were disrupted with a PlusOne sample grinding kit (GE Healthcare, Munich, Germany), and protein extracts were prepared as recently reported (14). The protein concentration was determined as described by Bradford (5). Isoelectric focusing was performed using an IPGphor (GE Healthcare) and commercial 24-cm IPG strips with a nonlinear pH 3 to 10 gradient (GE Healthcare) as described previously (14). The EttanDalt II system (GE Healthcare) was used for separation according to molecular mass in 12.5% Duracryl (Genomic Solution, Ann Arbor, MI) or acrylamide gels. Two-dimensional difference gel electrophoresis (2D DIGE) was carried out essentially as described previously (14). Preelectrophoretic labeling with different fluorescent dyes allowed coseparation of three samples in a single gel, representing the reference state, the test state, and an internal standard. The compositions of these three states varied in the two different lines of experiments. (i) Protein extracts from cultures grown with 0.21 mM ethylbenzene or 0.24 mM toluene ("standard" equilibrium concentrations in the aqueous phase) served as reference states and were labeled with Cy5. Protein extracts from cultures adapted to 0.08 or 0.32 mM ethylbenzene and to 0.07 or 0.74 mM toluene represented the four test states and were each labeled with Cy3. Each test state was related to the corresponding reference state. (ii) Protein extracts from untreated succinate-utilizing cultures harvested 45 min after addition of the aromatic stressor(s) to the test cultures served as the reference state and were labeled with Cy5. This reference state was chosen since only a few proteins displayed changed abundances relative to those in untreated cells harvested after 250 min (see Table S3 in the supplemental material). In each experiment protein extracts obtained at two or three different time points (45, 250, and 600 min) after addition of the aromatic stressor(s) served as test states. For example, succinate-utilizing cultures harvested 45, 250, and 600 min after addition of 0.5 mM ethylbenzene represented three independent test states that were each related to the reference state. Each test state was labeled with Cy3. The preparations in the two lines of experiments also contained different internal standards, which consisted of equal amounts of the corresponding reference and test states and were labeled with Cy2. To achieve statistical confidence, a minimum of four parallel gels were included in the analysis for each test state.

2D DIGE gels were scanned immediately after electrophoresis with a Typhoon 9400 scanner (GE Healthcare). Cropped images were analyzed with the DeCyder software (version 5.0; GE Healthcare). The parameters used for codetection of spots were described recently (54). Spot matching was manually controlled for differentially regulated spots, which had to fulfill the following criteria: average ratio (fold change) of less than −2.5-fold or more than 2.5-fold, *t* test value of less than 10^{-4}, and matched in at least 75% of the gels. Altogether, 23 and 52 2D DIGE gels (corresponding to 69 and 156 gel images) were analyzed for alkylbenzene- and succinate-utilizing cells, respectively. Differentially regulated pro-

TABLE 1. Proteomic responses of strain EbN1 to semi-inhibitory concentrations of aromatic solvents

Protein[b]	Predicted function[b]	Fold change[a]						
		Alkylbenzene-utilizing cells[c]		Succinate-utilizing cells 250 min after exposure to[c]:				
		Ethylbenzene (0.32 mM)[d]	Toluene (0.74 mM)[d]	Ethylbenzene (0.5 mM)[e]	Toluene (1.2 mM)[e]	*p*-Cresol (3.0 mM)[f]	Phenol (6.5 mM)[f]	Solvent mixture (2.7 mM)[f,g]
Denitrification								
NirS	Cytochrome cd_1 nitrite reductase	**3.0**	**3.2**	2.3	1.9	1.7	1.1	**3.0**
NorQ	Putative chaperone required for nitric oxide reductase (NorCB)	(−)	(−)	1.6	1.3	1.0	1.0	**2.6**
NosZ	Nitrous oxide reductase	(−)	(−)	**5.1**	**5.2**	1.9	2.0	**2.6**
Ethylbenzene degradation								
EbdA	Ethylbenzene dehydrogenase, alpha subunit	−1.3	**2.7**	–	–	–	–	–
Apc1	Acetophenone carboxylase, subunit 1	−2.4	1.7	–	–	–	–	**5.9**
Apc3	Acetophenone carboxylase, subunit 3	−1.9	1.6	(−)	(−)	–	–	**2.6**
Apc4	Acetophenone carboxylase, subunit 4	−1.6	1.8	(−)	(−)	–	–	**6.1**
Orf84	Putative methyltransferase	1.6	**3.6**	–	–	–	–	**8.3**
Toluene degradation								
BssA	Benzylsuccinate synthase, alpha subunit	1.6	**3.3**	–	–	–	–	–
BssE	Benzylsuccinate synthase, chaperone	2.0	**3.1**	–	–	–	–	–
BbsG	(*R*)-Benzylsuccinyl-CoA dehydrogenase	−1.0	1.5	–	–	–	–	–
BbsH	Phenylitaconyl-CoA hydratase	−1.3	−1.1	–	–	–	–	–
BbsA	Benzoylsuccinyl-CoA thiolase, alpha subunit	1.0	−1.1	–	–	–	–	–
PHA synthesis								
PhbB	Acetoacetyl-CoA reductase	1.3	**3.1**	–	–	–	–	(−)
PhaC	Putative poly(3-hydroxyalkanoate) synthase	1.0	**3.5**	–	–	–	(−)	(−)
EbA1323	Predicted phasin	**5.7**	**5.5**	−1.6	−1.3	1.2	1.3	1.4
EbA6852	Probable phasin	**4.0**	**27.4**	(−)	(−)	(−)	(−)	(−)
EbA5033	Probable phasin	**5.4**	**6.6**	−1.2	−1.2	–	–	–
Oxidative stress-related proteins								
SodB	Superoxide dismutase (Fe)	1.6	**2.8**	−1.1	−1.1	−1.6	2.1	2.3
KatA	Catalase	(−)	(−)	**2.5**	1.4	2.0	1.6	**5.3**
Dps	DNA-binding ferritin-like protein	1.3	1.4	2.0	2.0	2.4	1.3	**2.6**
NorVW	Flavorubredoxin with associated reductase	(−)	(−)	2.3	−1.3	(−)	2.4	**7.0**
EbA1861	Putative high-affinity iron transporter	**−9.9**	**−37.3**	–	–	–	–	–
EbA4918	Putative iron-binding protein of ABC iron transporter	**−2.6**	**−4.5**	–	–	–	–	–
AcnA	Aconitase A	2.1	**5.9**	–	–	–	–	–
AcnA2	Aconitase A2	1.8	**4.5**	–	–	–	–	–
General stress-related proteins								
BetB	Betaine aldehyde dehydrogenase	2.3	**3.5**	(−)	(−)	(−)	(−)	2.2
WbjB	Involved in O-antigen chain biosynthesis	–	–	**4.5**	2.0	–	–	–
HtpG	Heat shock protein	(−)	(−)	**2.6**	1.9	1.3	1.5	1.2
EbB88	Putative heat shock protein	1.0	1.2	**3.3**	2.1	1.5	**4.8**	**3.6**
GrpE	Putative Hsp-70 cofactor	(−)	(−)	1.8	1.4	–	–	**4.7**
ClpB	Chaperone	1.3	1.4	**3.6**	2.0	2.2	**4.1**	**3.4**
OmpC	Outer membrane protein (porin)	–	–	1.0	−1.3	**32.4**	**21.9**	**23.4**

[a] Fold change in protein abundance as determined by 2D DIGE (see Fig. 2).
[b] Protein designations and predicted functions as described previously (35; http://www.micro-genomes.mpg.de/ebn1). Proteins may belong to more than one group.
[c] Comparison of fold changes between alkylbenzene- and succinate-utilizing cells in absolute terms is not recommended, since the 2D DIGE working packages had different compositions. Visual comparison of relative spot positions of identified proteins (>|2.5|-fold change) was used to match protein spots with fold changes below the threshold in other DIGE work spaces. –, proteins that were absent on 2D electrophoresis gels; (−), uncertain matches due to ambiguous protein spot patterns. Values marked in bold represent changes above the set threshold of significance.
[d] See Tables S1 and S2 in the supplemental material.
[e] See Tables S1 and S3 in the supplemental material.
[f] See Tables S1 and S4 in the supplemental material.
[g] The total solvent concentration of a mixture of ethylbenzene, toluene, *p*-cresol, and phenol in the culture medium was 2.7 mM.

tein spots were manually excised either from the same 2D DIGE gel (alkylbenzene-utilizing and -shocked cells and phenol-shocked cells) or from separate preparative gels (*p*-cresol- and solvent mixture-shocked cells), both stained with colloidal Coomassie brilliant blue using the method described by Doherty et al. (11). The high reproducibility of spot patterns between 2D DIGE and colloidal Coomassie brilliant blue-stained gels was demonstrated previously with strain EbN1 (54).

Protein identification by mass spectrometry. Tryptic digestion of excised proteins was performed as described previously (23). Peptide masses were determined by matrix-assisted laser desorption ionization–time of flight mass spectrometry (TopLab GmbH, Martinsried, Germany). The protein identification and genome analysis were based on the previously published list of annotated genes in the genome sequence of strain EbN1 (35). Peptide mass fingerprints were mapped to the in silico digests of the predicted proteins by using the MS-Digest program (9).

Analysis of PHA. PHA were analyzed using whole-cell samples. For this, about 5 mg (dry weight) of cell matter was subjected to methanolysis in the presence of 15% (wt/vol) sulfuric acid. The methyl esters of 3-hydroxybutyric acid were analyzed by gas chromatography with a Hewlett Packard type GC6850 gas chromatograph (Hewlett Packard, Waldbronn, Germany) equipped with a hydrophobic capillary column (SGE GmbH, Darmstadt, Germany), an autosampler (Agilent Technologies Inc., Palo Alto, CA), and a flame ionization detector (6, 44). Retention times and peaks were analyzed using the Agilent Cerity software (version A.02.01; Agilent Technologies Inc.), and the total amount of poly(3-hydroxybutyrate) (PHB) in samples was analyzed in relation to methyl esters obtained from purchased sodium salt of 3-hydroxybutyric acid.

To analyze polyesters other than PHB, samples were also analyzed by combined gas chromatography and mass spectrometry. The solutions of 1-methyl-esters were injected into a Hewlett Packard series 6890 GC capillary gas chromatograph equipped with a Hewlett Packard series 5973 electron ionization mass selective detector and a capillary column (identical to the column described above). Data were collected using the program HP MSD Productivity ChemStations (revision B.01.00). Single fragments were analyzed with the program AMDIS (automated mass spectral deconvolution and identification system; version 2.1) and by the NIST mass spectral search program (version 1.6d ed.; S. Stein, A. Levitsky, O. Fateev, and G. Mallard).

RESULTS AND DISCUSSION

The ability to utilize ethylbenzene and other aromatic compounds under nitrate-reducing conditions is a distinct feature of strain EbN1. In this study we investigated the stress response of strain EbN1 exposed to experimentally determined semi-inhibitory (about 50% growth inhibition) (see Table S1 in the supplemental material) concentrations of aromatic growth substrates under two different conditions. First, during anaerobic growth with 0.32 mM ethylbenzene or 0.74 mM toluene the corresponding degradation pathways were operative. Second, when succinate-utilizing cells were suddenly exposed to 0.5 mM ethylbenzene, 1.2 mM toluene, 3.0 mM *p*-cresol, and 6.5 mM phenol applied as single stressors or as a mixture (total solvent concentration, 2.7 mM), the corresponding degradation pathways were not operative. Additional physiological and proteomic data for each individual experiment are provided in the supplemental material, as indicated in Table 1.

Growth behavior. Concentration-dependent impairment of growth was observed under all stressor conditions applied (Fig. 1; see Table S1 in the supplemental material). The growth rates and maximum optical densities were significantly reduced at semi-inhibitory concentrations of aromatic solvents, as exemplified by growth with ethylbenzene (Fig. 1A) and by the growth of succinate-utilizing cultures shocked with the solvent mixture (Fig. 1C). Remarkably, in the latter case about one-quarter of the applied semi-inhibitory concentration of each single solvent sufficed to reduce growth by one-half, suggesting an additive effect of the different toxic properties of the individual solvents.

Growth with alkylbenzenes was completely inhibited at 0.48 mM ethylbenzene and 0.86 mM toluene (equilibrium concentrations in aqueous medium) (see Table S1 in the supplemental material). Complete growth inhibition of succinate-utilizing cultures was observed upon shock with 0.6 mM ethylbenzene, 3.0 mM toluene, 4.0 mM *p*-cresol, or 8.0 mM phenol singly or with 3.0 mM solvent mixture. The growth-inhibiting concentrations determined indicated that ethylbenzene was the most toxic and phenol was the least toxic of the four tested compounds, agreeing well with the theoretical membrane concentrations of the compounds (40) (e.g., 175 mM ethylbenzene and 48 mM phenol when these compounds were present in the aqueous medium at concentrations of 0.6 and 8.0 mM, respectively).

Petroleum-contaminated groundwater often contains ethylbenzene and toluene at a concentration of 1 mg liter^{-1} (9.4 and 10.9 μM, respectively) (17). In addition, combined concentrations of phenol and alkyl-substituted phenols up to 68 g liter^{-1} have been determined for industrial wastewaters (4). Considering its markedly higher solvent tolerance (e.g., growth with <0.48 mM ethylbenzene), strain EbN1 should be able to survive and proliferate in most contaminated environments. Interestingly, the semi-inhibitory alkylbenzene concentrations determined for succinate-utilizing cells of strain EbN1 were higher (at least 2.5-fold) than those reported for other anaerobic degraders (*Thauera aromatica*, *Desulfococcus multivorans*, and *Geobacter sulfurreducens*) (12). Indeed, the ethylbenzene tolerance of strain EbN1 is more similar to that of the aerobic bacterium *Pseudomonas putida* (12).

Denitrification. During anaerobic growth with increasing concentrations of ethylbenzene, nitrate consumption and turnover of intermediary formed nitrite decelerated. With 0.21 and 0.32 mM ethylbenzene (Fig. 1B), as well as with 0.74 mM toluene (data not shown), the transition to stationary growth phase coincided with complete depletion of nitrate and maximum formation of nitrite. In contrast, succinate-utilizing, solvent-shocked cultures (Fig. 1D) continued to grow when nitrate was depleted, although denitrification was similarly impaired.

In accordance with the physiological behavior described above, the abundance of several denitrification enzymes changed in response to solvent stress (Table 1 and Fig. 2). In cells growing with 0.32 mM ethylbenzene and 0.74 mM toluene, periplasmic cytochrome cd_1 nitrite reductase (NirS) was 3.0- and 3.2-fold more abundant, respectively. Notably, NirS was among the most abundant protein spots observed in 2D DIGE gels generated from alkylbenzene-utilizing cultures, while it was far less abundant in succinate-utilizing cultures. Recently, increased NirS abundance was also observed during anaerobic growth of strain EbN1 with other aromatic substrates (54). One may speculate that aromatic compounds could interfere with electron flow to or within NirS, e.g., by inhibiting NirS dimerization or the NirS-cytochrome *c* interaction. An impaired electron flow was reported to result in NirS inactivation by irreversible NO binding in *Pseudomonas nautica* (29). Hence, an increased NirS concentration might compensate for solvent-induced NirS inactivation in strain EbN1.

Unstressed, succinate-utilizing cells displayed growth phase-

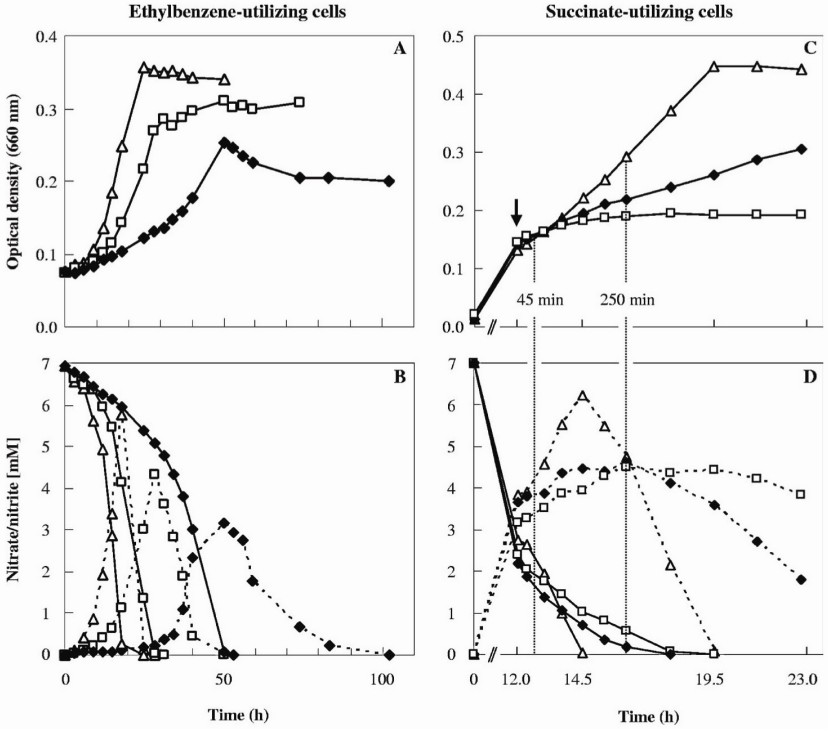


FIG. 1. Physiological response of strain EbN1 to solvent stress during anaerobic growth with ethylbenzene (A and B) and succinate (C and D). (A) Growth of ethylbenzene-adapted cells with 0.08 mM △, 0.21 mM □, and 0.32 mM ◆ ethylbenzene. These values represent the equilibrium concentrations in the aqueous medium when the ethylbenzene was supplied in an inert carrier phase. (C) Growth of succinate-utilizing cells suddenly exposed (indicated by an arrow) to a solvent mixture (containing ethylbenzene, toluene, *p*-cresol, and phenol) having a total solvent concentration of 0 mM △, 2.7 mM ◆, or 3.0 mM □ in the culture medium. The vertical dotted lines indicate time points when mass cultures were harvested for proteomic studies (45 or 250 min after solvent addition). Concentrations of nitrate (solid lines) and nitrite (dashed lines) are indicated in panels B and D.

dependent increases in the abundance of NirS (5.7- and 5.9-fold increases in the linear [250 min] and stationary [600 min] growth phases, respectively) (see Table S3 in the supplemental material). Interestingly, upon shock with the single aromatic stressors the NirS abundance remained unchanged, whereas only the solvent mixture led to a 3.0-fold increase in NirS abundance 250 min after solvent addition. Moreover, periplasmic nitrous oxide reductase (NosZ), which performs the last reaction in denitrification, was 5.1-, 5.2-, and 2.6-fold up-regulated after shock with ethylbenzene, toluene, and the solvent mixture, respectively. The abundance of the NO reductase-related chaperone NorQ also was increased (2.6-fold) in succinate-utilizing cells shocked with the solvent mixture. Inactivation of the *norQ* homolog *nirQ* in *Pseudomonas stutzeri* resulted in an inactive NO reductase complex (24). Thus, up-regulation of NorQ in strain EbN1 might point to an impaired NO reductase and elevated NO levels. Accordingly, a 7.0-fold-increased abundance of the NO-detoxifying flavorubredoxin (NorVW) was observed in cells shocked with the solvent mixture. In *Escherichia coli norVW* expression was induced at submicromolar NO concentrations (15). Generally, fine-tuned cooperation of denitrification enzymes is thought to ensure low concentrations of toxic NO in unchallenged cells (16).

Degradation pathways. Proteins involved in the anaerobic degradation of ethylbenzene, toluene, *p*-cresol, and phenol have been identified in previous proteomic studies with strain EbN1 (28, 54). Here, 11 and 12 2D electrophoresis-separated protein spots could be correlated with ethylbenzene and toluene degradation pathways, respectively (see Table S2 in the supplemental material). Interestingly, only in cells utilizing 0.74 mM toulene did a few of these protein spots reveal slightly increased abundances (e.g., 3.3- and 3.1-fold increases for the catalytic subunit [BssA] and the chaperone [BssE] of benzylsuccinate synthase, respectively). Ethylbenzene-utilizing cells did not show increased abundance of catabolic enzymes. It is noteworthy that the initial enzyme of anaerobic ethylbenzene degradation, ethylbenzene dehydrogenase, exhibits a very high affinity (K_m, 0.4 μM) for ethylbenzene (43). Indeed, the lowest ethylbenzene concentration (0.08 mM) used in this study is 200 times higher than this K_m. Thus, alkylbenzene degradation should not be enhanced by slightly increased levels of the catabolic enzymes and therefore should not contribute to the solvent tolerance of strain EbN1 under the conditions used. Remarkably, the toluene tolerance of *P. putida* strain DOT-T1E did not change when toluene degradation was disabled by deletion of the alpha subunit of toluene dioxygenase (30).

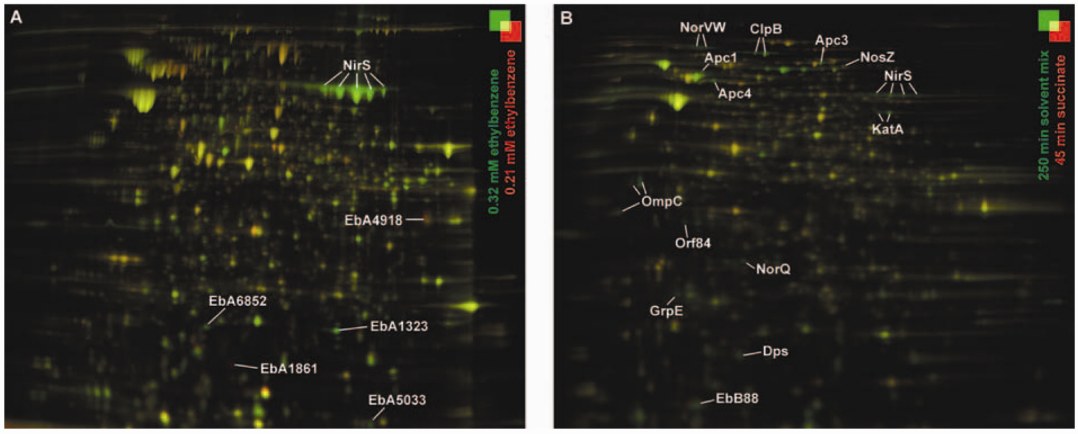


FIG. 2. Proteomic response of anaerobically growing strain EbN1 to solvent stress. (A) Ethylbenzene-utilizing cells (0.21 mM ethylbenzene; reference state) were shifted to a semi-inhibitory ethylbenzene concentration (0.32 mM). Cells were harvested at an optical density at 660 nm of 0.2. (B) Succinate-utilizing cells were shocked with a solvent mixture containing ethylbenzene, toluene, *p*-cresol, and phenol (total concentration, 2.7 mM). The solvent-shocked cells were harvested 250 min after solvent addition. Untreated, succinate-utilizing cells were harvested 45 min (reference state) after addition of the solvent mixture to test cultures. Proteins whose abundance was increased or decreased are indicated by green or red spots, respectively. Selected, mass spectrometrically identified proteins regulated more than |2.5|-fold are indicated. Fold changes in abundance are shown in Table 1.

In succinate-utilizing, solvent-shocked cells enzymes involved in the degradation of the aromatic compounds were not detected during the incubation time. The only exception was observed with cells exposed to the solvent mixture, where the levels of three subunits of acetophenone carboxylase (Apc1, Apc3, and Apc4; 5.9-, 2.6-, and 6.1-fold, respectively) and of a putative methyltransferase (Orf84; 8.3-fold) were increased; both proteins are involved in ethylbenzene degradation. The physiological meaning of this regulatory behavior is unclear, since it was not observed when the single aromatic stressors were used.

PHA formation. Many bacteria accumulate PHA as an insoluble energy and carbon storage compound when an excess of a carbon source is accompanied by a limitation in another nutrient. In *Ralstonia eutropha*, the initial formation of acetoacetyl-coenzyme A (acetoacetyl-CoA) from two acetyl-CoA units is catalyzed by acetyl-CoA acetyltransferase (PhbA). Subsequent reduction catalyzed by acetoacetyl-CoA reductase (PhbB) yields D-3-hydroxyacyl-CoA, which is polymerized to PHB by PHA synthase (PhaC). The product accumulates as granules which are coated with phospholipids and proteins, mostly phasins. PHA-forming cells synthesize phasins only in amounts that can be bound to the granules (52). Therefore, the occurrence of phasins can be used as a marker for PHA synthesis (55). In the present proteomic analysis, the levels of PhbB and PhaC were increased 3.0-fold during growth with 0.74 mM toluene. In addition, three phasin-like proteins (EbA1323, EbA6852, and EbA5033) were up-regulated in the same cultures (up to 27.4-fold) and also during growth with 0.32 mM ethylbenzene (up to 5.7-fold). The sequence similarities of these predicted phasins to their orthologs PhaP1 to PhaP4 in *R. eutropha* (33) are 42 to 66%. In succinate-utilizing, solvent-shocked cultures of strain EbN1, where the aromatic compounds were not catabolized, either PHB-related proteins were not detected or their abundances were not increased.

The proteomic findings were corroborated by chemical determination of PHA levels. Only during growth with 0.21 and 0.32 mM ethylbenzene and with 0.74 mM toluene did PHB accumulate at levels that accounted for up to 2.0, 10.3, and 5.2% of the cell dry weight, respectively. PHAs other than PHB were not observed. However, the absence of PHB in ethylbenzene- and toluene-shocked cells may have been due to an insufficient incubation time (maximum of 10 h after alkylbenzene addition) (see Table S3 in the supplemental material), since phasin-like proteins were first detected after 20 h in cultures utilizing 0.07 mM toluene.

Under all applied growth conditions, the potential electron supply provided by the organic substrate exceeded the electron-accepting capacity of nitrate (>19-fold, >4-fold, and 4-fold in ethylbenzene-, toluene-, and succinate-utilizing cells, respectively). PHB accumulation was observed only in alkylbenzene-utilizing cultures displaying reduced growth, nitrate consumption, and nitrite turnover. Importantly, in these cultures the electron-accepting nitrate and nitrite were not limiting. Thus, PHB formation presumably does not result from imbalanced nutrient supply but rather results from an impaired coupling of alkylbenzene catabolism and denitrification (see above). Thus, one may speculate that in alkylbenzene-utilizing cells of strain EbN1 alkylbenzene-derived acetyl-CoA is rerouted from oxidation via the tricarboxylic acid cycle to PHB synthesis accompanied by a decrease in the NAD(P)H pool and recycling of free CoA. In such a scenario, PHB could be considered a sink for reducing equivalents, ensuring continuous alkylbenzene degradation.

PHB could also function as a kind of hydrophobic trap or sink for aromatic compounds, since PHB granules are known to accumulate hydrophobic compounds, such as the fluorescent dye Nile red (42). Considering the absence of PHB and related proteins in succinate-utilizing, solvent-shocked cells of

strain EbN1, this would be restricted to long-term solvent exposure and/or utilization, if it occurs at all.

Oxidative stress-related proteins. Oxidative stress responses have been reported in aerobic bacteria challenged with aromatic solvents (e.g., phenol-stressed *P. putida* [38]), where highly reactive oxygen species can be generated from impaired oxygen respiration. Even though dioxygen and superoxide are not present in anoxic media, highly reactive NO compounds may also be generated during denitrification. In particular, the radical-containing intermediate NO may be accidentally converted to the highly reactive nitroxyl anion (NO^-) via several intracellular routes (21), resulting in oxidative stress. The observed increased abundance of superoxide dismutase (SodB; 2.8-fold) during anaerobic growth with 0.74 mM toluene, as well as the increased abundance of catalase (KatA; 5.3-fold), a DNA-binding protein related to oxidative stress (Dps; 2.6-fold), and NorVW (7.0-fold) (see above), in succinate-utilizing cultures shocked with the solvent mixture may have been due to the presence of such reactive NO species. Accordingly, nitrate has been reported to increase SodB activity in *Porphyromonas gingivalis* (1) and to enhance *sodB* expression in *E. coli* (34). The latter result was not observed in a nitrate reductase-deficient *E. coli* strain (34). Indeed, NO^- is a known substrate of superoxide dismutase, which reoxidizes it to NO (37). Notably, constitutive formation of SodB and KatA in anaerobically growing strain EbN1 seems likely, since the corresponding protein spots were detected on almost all 2D electrophoresis gels in this and previous studies (28, 54).

Besides up-regulation of antioxidative defense proteins, large decreases in the abundances of two predicted iron uptake proteins, EbA1861 and EbA4918, were observed in alkylbenzene-utilizing cells (up to a 37.3-fold decrease with 0.74 mM toluene). The current evidence for coordinated regulation of antioxidative defense and cellular iron homeostasis in bacteria (45) suggests that a similar regulatory circuit might be present in strain EbN1. EbA1861 is related to the small COG 3470 group, which comprises uncharacterized proteins assumed to function in high-affinity Fe^{2+} uptake. EbA4918 is similar to the multimember COG 1840 group of periplasmic components of ABC-type iron/thiamine transporters. In *P. putida* KT2440 the abundance of a similar protein (HitA; 46% similarity to EbA4918) decreased upon phenol exposure (38). Down-regulation of EbA1861 and EbA4918 in strain EbN1 might result in reduced intracellular iron availability, which would agree with the up-regulation of aconitase A and A2 (5.9- and 4.5-fold, respectively, during growth with 0.74 mM toluene). AcnA is known to replace the primary housekeeping aconitase AcnB in *E. coli* under oxidative stress or iron starvation conditions, since it contains a more stable [4Fe-4S] cluster (47).

General stress-related proteins. Proteins generally associated with various types of stresses (e.g., osmotic or temperature stress) were identified mainly in succinate-utilizing, solvent-shocked cultures. Only betaine aldehyde dehydrogenase (BetB), catalyzing the last biosynthetic reaction of osmotically active betaine, displayed a 3.5-fold increase in abundance during growth with 0.74 mM toluene. Osmotically stressed cells of *Oceanomonas baumannii* also increased betaine synthesis when they were treated with phenol (7).

The abundance of WbjB in succinate-utilizing cultures shocked with 0.5 mM ethylbenzene increased maximally (6.5-fold) 45 min after addition of the stressor (see Table S3 in the supplemental material). WbjB orthologs in *Pseudomonas aeruginosa* (WbjB) and *Staphylococcus aureus* (Cap5E) are involved in biosynthesis of *N*-acetyl-L-fucosamine (26), which is a constituent of surface polysaccharide structures (O antigens of lipopolysaccharide [LPS] and capsules, respectively). Differences in solvent tolerance have been correlated with LPS structure or composition in *Pseudomonas* strains; e.g., *o*-xylene-tolerant *P. putida* strain Idaho had rough LPS, while *o*-xylene-sensitive *P. putida* strain MW1200 had smooth LPS (32). This and similar observations with other microorganisms led to the assumption that outer membrane LPS might confer a certain degree of solvent tolerance (50). It is noteworthy, however, that knockout of *wbpL*, encoding the initial enzyme in O-antigen biosynthesis in *P. putida* strain DOT-T1E, did not alter toluene tolerance (25).

Solvent-induced formation of protein folding catalysts has been observed in bacteria exposed to, e.g., phenol (38) or toluene (39). In this study, the abundance of several heat shock proteins (HtpG, GrpE, EbB88, and EbA2730) and the chaperone ClpB was increased in succinate-utilizing cultures upon shock with all aromatic compounds except *p*-cresol (Table 1; see Tables S3 and S4 in the supplemental material). In alkylbenzene-utilizing cells (at least) ClpB is constitutively formed, which was also observed during aerobic and anaerobic growth of strain EbN1 with other aromatic compounds (54).

The abundance of the outer membrane porin OmpC was increased 32.4-, 21.9-, and 23.4-fold in succinate-utilizing cells of strain EbN1 shocked with *p*-cresol, phenol, and the solvent mixture, respectively. Similarly, solvent-tolerant *P. putida* S12 up-regulated the OprH porin (12-fold) upon toluene stress, probably to stabilize the outer membrane (48). Furthermore, the occurrence of OmpC as several 2D electrophoresis-separated spots with different pI values, as well as molecular weights (Fig. 2), might indicate that there was proteolysis of OmpC in the *p*-cresol-, phenol-, and solvent mixture-shocked cells of strain EbN1. Notably, misfolded or unfolded outer membrane porins were reported to trigger the σ^E-dependent envelope stress response in *E. coli* (49). One may speculate that OmpC or its degradation products might be involved in a similar mechanism in solvent-stressed cells of strain EbN1. The solvent shock specificity of increased OmpC abundance is supported by the unchanged OmpC abundance and 2D electrophoresis separation as a single spot in cultures of strain EbN1 adapted to anaerobic growth with noninhibitory concentrations of *p*-cresol or phenol (54).

ACKNOWLEDGMENTS

We are grateful to D. Lange for experimental assistance and to K. Affeldt, R. Meyer, J. Palfi, M. Wietz, and D. Radovan for laboratory assistance.

This work was supported by the Max-Planck-Gesellschaft and the Deutsche Forschungsgemeinschaft.

REFERENCES

1. **Amano, A., T. Ishimoto, H. Tamagawa, and S. Shizukuishi.** 1992. Role of superoxide dismutase in resistance of *Porphyromonas gingivalis* to killing by polymorphonuclear leukocytes. Infect. Immun. **60:**712–714.
2. **Bauer, R. D., P. Maloszewski, Y. Zhang, R. U. Meckenstock, and C. Griebler.** 2008. Mixing-controlled biodegradation in a toluene plume—results from two-dimensional laboratory experiments. J. Contam. Hydrol. **96:**150–168.
3. **Beller, H. R.** 2002. Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring *in situ* BTEX biodegradation. Environ. Sci. Technol. **36:**2724–2728.

4. **Berne, F., and J. Cordonnier.** 1995. Industrial water treatment: refining, petrochemicals and gas processing techniques, p. 79–87. Gulf Publishing Company, Houston, TX.
5. **Bradford, M. M.** 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54:**1977–1982.
7. **Brown, G. R., I. C. Sutcliffe, D. Bendell, and S. P. Cummings.** 2000. The modification of the membrane of *Oceanomonas baumannii*^{T} when subjected to both osmotic and organic solvent stress. FEMS Microbiol. Lett. **189:**149–154.
8. **Champion, K. M., K. Zengler, and R. Rabus.** 1999. Anaerobic degradation of ethylbenzene and toluene in denitrifying strain EbN1 proceeds via independent substrate-induced pathways. J. Mol. Microbiol. Biotechnol. **1:**157–164.
9. **Clauser, K. R., P. Baker, and A. L. Burlingame.** 1999. Role of accurate mass measurement (±10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Anal. Chem. **71:**2871–2882.
10. **Cline, P. V., J. J. Delfino, and P. S. C. Rao.** 1991. Partitioning of aromatic constituents into water from gasoline and other complex solvent mixtures. Environ. Sci. Technol. **25:**914–920.
11. **Doherty, N., B. Littman, K. Reilly, A. Swindell, J. Buss, and N. Anderson.** 1998. Analysis of changes in acute-phase plasma proteins in an acute inflammatory response and in rheumatoid arthritis using two-dimensional gel electrophoresis. Electrophoresis **19:**355–363.
12. **Duldhardt, I., I. Nijenhuis, F. Schauer, and H. J. Heipieper.** 2007. Anaerobically grown *Thauera aromatica*, *Desulfococcus multivorans*, *Geobacter sulfurreducens* are more sensitive towards organic solvents than aerobic bacteria. Appl. Microbiol. Biotechnol. **77:**705–711.
13. **Environmental Protection Agency.** 1986. Underground motor fuel storage tanks: a national survey. U.S. Environmental Protection Agency, Washington, DC.
14. **Gade, D., J. Thierman, D. Markowsky, and R. Rabus.** 2003. Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium *Pirellula* sp. strain 1. J. Mol. Microbiol. Biotechnol. **5:**240–251.
15. **Gardner, A. M., C. R. Gessner, and P. R. Gardner.** 2003. Regulation of the nitric oxide reduction operon (*norRVW*) in *Escherichia coli*. J. Biol. Chem. **278:**10081–10086.
16. **Goretski, J., O. Zafiriou, and T. Hollocher.** 1990. Steady-state nitric oxide concentrations during denitrification. J. Biol. Chem. **265:**11535–11538.
17. **Gülensoy, N., and P. J. J. Alvarez.** 1999. Diversity and correlation of specific aromatic hydrocarbon biodegradation capabilities. Biodegradation **10:**331–340.
18. **Heider, J.** 2007. Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. Curr. Opin. Chem. Biol. **11:**188–194.
19. **Heipieper, H. J., G. Neumann, S. Cornelissen, and F. Meinhardt.** 2007. Solvent-tolerant bacteria for biotransformations in two-phase fermentation systems. Appl. Microbiol. Biotechnol. **74:**961–973.
20. **Hess, A., B. Zarda, D. Hahn, A. Häner, D. Stax, P. Höhener, and J. Zeyer.** 1997. *In situ* analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. **63:**2136–2141.
21. **Hughes, M. N.** 1999. Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. Biochim. Biophys. Acta **1411:**263–272.
22. **Isken, S., and J. A. M. de Bont.** 1998. Bacteria tolerant to organic solvents. Extremophiles **2:**229–238.
23. **Jenö, P., T. Mini, S. Moes, E. Hintermann, and M. Horst.** 1995. Internal sequences from proteins digested in polyacrylamide gels. Anal. Biochem. **224:**75–82.
24. **Jüngst, A., and W. G. Zumft.** 1992. Interdependence of respiratory NO reduction and nitrite reduction revealed by mutagenesis of *nirQ*, a novel gene in the denitrification gene cluster of *Pseudomonas stutzeri*. FEBS Lett. **314:**308–314.
25. **Junker, F., J. J. Rodriguez-Herva, E. Duque, M. Ramos-Gonzalez, M. Llamas, and J. L. Ramos.** 2001. A WbpL mutant of *Pseudomonas putida* DOT-T1E strain, which lacks the O-antigenic side chain of lipopolysaccharides, is tolerant to organic solvent shocks. Extremophiles **5:**93–99.
26. **Kneidinger, B., K. O'Riordan, J. Li, J.-R. Brisson, J. C. Lee, and J. S. Lam.** 2003. Three highly conserved proteins catalyze the conversion of UDP-*N*-acetyl-D-glucosamine to precursors for the biosynthesis of O antigen in *Pseudomonas aeruginosa* O11 and capsule in *Staphylococcus aureus* type 5. J. Biol. Chem. **278:**3615–3627.
27. **Koch, R., and B. O. Wagner.** 1989. Umweltchemikalien: physikalisch-chemische Daten, Toxizitäten, Grenz- und Richtwerte, Umweltverhalten. VCH-Verlagsgesellschaft, Weinheim, Germany.
28. **Kühner, S., L. Wöhlbrand, I. Fritz, W. Wruck, C. Hultschig, P. Hufnagel, M. Kube, R. Reinhardt, and R. Rabus.** 2005. Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1. J. Bacteriol. **187:**1493–1503.
29. **Lopes, H., S. Besson, I. Moura, and J. J. G. Moura.** 2001. Kinetics of inter- and intramolecular electron transfer of *Pseudomonas nautica* cytochrome *cd1* nitrite reductase: regulation of the NO-bound end product. J. Biol. Inorg. Chem. **6:**55–62.
30. **Mosqueda, G., M.-I. Ramos-Gonzalez, and J. L. Ramos.** 1999. Toluene metabolism by the solvent-tolerant *Pseudomonas putida* DOT-T1 strain, and its role in solvent impermeabilization. Gene **232:**69–76.
31. **Pelz, O., A. Chatzinotas, N. Andersen, S. M. Bernasconi, C. Hesse, W.-R. Abraham, and J. Zeyer.** 2001. Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations. Arch. Microbiol. **175:**270–281.
32. **Pinkart, H., J. Wolfram, R. Rogers, and D. White.** 1996. Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. Appl. Environ. Microbiol. **62:**1129–1132.
33. **Pötter, M., H. Müller, F. Reinecke, R. Wieczorek, F. Fricke, B. Bowien, B. Friedrich, and A. Steinbüchel.** 2004. The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. Microbiology **150:**2301–2311.
34. **Privalle, C. T., and I. Fridovich.** 1991. Anaerobic inductions of the active forms of superoxide dismutases in *Escherichia coli*. Free Radic. Res. Commun. **12-13:**419–428.
35. **Rabus, R., M. Kube, J. Heider, A. Beck, K. Heitmann, F. Widdel, and R. Reinhardt.** 2005. The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Arch. Microbiol. **183:**27–36.
36. **Rabus, R., and F. Widdel.** 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. **163:**96–103.
37. **Reif, A., L. Zecca, P. Riederer, M. Feelisch, and H. H. H. W. Schmidt.** 2001. Nitroxyl oxidizes NADPH in a superoxide dismutase inhibitable manner. Free Radic. Biol. Med. **30:**803–808.
38. **Santos, P. M., D. Benndorf, and I. Sá-Correia.** 2004. Insights into *Pseudomonas putida* KT2440 response to phenol-induced stress by quantitative proteomics. Proteomics **4:**2640–2652.
39. **Segura, A., P. Godoy, P. van Dillewijn, A. Hurtado, N. Arroyo, S. Santacruz, and J.-L. Ramos.** 2005. Proteomic analysis reveals the participation of energy- and stress-related proteins in the response of *Pseudomonas putida* DOT-T1E to toluene. J. Bacteriol. **187:**5937–5945.
40. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1994. Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. **269:**8022–8028.
41. **Sikkema, J., J. A. M. de Bont, and B. Poolman.** 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. **59:**201–222.
42. **Spiekermann, P., B. H. A. Rehm, R. Kalscheuer, D. Baumeister, and A. Steinbüchel.** 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch. Microbiol. **171:**73–80.
43. **Szaleniec, M., C. Hagel, M. Menke, P. Nowak, M. Witko, and J. Heider.** 2007. Kinetics and mechanism of oxygen-independent hydrocarbon hydroxylation by ethylbenzene dehydrogenase. Biochemistry **46:**7637–7646.
44. **Timm, A., D. Byrom, and A. Steinbüchel.** 1990. Formation of blends of various poly(3-hydroxyalkanoic) acids by a recombinant strain of *Pseudomonas oleovorans*. Appl. Microbiol. Biotechnol. **33:**296–301.
45. **Touati, D.** 2000. Iron and oxidative stress in bacteria. Arch. Biochem. Biophys. **373:**1–6.
46. **Tschech, A., and G. Fuchs.** 1987. Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. Arch. Microbiol. **148:** 213–217.
47. **Varghese, S., Y. Tang, and J. A. Imlay.** 2003. Contrasting sensitivities of *Escherichia coli* aconitases A and B to oxidation and iron depletion. J. Bacteriol. **185:**221–230.
48. **Volkers, R. J. M., A. L. de Jong, A. G. Hulst, B. L. M. van Baar, J. A. M. de Bont, and J. Wery.** 2006. Chemostat-based proteomic analysis of toluene-affected *Pseudomonas putida* S12. Environ. Microbiol. **8:**1674–1679.
49. **Walsh, N. P., B. M. Alba, B. Bose, C. A. Gross, and R. T. Sauer.** 2003. OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. Cell **113:**61–71.
50. **Weber, F. J., and J. A. M. de Bont.** 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim. Biophys. Acta **1286:**225–245.
51. **Widdel, F., and R. Rabus.** 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr. Opin. Biotechnol. **12:**259–276.
52. **Wieczorek, R., A. Pries, A. Steinbüchel, and F. Mayer.** 1995. Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. J. Bacteriol. **177:**2425–2435.
53. **Winderl, C., S. Schaefer, and T. Lueders.** 2007. Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker. Environ. Microbiol. **9:**1035–1046.
54. **Wöhlbrand, L., B. Kallerhoff, D. Lange, P. Hufnagel, J. Thiermann, R. Reinhardt, and R. Rabus.** 2007. Functional proteomic view of metabolic regulation in "*Aromatoleum aromaticum*" strain EbN1. Proteomics **7:**2222–2239.
55. **York, G. M., B. H. Junker, J. Stubbe, and A. J. Sinskey.** 2001. Accumulation of the PhaP phasin of *Ralstonia eutropha* is dependent on production of polyhydroxybutyrate in cells. J. Bacteriol. **183:**4217–4226.

3

Anaerobic degradation of *p*-ethylphenol by "*Aromatoleum aromaticum*" strain EbN1: pathway, regulation and involved proteins.

Lars Wöhlbrand[1], Heinz Wilkes[2], Thomas Halder[3], and Ralf Rabus[1, 4]*

Journal of Bacteriology (2008)

doi:10.1128/JB.00409-08

[1] Max Planck Institute for Marine Microbiology, Bremen, Germany

[2] GeoForschungsZentrum Potsdam, Potsdam, Germany

[3] TopLab GmbH, Martinsried, Germany

[4] I nstitute for Chemistry and Biology of the Marine Environment (ICBM), University Oldenburg, Oldenburg, Germany

* Corresonding author

ABSTRACT

The denitrifying "*Aromatoleum aromaticum*" strain EbN1 was demonstrated to utilize *p*-ethylphenol under anoxic conditions and suggested to employ a degradation pathway which is reminiscent of known anaerobic ethylbenzene degradation: initial hydroxylation of *p*-ethylphenol to 1-(4-hydroxyphenyl)-ethanol followed by dehydrogenation to *p*-hydroxyacetophenone. Possibly, subsequent carboxylation and thiolytic cleavage yield *p*-hydroxybenzoyl-CoA, which is channelled into the central benzoyl-CoA pathway. Substrate-specific formation of 3 of the 4 proposed intermediates was confirmed by GC-MS analysis, also applying deuterated *p*-ethylphenol. Proteins involved in this degradation pathway are encoded in a single large operon-like structure (~15 kb). Among them are a *p*-cresol methylenehydroxylase-like protein (PchCF), two predicted dehydrogenases (ChnA and EbA309), a biotin-dependent carboxylase (XccABC) and a thiolase (TioL). Proteomic analysis (2D DIGE) revealed their specific and coordinated up-regulation in cells adapted to anaerobic growth with *p*-ethylphenol and *p*-hydroxyacetophenone (e.g. PchF up to 29-fold). Co-regulated proteins of to date unknown function (e.g. EbA329) are possibly involved in *p*-ethylphenol and *p*-hydroxyacetophenone specific solvent stress response and related to other aromatic solvent induced proteins of strain EbN1.

INTRODUCTION

Alkylphenols, such as *p*-ethylphenol, are present in coal tars and crude oils (2, 41). Besides accidental release of fuel-derived alkylphenols to the environment, phenolic compounds are prominent constituents of petrochemical wastewaters arising from spent caustic and coal gasification. Alkylphenol concentrations in these effluents (mainly phenol, cresols and ethylphenols) range from 100 to 68 000 mg l^{-1} depending on the source (2). Moreover, *p*-ethylphenol can also be plant-derived. E.g., native olive oils contain up to 52 mg *p*-ethylphenol per kg oil and the content can reach 470 mg kg^{-1} oil during storage (5). *p*-Ethylphenol can also be formed from *p*-coumaric acid, a major component of cereal cell walls, by several yeast and *Lactobacillus* species (10, 46). Due to their cyto-toxicity and relatively high water solubility (www.epa.gov/safewater/mcl.html), alkylphenols are of environmental concern.

Anaerobic degradation of aromatic compounds requires reactions independent of molecular oxygen. They are fundamentally different from the oxygenase-catalyzed reactions employed under oxic conditions (for overview see ref. 15). A variety of anaerobic aromatic compound-degrading bacteria were newly isolated during the last decades (for overview see refs. 42, 47). Most of them are denitrifiers belonging to the "*Aromatoleum*"/*Azoarcus*/*Thauera* cluster within *Betaproteobacteria* (Rabus et al. unpublished). "*Aromatoleum aromaticum*" strain EbN1, a metabolically versatile representative of this group, was originally isolated with ethylbenzene under anoxic conditions and demonstrated to utilize a large variety of aromatic compounds (35). The complete genome sequence of strain EbN1 (34) and several proteomic studies (23, 45, 48) revealed a fine-tuned, substrate-specific regulation of its known catabolic pathways.

Anaerobic ethylbenzene degradation (Fig. 1) in strain EbN1 proceeds via initial oxidation of ethylbenzene to (*S*)-1-phenylethanol by ethylbenzene dehydrogenase (EbdABC; ref. 20). A second oxidation step, catalyzed by (*S*)-1-phenylethanol dehydrogenase (Ped; refs. 17, 21) then yields acetophenone, which is subsequently converted to benzoyl-CoA via carboxylation (acetophenone carboxylase, Apc1-5), CoA-activation (benzoylacetate CoA-ligase, Bal) and thiolytic removal (benzoylacetyl-CoA thiolase) of acetyl-CoA (6). All proteins involved in anaerobic degradation of ethylbenzene are encoded in a gene cluster at approx. 1.4 Mb of the chromosome (33, 34). The "upper" (including *ebd* and *ped* genes) and "lower" parts (including *apc* and *bal* genes) are sequentially regulated in response to the presence of their specific substrates, i.e. ethylbenzene and acetophenone (23).

p-Ethylphenol has not been tested as substrate for anaerobic growth of strain EbN1 before, but was reported to support anaerobic growth of phylogenetically related strain EB1 (3). A pathway for anaerobic degradation of *p*-ethylphenol is presently unknown. Oxygen independent hydroxylation of *p*-ethylphenol occurs during its aerobic degradation in *Pseudomonas putida* JD1 (9). However, subsequent reaction steps include monooxygenase activity (44), necessitating a different pathway under anoxic conditions.

In the present study we investigated anaerobic degradation of *p*-ethylphenol in strain EbN1 to elucidate the degradation pathway, study substrate-specific regulation and analyze involved proteins.

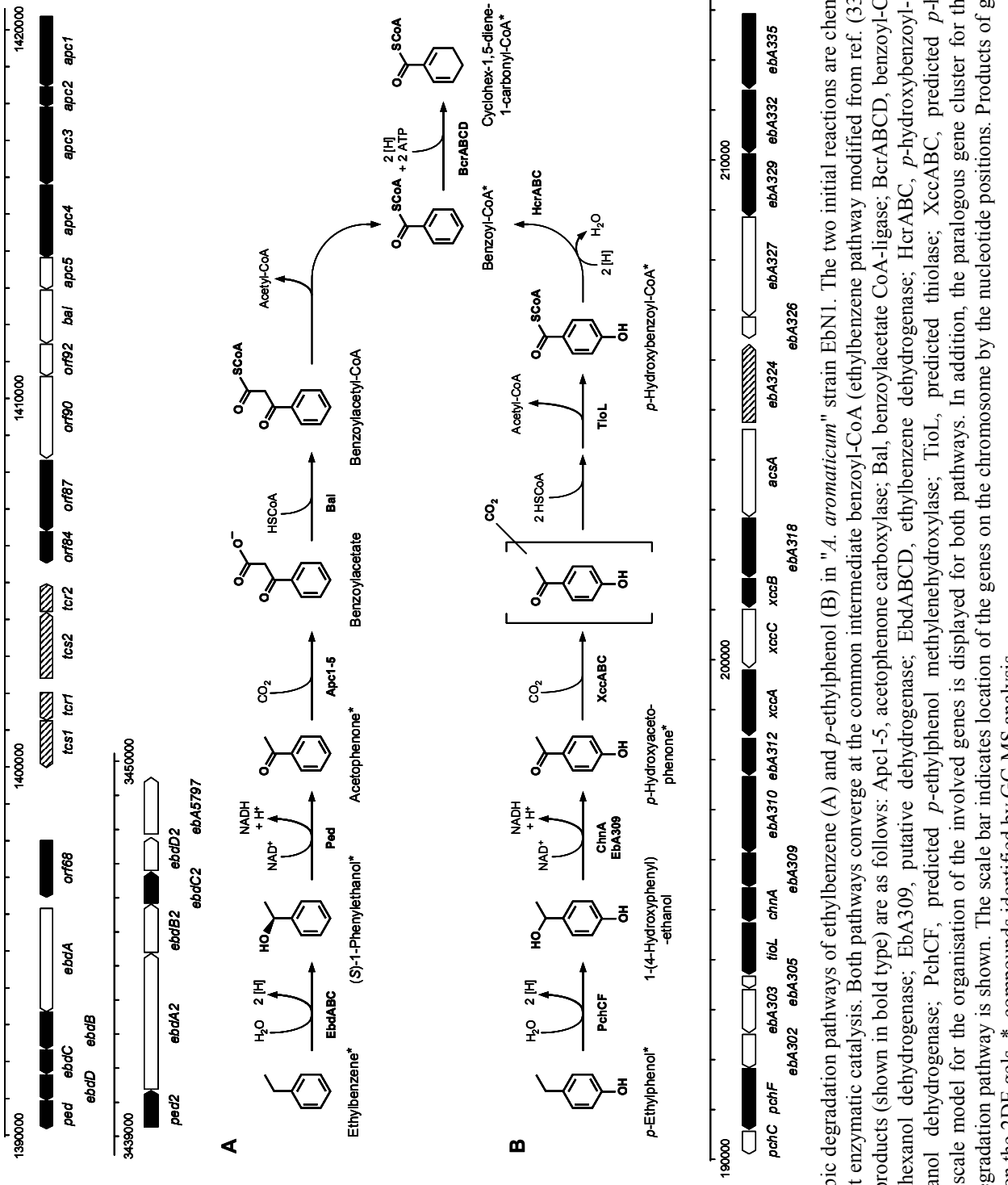


FIG. 1. Anaerobic degradation pathways of ethylbenzene (A) and *p*-ethylphenol (B) in "*A. aromaticum*" strain EbN1. The two initial reactions are chemically analogous, but involve different enzymatic catalysis. Both pathways converge at the common intermediate benzoyl-CoA (ethylbenzene pathway modified from ref. (33)). Enzyme names of indicated gene products (shown in bold type) are as follows: Apc1-5, acetophenone carboxylase; Bal, benzoylacetate CoA-ligase; BcrABCD, benzoyl-CoA reductase; ChnA, predicted cyclohexanol dehydrogenase; EbA309, putative dehydrogenase; EbdABCD, ethylbenzene dehydrogenase; HcrABC, *p*-hydroxybenzoyl-CoA reductase; Ped, (*S*)-1-phenylethanol dehydrogenase; PchCF, predicted *p*-ethylphenol methylenehydroxylase; TioL, predicted thiolase; XccABC, predicted *p*-hydroxyacetophenone carboxylase. A scale model for the organisation of the involved genes is displayed for both pathways. In addition, the paralogous gene cluster for the "upper part" of the alkylbenzene degradation pathway is shown. The scale bar indicates location of the genes on the chromosome by the nucleotide positions. Products of genes marked in black were identified on the 2DE gels. *, compounds identified by GC-MS analysis.

MATERIALS AND METHODS

Media and cultivation. "*A. aromaticum*" strain EbN1 was cultivated under nitrate-reducing conditions as previously described (35). The soluble substrates were added from sterile stock solutions. The poorly water soluble substrates ethylbenzene and *p*-ethylphenol were provided as dilutions in 2,2,4,4,6,8,8-heptamethylnonane (HMN) as inert carrier phase. The used chemicals were of analytical grade.

Cultivation was carried out in 500 ml flat bottles containing 400 ml of medium and 10 ml carrier phase when required. Bottles were anoxically sealed with rubber stoppers under a N_2/CO_2 (9:1; v/v) atmosphere. Cells were adapted to anaerobic growth with acetophenone (2 mM), benzoate (4 mM), *p*-hydroxyacetophenone (2 mM), ethylbenzene (2 % in HMN; v/v) and *p*-ethylphenol (0.5 % in HMN; w/v) for at least five passages.

Mass cultivation was performed to supply sufficient cell material for RNA and proteomic analysis. Cells were harvested at midlinear growth phase as described (6). Cell pellets were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. For each growth condition, a set of twelve parallel cultures generated independent biological samples.

Analysis of metabolites. Formation of metabolites was studied in substrate-adapted cultures (see above) with benzoate-adapted cultures serving as reference state. Unambiguous evidence for identified metabolites to be derived directly from *p*-ethylphenol was obtained from experiments using deuterated *p*-ethylphenol (2,3,5,6-d_4-*p*-ethylphenol; Campro Scientific, Berlin, Germany) as growth substrate. Metabolites of at least two independent cultures were extracted and analyzed per adaptation condition.

Respective cultures were incubated until nitrate and nitrite were nearly depleted, as determined by means of Merckoquant test stripes (Merck, Darmstadt, Germany). Subsequently, cultures were heat-inactivated, chilled on ice and the carrier phase was removed via a separatory funnel after heating as described (36). The inactivated cultures were extracted repeatedly at pH 1.5 with diethyl ether. Extracts were derivatized prior to gas chromatographic-mass spectrometric (GC-MS) analysis using a solution of diazomethane in diethyl ether. GC-MS measurements were performed using a Trace GC-MS (Thermoelectron, Dreieich, Germany). The GC was equipped with a temperature-programmable injection system and a BPX5 fused silica capillary column (length 50 m, inner diameter 0.22 mm, film thickness 0.25 μm). Helium was used as carrier gas. The GC oven temperature was

programmed from 50 °C (1 min isothermal) to 310 °C (20 min isothermal) at a rate of 3 °C min^{-1}. The mass spectrometer was operated in electron impact mode at an ion source temperature of 230 °C. Full scan mass spectra were recorded over the mass range of 50 – 600 Dalton at a rate of 2.5 scans s^{-1}. Identification of metabolites is based on comparison of GC retention times and mass spectra with those of authentic standards.

Physiological adaptation experiments. Substrate-adaptation experiments were performed with cells of strain EbN1 adapted to anaerobic growth with ethylbenzene, acetophenone, *p*-ethylphenol or *p*-hydroxyacetophenone shifted to any of these four substrates. Controls lacked either organic substrate or inoculum. During incubation samples were withdrawn from the aqueous phase (1 ml) using N_2-flushed sterile syringes at intervals of 2-3 h. Samples were directly used to monitor growth by measuring the optical density at 660 nm (UV-1202, Shimadzu, Duisburg, Germany). The end of incubation was indicated by the depletion of the electron acceptor nitrate and intermediary formed nitrite as well as lack of increase in optical density. Nitrate and nitrite concentrations were monitored with Merckoquant test strips (Merck). For each of the four test conditions, four replicate shift experiments were conducted (two replicates for each of two independently inoculated cultures). In all cases, the four replicates yielded highly similar time courses of growth.

2D DIGE. Cell disruption with the PlusOne Sample grinding kit (GE Healthcare, Munich, Germany) and preparation of protein extracts was performed as previously described (13). Protein concentration was determined according to the method described by Bradford (4).

Isoelectric focussing (IEF) was carried out as described (13) using the IPG-phor system (GE Healthcare) and 24 cm IPG strips with a nonlinear pH-gradient of 3-11 (GE Healthcare). DeStreak rehydration solution (GE Healthcare) was used to enhance resolution and reproducibility in the alkaline pH range. The EttanDalt II system (GE Healthcare) was used to carry out separation according to molecular mass in 12.5 % acrylamide gels as described recently (48).

2D DIGE was essentially carried out as described by Gade et al. (13). A total of 200 pmol of CyDye Fluors were used to label 50 µg of protein sample. Pre-electrophoretic labelling with different cyanine dyes allows co-separation of up to three samples in a single gel. An individual experiment in the present study contained (per gel) equal amounts of reference state, test state and internal standard.

To account for biological variation, protein extracts from three independently grown cultures were prepared for each substrate condition. Three parallel gels for each protein extract were performed to also consider technical variation. Protein extracts from cells anaerobically grown with benzoate served as reference state since its activated form, benzoyl-CoA, is the central intermediate of anaerobic aromatic compound degradation in strain EbN1. The reference state was labelled with Cy5. Protein extracts from cells anaerobically grown with ethylbenzene, acetophenone, *p*-ethylphenol or *p*-hydroxyacetophenone served as test states and were each labelled with Cy3. All performed experiments contained the same internal standard, which was composed of equal amounts of all protein preparations of the reference and all test states and labelled with Cy2.

2D DIGE gels were scanned immediately after electrophoresis with a Typhoon 9400 scanner (GE Healthcare). Analysis of cropped images was performed with the DeCyder software (version 5.0, GE Healthcare). Parameters for co-detection of protein spots were as described by Wöhlbrand et al. (48). Protein spots, that were defined as significantly regulated fulfilled the following criteria: an average ratio (fold change) of <-2.5 or >2.5, an ANOVA *p* value of <0.05, a *t*-test value of $<10^{-4}$, and matched in at least 27 gels. Overall, the DeCyder analysis included 36 gels (representing 108 gel images), which contained on average 1063 detected protein spots.

In order to identify differentially regulated spots by mass spectrometry (MS), separate preparative gels (300 µg protein load) were run for all test states. These gels were stained with colloidal Coomassie Brilliant Blue (cCBB) according to the method described by Doherty et al. (11). The high reproducibility of spot patterns allowed MS-based identification of differentially regulated proteins as determined by 2D DIGE/DeCyder. Only unambiguously matching spots were included in this analysis.

Protein identification by mass spectrometry. Tryptic digest of excised proteins was performed as described before (18). Peptide masses were determined by MALDI-TOF-MS. Protein identification and genome analysis were based on the published list of annotated genes from the genome of strain EbN1 (34). Peptide mass fingerprints were mapped to the *in silico* digests of the predicted proteins by using the MS-digest program (7).

Preparation of mRNA and real-time RT-PCR. Total RNA was prepared from substrate-adapted cells as described by Oelmüller et al. (30). RNA was prepared from two independent cultures for each growth condition to account for biological variation. Total

removal of DNA by DNase treatment was confirmed by PCR as described (23). The quality of RNA preparation was controlled with a RNA 6000 Nano assay using the 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). Antisense primers of gene-specific primer pairs (Table 1) were used for reverse transcription of 2.5 µg total RNA using H minus M-MLuV reverse transcriptase (MBI Fermentas, St. Leon-Roth, Germany) according to the manufacturer's instructions. Real-time PCR was performed as described (23) using an iQ5 Real-Time PCR Detection System (Biorad, Munich, Germany) and the qPCR Mastermix Plus for SYBR Green I (Eurogentec, Cologne, Germany). PCR efficiencies were determined as described by Ramakers et al. (37) and relative expression levels were calculated as described by Pfaffl (31). For each individual RNA preparation, at least two independent reverse transcriptions were performed. For each generated cDNA at least two real-time RT-PCR experiments with three parallels each were conducted.

TABLE 1. Primer sequences of genes involved in anaerobic ethylbenzene and *p*-ethylphenol degradation of "*A. aromaticum*" strain EbN1.

Primer[a]	Sequence (5´ → 3´)	Target gene	Product length (bp)
bcrC 108F	CAAGTGGTGGCAACGATGTGT	*bcrC*	191
bcrC 299R	GAAGGTCTGGCGATACTGGATGC		
hcrB 405F	GCCATCGTCAACATAGAATT	*hcrB*	202
hcrB 607R	TGCCAGTACTTCAACCAGAGC		
ebdA 300F	CTCCGCGGCGTCCTTGCT	*ebdA*	186
ebdA 486R	CGCGCCGTGCCCAGTTCTACC		
ebdA2 2666F	GGCGGAGCTCGGTATCAA	*ebdA2*	190
ebdA2 2856R	GGGCTTCCATTCAGGTAGTA		
apc1 1441F	AGCGGCGCCATCTCACTG	*apc1*	153
apc1 1594R	ACCGCCGTCGACATTCTCT		
pchF 1099F	CCATCCGGGAGCACCACT	*pchF*	237
pchF 1336R	GGCCGGCAACGTCATCATC		
xccA 1215F	GAATTCCGGGTGCTGGCTCTC	*xccA*	198
xccA 1413R	GCGCGACGGGGCTCAATA		

[a] Antisense primers (R) were used for reverse transcription.

Expression of the catalytic subunits of the initial enzymes of both pathways, i.e. *ebdA* (encoding the α-subunit of ethylbenzene dehydrogenase) and *pchF* (encoding the α-subunit of the *p*-cresol methylenehydroxylase-like protein) as well as subunits of the enzymes involved in carboxylation of acetophenone (*apc1*, encoding the β-subunit of acetophenone carboxylase) and *p*-hydroxyacetophenone (*xccA*, encoding the carboxyltransferase subunit of the biotin carboxylase), were studied. Also transcription of *hcrB* (encoding the β-subunit of the predicted *p*-hydroxybenzoyl-CoA reductase) was analyzed, since reductive dehydroxylation of *p*-hydroxybenzoyl-CoA is assumed to feed into the central benzoyl-CoA pathway during *p*-ethylphenol degradation (Fig. 1). Benzoate-adapted cells served as reference state and *bcrC* (encoding the γ-subunit of benzoyl-CoA reductase) was selected as reference gene, since benzoyl-CoA reductase represents the first common enzyme of the ethylbenzene and *p*-ethylphenol degradation pathways.

RESULTS AND DISCUSSION

p-Ethylphenol and *p*-hydroxyacetophenone represent only recently discovered substrates for anaerobic growth of "*A. aromatoleum*" strain EbN1. Their anaerobic degradation pathways have been unknown to date. Based on structural considerations, a reaction sequence similar to that of anaerobic ethylbenzene degradation could be envisioned (Fig. 1). Thus, *p*-ethylphenol may be converted to benzoyl-CoA via 1-(4-hydroxyphenyl)-ethanol, *p*-hydroxyacetophenone and *p*-hydroxybenzoyl-CoA (Fig. 1). To test this hypothesis, substrate-specific metabolites were analyzed (Fig. 2). Moreover, cultures of strain EbN1 adapted to anaerobic growth with *p*-ethylphenol, *p*-hydroxyacetophenone, ethylbenzene and acetophenone provided the basis for (i) determining adaptation times to growth with either of these substrates (Fig. 3), (ii) investigating substrate dependent regulation of the *p*-ethylphenol pathway on the molecular level (Table 2 and Figs. 4 to 6) and (iii) elucidating possible functions of the specifically regulated proteins.

Metabolite formation during anaerobic *p*-ethylphenol degradation. Formation of *p*-hydroxy-acetophenone, *p*-hydroxybenzoate and benzoate in *p*-ethylphenol-utilizing cultures could be demonstrated by GC-MS analysis. This finding was further corroborated by identification of corresponding 2,3,5,6-d_4-isotopomers upon incubation with 2,3,5,6-d_4-*p*-ethylphenol (Fig. 2). Accordingly, *p*-hydroxybenzoate and benzoate were identified when cells were grown with *p*-hydroxyacetophenone. Overall, the observed metabolites are in agreement with the proposed pathway (Fig. 1).

Adaptation to aromatic growth substrates. Under all four substrate-shift conditions, the primary adaptation substrate (see above) was readily utilized, while anaerobic growth with the three other aromatic substrates occurred in most cases only after prolonged incubation (Fig. 3), indicating that the respective degradation capacities had to be induced. Ethylbenzene- as well as acetophenone-adapted cells required pronounced adaptation times (~50 h) to start utilizing *p*-ethylphenol and *p*-hydroxyacetophenone for growth (Fig. 3 A and C). Conversely, *p*-ethylphenol-adapted cells displayed distinct adaptation times for the utilization of ethylbenzene and acetophenone, respectively (Fig. 3B). *p*-Hydroxyacetophenone-adapted cells required even an adaptation time of >160 h to start growing with acetophenone (Fig. 3D). Thus, these adaptation experiments already indicate that the two degradation pathways are operative only in the presence of their respective substrates. The only exception to these

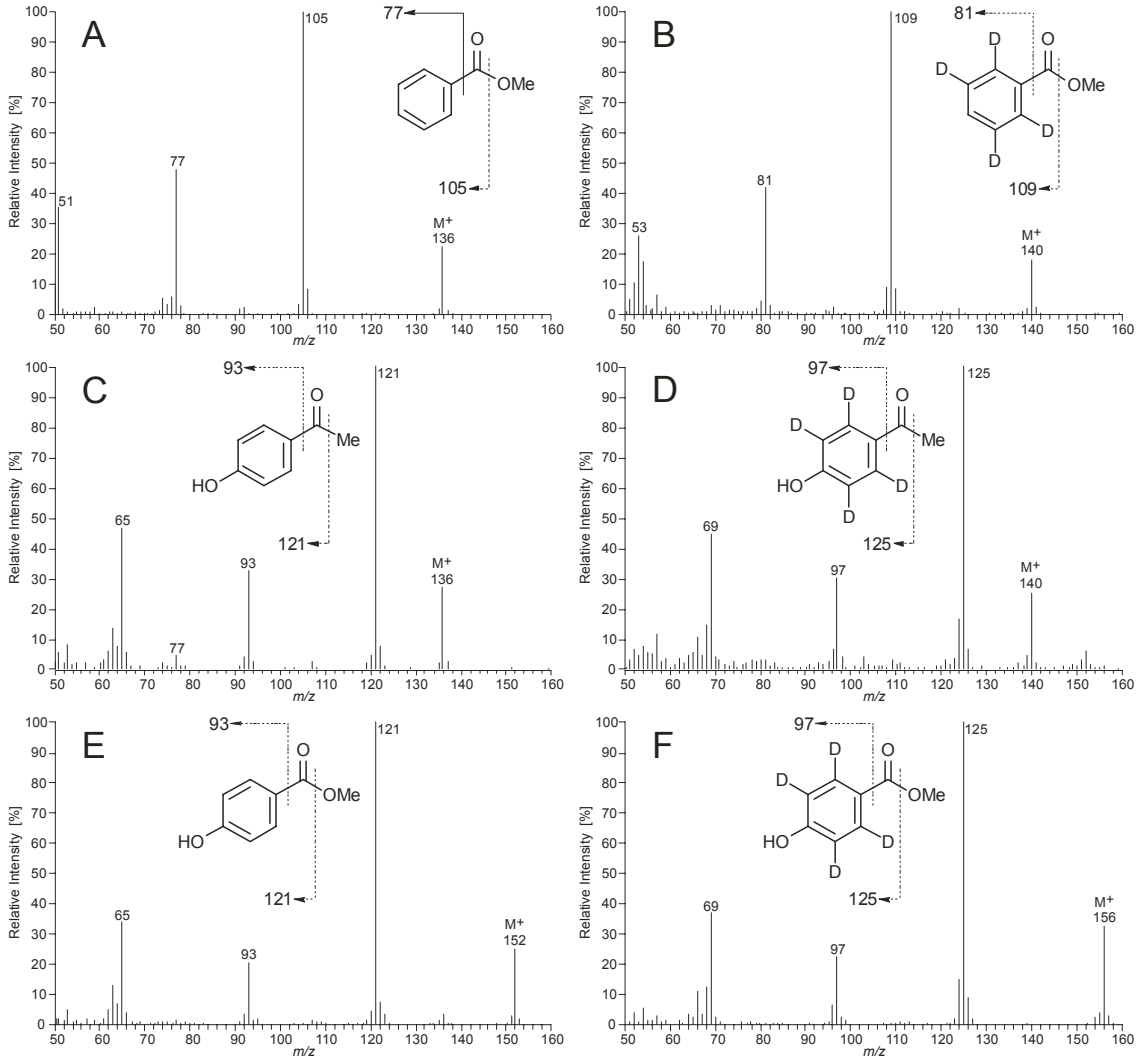


FIG. 2. Mass spectra of identified metabolites of anaerobic *p*-ethylphenol degradation and their 2,3,5,6-d_4-isotopomers derived from 2,3,5,6-d_4-*p*-ethylphenol utilization: (A) benzoic acid methyl ester, (B) 2,3,5,6-d_4-benzoic acid methyl ester (please note that the mass spectrometric evidence demonstrates 4 *d*-atoms on the aromatic ring, but does not necessarily prove the depicted labelling pattern), (C) *p*-hydroxyacetophenone, (D) 2,3,5,6-d_4-*p*-hydroxyacetophenone, (E) *p*-hydroxybenzoic acid methyl ester and (F) 2,3,5,6,-d_4-*p*-hydroxybenzoic acid methyl ester.

congruent adaptation patterns was the simultaneous adaptation of *p*-hydroxyacetophenone-adapted cells to the utilization of ethylbenzene (Fig. 3D), which contrasts the absence of expression of ethylbenzene-related genes under this adaptation condition (Table 2 and Fig. 5).

Regulation of the degradation pathways. The soluble proteomes of substrate-adapted cells of strain EbN1 were analyzed by two-dimensional difference gel electrophoresis (2D DIGE). In total, 87 protein spots were differentially regulated (fold change $> |2.5|$ compared to benzoate-adapted cells) of which 71 were identified by mapping of peptide masses. The identified proteins represented 51 different protein species, 25 of which could be

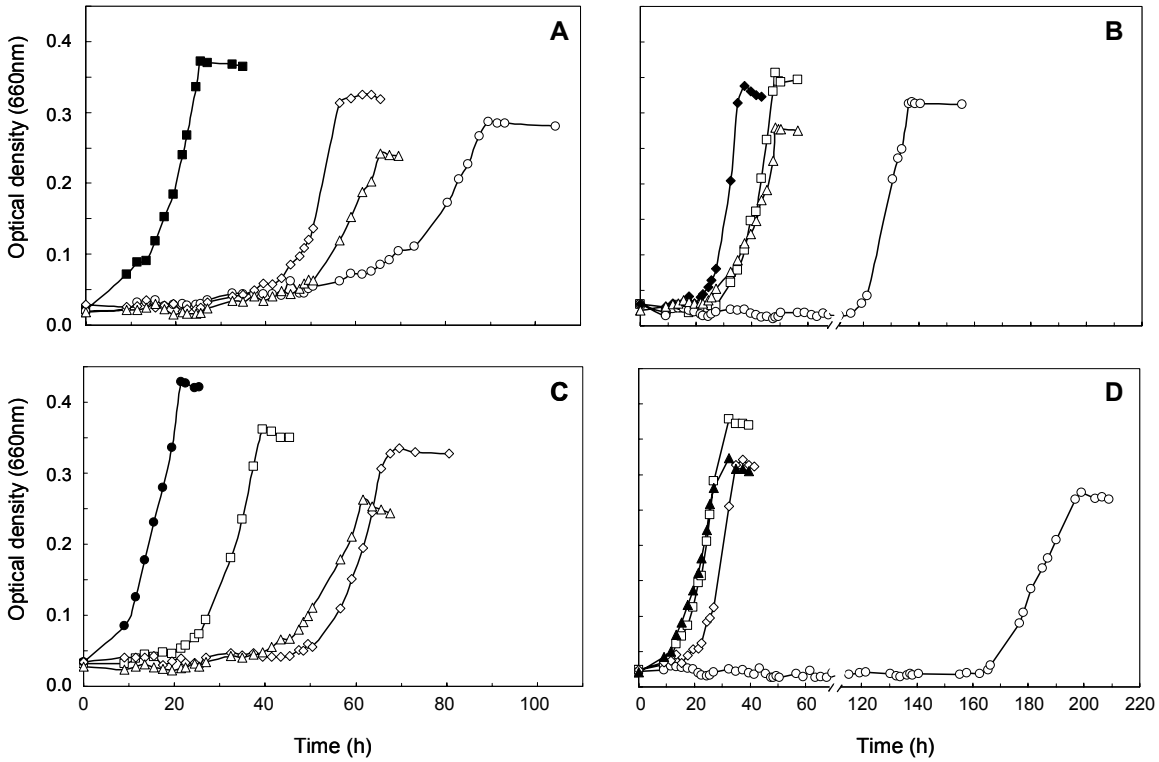


FIG 3. Anaerobic growth of "*A. aromaticum*" strain EbN1 with aromatic substrates under nitrate-reducing conditons. Cultures were inoculated with cells adapted to anaerobic growth with ethylbenzene (A), *p*-ethylphenol (B), acetophenone (C) or *p*-hydroxyacetophenone (D). Inoculated fresh anoxic media contained as single aromatic substrate ethylbenzene (□), acetophenone (○), *p*-ethylphenol (◇) or *p*-hydroxyacetophenone (△). Filled symbols represent the adaptation-substrate of the respective inoculum culture.

assigned to the two studied degradation pathways (Table 2 and Fig. 4). In addition to the proteomic analysis, gene expression of the catalytic subunits of the initial enzymes of both pathways as well as the respective carboxylating enzymes was investigated on the RNA-level (Fig. 5).

(i) Substrate specific regulation of the ethylbenzene degradation pathway. Subunits of both enzymes of the "upper" part of the ethylbenzene degradation pathway (EbdBCD, ethylbenzene dehydrogenase; Ped, (*S*)-1-phenylethanol dehydrogenase) were specifically increased in abundance (17- to 154-fold) during anaerobic growth with ethylbenzene, but not with *p*-ethylphenol (Table 2). Accordingly, the relative expression of *ebdA* was strongly increased in cells grown with ethylbenzene (> 460-fold) and to lower extent with acetophenone (17-fold), agreeing well with previously reported ethylbenzene specific expression of *ebdA* and formation of EbdC and EbdD in strain EbN1 (23). In *p*-ethylphenol- and *p*-hydroxyacetophenone-adapted cells, however, only a low level

induction of *ebdA* transcription was observed (30- and 9-fold, respectively), agreeing with the concurrent lack of significant EbdBCD and Ped formation. Notably, the abundance of

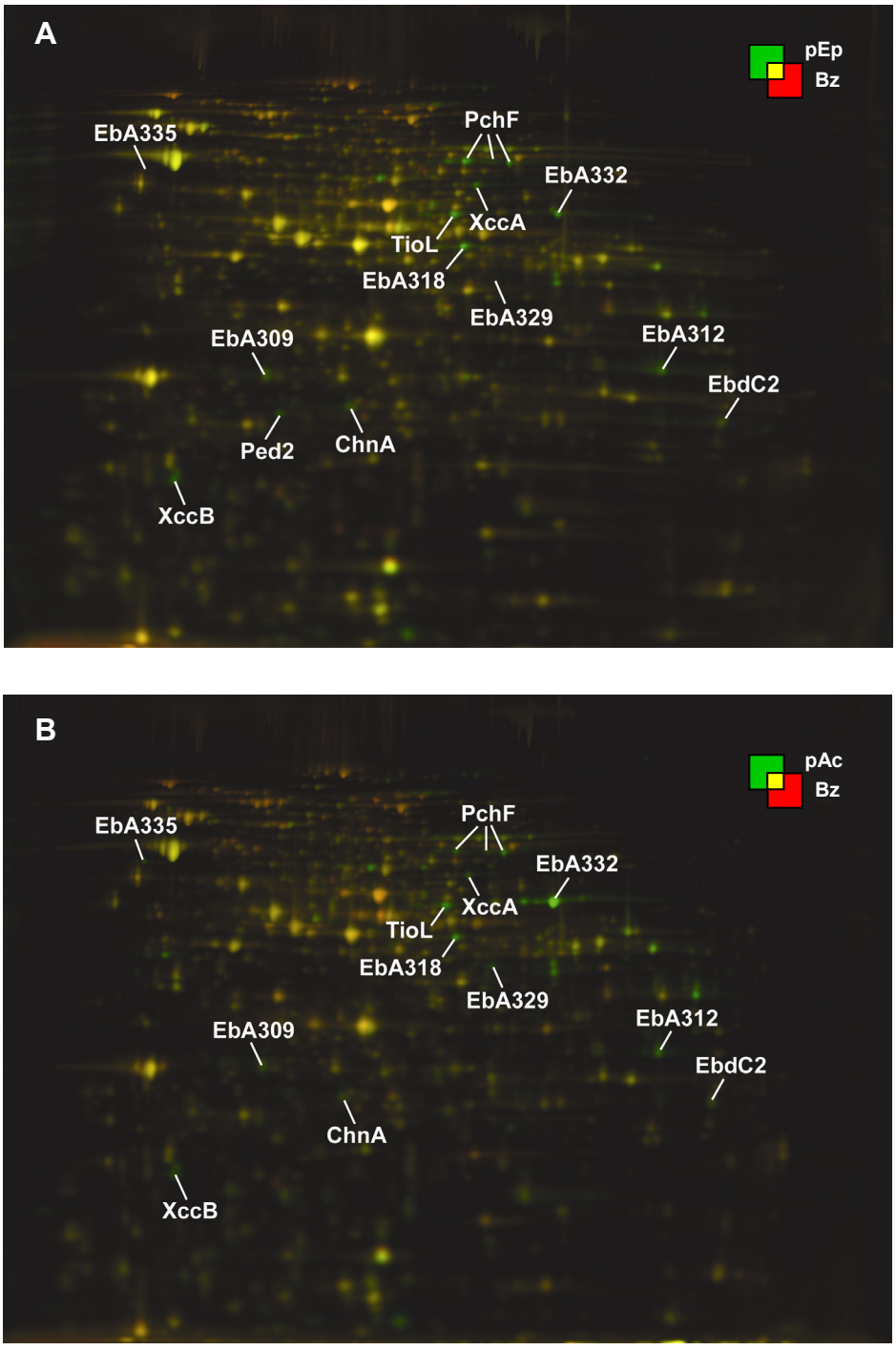


FIG 4. Differential protein profiles (2D DIGE) of substrate-adapted cells of "*A. aromaticum*" strain EbN1. Adaptation substrates for anaerobic growth were *p*-ethylphenol (A; pEp) and *p*-hydroxyacetophenone (B; pAc). Cultures adapted to anaerobic growth with benzoate (Bz) served as reference state. Protein abbreviations are as described in legend to Fig. 1. Fold changes in protein abundance of annotated proteins are indicated in Table 2.

TABLE 2. Fold changes in abundance of proteins involved in anaerobic ethylbenzene and *p*-ethylphenol degradation in substrate-adapted cells of "*A. aromaticum*" strain EbN1 as determined by 2D DIGE.

Identified proteins[a]	Adaptation substrate[b]			
	Etb	Acp	pEp	pAc
Ethylbenzene degradation				
EbdB (c1A63)	**60.3**	1.3	2.4	1.6
EbdC (c1A62)[c]	**154.0**	1.3	1.7	1.4
EbdD (c1A60)	**17.8**	1.4	1.4	1.4
Ped (c1A58)	**17.5**	1.7	1.5	1.8
Orf68 (c1A68)[c]	**311.2**	**3.0**	**21.0**	**39.1**
Acetophenone degradation				
Apc1 (c1A105)[c]	**32.2**	**21.6**	1.3	-1.4
Apc2 (c1A200)[c]	**73.4**	**29.9**	-1.0	1.1
Apc3 (c1A102)[c]	**35.8**	**33.9**	-2.4	**-2.6**
Apc4 (c1A100)[c]	**95.9**	**45.7**	1.4	-1.4
Orf84 (c1A84)	**53.8**	1.3	2.1	1.4
Orf87 (c1A87)	**101.3**	1.7	-1.1	1.2
p-Ethylphenol degradation				
PchF (EbA300)	1.9	1.4	**18.9**	**23.1**
ChnA (EbA307)	1.1	-1.0	**7.2**	**6.9**
EbA309	1.5	1.6	**12.4**	**13.4**
EbA310	-1.5	-1.4	**9.1**	**8.7**
EbA312				
p-Hydroxyacetophenone degradation				
XccA (EbA314)	1.6	1.9	**35.5**	**35.6**
XccB (EbB9)	1.7	1.6	**88.3**	**85.3**
TioL (EbA306)	1.3	1.2	**28.4**	**32.5**
EbA318	1.7	1.6	**41.6**	**40.7**
pEp and pAc related proteins				
EbA329	**2.7**	**2.6**	**15.7**	**32.3**
EbA332	**4.2**	2.1	**102.1**	**259.7**
EbA335	**6.7**	1.3	**4.9**	**9.4**
Paralogous ethylbenzene gene cluster				
EbdC2 (EbA5793)	2.0	1.4	**3.5**	**3.4**
Ped2 (EbA5789)	**2.9**	1.6	**20.6**	-1.0

[a] Proteins are listed according to the reaction sequence. Protein names and ORF numbers (e.g. EbA309) are as described earlier (34).

[b] Cells were adapted to anaerobic growth with the selected substrates for at least 5 passages. Substrate abbreviations: Acp, acetophenone; Etb, ethylbenzene; pAc, *p*-hydroxyacetophenone; pEp, *p*-ethylphenol. Fold changes marked in bold are above the set threshold of significance ($> |2.5|$).

[c] For protein species separated as two spots with different pI, the fold change of the most abundant spot is indicated. Not shown data revealed a similar ratio of fold changes.

hypothetical protein Orf68 was not only strongly increased (>300-fold) in ethylbenzene grown cells as reported previously (23), but also markedly in *p*-ethylphenol and *p*-hydroxyacetophenone grown cells (21- and 39-fold, respectively), suggesting a pathway independent function (for further details see supplementary material).

Significantly elevated abundances (up to 95-fold; Table 2) of identified subunits of acetophenone carboxylase (Apc1234) as well as an increased *apc1* expression level (up to 66-fold; Fig. 5) were only observed in cells grown with ethylbenzene and acetophenone, agreeing with previous observations (23). The lack of significant formation of the Apc-proteins during anaerobic growth with *p*-ethylphenol and *p*-hydroxyacetophenone (-2.6 and 1.4-fold, respectively) and the marginally increased expression of *apc1* (7- and 6-fold, respectively) points to a narrow effector spectrum of the predicted sensor/regulator system Tcs1/Tcr1 for acetophenone. Interestingly, the hypothetical proteins Orf84 and Orf87, encoded downstream of the apc genes, were only increased in abundance in ethylbenzene-, but not in acetophenone-adapted cells. This regulatory pattern indicates that both genes (orf84 and orf87) may not represent an assumed extension of the apc-operon (23), but rather serve an ethylbenzene-related, thus far unclear function.

(ii) Paralogous ethylbenzene dehydrogenase (EbdABC2) not involved in anaerobic p-ethylphenol degradation. The chromosome of strain EbN1 contains a second gene cluster for the "upper" part of the ethylbenzene degradation pathway encoded at approx. 3.4 Mb of the chromosome (Fig. 1; ref. 34). The physiological functions of the encoded paralogs (EbdABCD2, Ped2 and EbA5797) are currently unknown. The induction of paralogous (S)-1-phenylethanol dehydrogenase (Ped2) during anaerobic growth with p-cresol, in analogy to induction of ebd-genes by toluene, lead to the assumption, that the paralogous ethylbenzene dehydrogenase (EbdABCD2) could be involved in p-ethylphenol degradation (48). Agreeing with such an assumption, p-ethylphenol was oxidized hat high rates by ethylbenzene dehydrogenase, even though with significantly higher K_m than ethylbenzene (40.5 µM versus 0.45 µM; ref. 43). However, the abundance of identified EbdC2 and Ped2 was only slightly increased during anaerobic growth with *p*-ethylphenol and *p*-hydroxyacetophenone (up to 3.5-fold for EbdC2 and 20.6-fold for Ped2, respectively; Table 2). Accordingly, expression of *ebdA2* (encoding the α-subunit of paralogous ethylbenzene dehydrogenase) was only marginally increased during growth with ethylbenzene (7.0-fold), but not with any other substrate (Fig. 5). Considering the strong increase in EbdC abundance (154.0-fold) and the high expression level of *ebdA* (>450-fold) in

ethylbenzene grown cells, a metabolic function of the paralogous enzyme in anaerobic *p*-ethylphenol degradation seems to be unlikely.

(iii) Coordinated and substrate specific regulation of the *p*-ethylphenol and *p*-hydroxyacetophenone degradation pathway. Twelve proteins could, for the first time, be related to anaerobic *p*-ethylphenol degradation, due to their specific up-regulation (up to 259-fold) in *p*-ethylphenol- and *p*-hydroxyacetophenone-adapted cells (Table 2 and Fig. 4). The predicted functions of these proteins are: subunit of a *p*-cresol methylenehydroxylase-like protein (PchF), thiolase (TioL), cyclohexanol dehydrogenase (ChnA), putative dehydrogenase (EbA309), FAD linked oxidase (EbA310), sugar phosphatase (EbA312), biotin-dependent carboxylase (XccAB) and four hypothetical proteins (EbA318, EbA329, EbA332 and EbA335). Analogously, expression analysis of *pchF* and *xccA* revealed a specific up-regulation as well as a highly similar relative expression level during anaerobic growth with *p*-ethylphenol (140- and >140-fold, respectively) and *p*-hydroxyacetophenone (107- and 97-fold, respectively; Fig. 5). In contrast, their expression was only slightly increased in

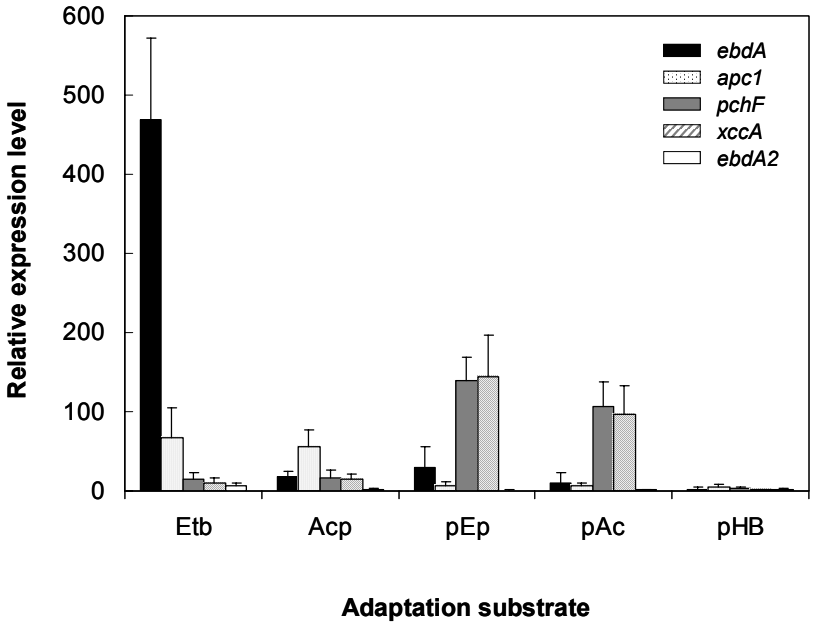


FIG 5. Relative expression levels of *ebdA*, *apc1*, *pchF* and *xccA* in substrate-adapted cells of "*A. aromaticum*" strain EbN1. The selected genes encode catalytic subunits ethylbenzene dehydrogenase (*ebdA*), acetophenone carboxylase (*apc1*), predicted *p*-ethylphenol methylenehydroxylase (*pchF*) and predicted *p*-hydroxyacetophenone carboxylase (*xccA*). Benzoate-adapted cells were used as reference state and *bcrC* (encoding γ-subunit of benzoyl-CoA reductase) served as reference gene. Relative expression levels were determined by real-time RT-PCR.

ethylbenzene- and acetophenone-adapted cells (<16-fold; Fig. 5), agreeing well with the proteomic data.

All of these proteins are encoded in a large operon-like structure (~15 kb) on the chromosome of strain EbN1 (34). The only exception are the hypothetical proteins EbA329, EbA332 and EbA335, which are encoded in a second operon-like structure, in a distance of only 2.5 kb downstream of the first one (Fig. 1). Interestingly, this interspace harbours the *ebA324* gene, encoding a putative sigma54-dependent transcriptional regulator *in trans*, possibly involved in gene regulation of the *p*-ethylphenol pathway.

The organisation of all genes of the *p*-ethylphenol degradation pathway in a single operon-like structure is indicative of a coordinated regulation, which contrasts the described sequential regulation of the two catabolic operons of the ethylbenzene degradation pathway (23). This assumption is supported by the highly similar *pchF* and *xccA* expression levels as well as the conspicuously similar increased abundances of all identified proteins encoded in the gene cluster (Table 2 and Fig. 5) during anaerobic growth with *p*-ethylphenol and *p*-hydroxyacetophenone. Moreover, reverse transcription applying only the *pchF* antisense primer enables PCR amplification of both *pchF* and *xccA*, pointing to expression of the whole operon as a polycistronic transcript (Fig. S1). Overall, the differential proteomic and gene expression data demonstrate a clear difference between the ethylbenzene and *p*-ethylphenol degradation pathways from a regulatory point of view.

(iv) Sigma54-dependent regulation of the anaerobic *p*-ethylphenol degradation pathway. Regulation of aromatic compound degradation pathways is known to occur on the transcriptional level, mediated by a variety of transcriptional regulators sensing the aromatic stimulus (14, 23). The two gene clusters, induced by *p*-ethylphenol and *p*-hydroxyacetophenone, are separated by *ebA324*, encoding a putative sigma54-dependent transcriptional regulator *in trans* (Fig. 1; ref. 34). Interestingly, both gene clusters harbour the -12/-24 consensus sequence of sigma54-dependent promotors (5´-TGGC-N_7-TTGCA-3´; ref. 16) approximately 100 bp up-stream of the translational start of *acsA* and *ebA335*, suggesting involvement of EbA324 in initiation of their transcription. Sequence analysis of EbA324 classified it as NtrC-type transcriptional regulator, comprising a C-terminal helix-turn-helix, a sigma54 interaction and an N-terminal receptor domain (27). NtrC-type regulators are known to be involved in transcription of genes with diverse physiological functions (24). The sigma54-dependent XylR regulator of aerobic toluene and xylene degradation in *Pseudomonas putida* is activated upon recognition of toluene and xylenes as well as their benzyl alcohol and benzaldehyde derivatives (1). Interestingly, the amino acid sequence of

EbA324 shares 40 % sequence identity with XylR, supporting its predicted function. Since *p*-ethylphenol and *p*-hydroxyacetophenone induce expression of the *p*-ethylphenol related gene cluster to the same extent, one may speculate that both molecules are recognized by EbA324, agreeing with the known broad effector spectrum of related XylR (1). However, chemically related ethylbenzene and acetophenone do apparently not belong to the effector spectrum of EbA324.

While the "upper" ethylbenzene pathway is gratuitously induced by toluene (23), previous comprehensive proteomic studies did not indicate induction of the *p*-ethylphenol pathway under anoxic conditions by other aromatic substrates than *p*-ethylphenol and *p*-hydroxyacetophenone (48). This regulatory difference may be due to two reasons: (i) Neither promoter regions nor the assigned regulators provided any evidence for a sigma54-dependent transcription of the genes of the ethylbenzene pathway. (ii) The involved transcriptional regulators differ fundamentally. The one-component sigma54-dependent EbA324 regulator combines effector sensing directly with initiation of transcription. In contrast, Tcs2/Tcr2 of the ethylbenzene pathway represents a two component sensor-regulator system. Apparently, the "single molecule system" of EbA324 does not allow cross induction of the regulator by another sensor, as speculated for Tcr2 being erroneously activated by toluene sensing TdiS (23).

Enzyme candidates of anaerobic *p*-ethylphenol and *p*-hydroxyacetophenone degradation. Based on their predicted functions, most of the specifically up-regulated proteins can be correlated to individual reactions within the proposed *p*-ethylphenol degradation pathway (Fig. 1).

(i) *p*-Ethylphenol methylenehydroxylase. The initial dehydrogenation of *p*-ethylphenol, forming 1-(4-hydroxyphenyl)-ethanol, could be catalyzed by the *p*-cresol methylenehydroxylase-like protein (PchCF). Dioxygen independent dehydrogenation of *p*-ethylphenol by an ethylphenol methylenehydroxylase (EPMH) has been reported for *Pseudomonas putida* JD1 (39) and *Aspergillus fumigatus* (19). Interestingly, *P. putida* JD1 possesses two different, but highly similar, hydroxylases for *p*-cresol and *p*-ethylphenol degradation (39), for which gene sequences are presently not available. Indeed, PchF of strain EbN1 shares 31 % sequence identity with the *p*-cresol methylhydroxylase (PchF; PCMH) from *P. putida* NCIMB 9866, which is also active on *p*-ethylphenol (26). Due to the hydroxyl group in *para* position, enabling intermediary formation of a quinone methide, the redox potential of the EPMH flavin cofactor (+280 mV) is sufficient for initial dehydrogenation of

p-ethylphenol (39). In contrast, dehydrogenation of ethylbenzene necessitates the molybdenum cofactor and an electron acceptor with a higher redox potential (+380 mV) to achieve reasonable oxidation rates of the chemically stable substrate (20). Thus, oxidation of *p*-ethylphenol by the PCMH-like protein (PchCF) in strain EbN1 seems reasonable.

(ii) 1-(4-Hydroxyphenyl)-ethanol dehydrogenase. The oxidation of 1-(4-hydroxyphenyl)-ethanol to *p*-hydroxyacetophenone might also be performed by PchCF, since purified EPMH from *P. putida* JD1 forms *p*-hydroxyacetophenone as major oxidation product from *p*-ethylphenol (39). However, in the initial reaction EPMH stereospecifically forms the *R*(+)-1-(4-hydroxyphenyl)-ethanol enantiomer in high excess (>90 %), which is only slowly oxidized by EPMH (40). Rapid oxidation of the *R*(+)-enantiomer in crude extracts of *P. putida* JD1 was suggested to result from the involvement of a second, so far unknown enzyme (40). In strain EbN1, the two predicted dehydrogenases (ChnA and EbA309) may catalyze this oxidation, since both display high sequence similarity to (*S*)-1-phenylethanol dehydrogenase of strain EbN1 (Ped; 33 % and 36 % identity, respectively), catalyzing the analogous reaction in the pathway for anaerobic ethylbenzene degradation (21).

(iii) Carboxylase. Carboxylation of *p*-hydroxyacetophenone is most probably catalyzed by the biotin-dependent carboxylase XccABC. This enzyme does not share any homology with acetophenone carboxylase (Apc1-5), acetone carboxylase (AcxABC) or biotin-independent, phenylphosphate carboxylase (PpcABCD) of strain EbN1 (34). Thus, it represents the fourth type of carboxylase in strain EbN1 and can be expected to employ a distinct catalytic mechanism. Biotin dependent carboxylases are a diverse group of enzymes found in various biosynthetic pathways in pro- and eukaryotes (8). The two-step catalysis involves (i) carboxylation of biotin and (ii) transcarboxylation from biotin to the respective acceptor molecules. Carboxylation of acetyl-CoA requires abstraction of a proton from the methyl group to form an enolate intermediate (8).

(iv) Further enzymes. Thiolytic cleavage of the so far unknown carboxylation product, yielding *p*-hydroxybenzoyl-CoA, might be performed by the predicted thiolase (TioL) encoded in the same gene cluster. Interestingly, TioL is apparently not homologous to the respective enzyme of the ethylbenzene pathway (benzoylacetate-CoA ligase, Bal). The final reductive dehydroxylation, yielding benzoyl-CoA is supposed to be catalyzed by the predicted hydroxybenzoyl-CoA reductase (HcrABC) as suggested for the phenol and *p*-cresol degradation pathways (34, 48). However, subunits of the enzyme could not be detected on 2DE gels until now and a transcript of *hcrB* could not be detected in significant amounts in

p-hydroxybenzoate grown cells (data not shown; also see supplementary material). Thus, the last step in the transformation of *p*-ethylphenol (also phenol and *p*-cresol) to benzoyl-CoA is presently not well understood.

It should be noted, however, that aerobic utilization of *m*-hydroxybenzoate also induced formation of the proteins of the *p*-ethylphenol pathway (PchF, TioL, ChnA, EbA310, XccAB, EbA318 and EbA332; ref. 48), though to markedly lower extent (maximum 30-fold). The physiological meaning of this regulatory pattern remains elusive at present.

Possible involvement of a specific solvent stress system in *p*-ethylphenol and *p*-hydroxyacetophenone metabolism. Besides the genes predicted to be involved in the anaerobic degradation of *p*-ethylphenol and *p*-hydroxyacetophenone, three further gene products, EbA329, EbA332 and EbA335, displayed strongly increased abundances during anaerobic growth of strain EbN1 with both aromatic substrates (Table 2 and Fig. 4). All of them represent proteins of unknown function, encoded together with another hypothetical protein (EbA326) and a putative transport protein (EbA327) in an operon-like structure shortly upstream of the *p*-ethylphenol gene cluster (Fig. 1; ref. 34).

Further analysis of EbA326 revealed domains related to a cytoplasmic universal stress protein (UpsA) from *E. coli* (27), the expression of which is enhanced during prolonged exposure to stress agents (29). Furthermore, analysis of EbA327 revealed two transmembrane and a sensory domain related to an RND-type efflux pump (AcrB) from *E. coli* (27). AcrB is assumed to capture its substrate either from within the inner membrane or from the cytoplasm (49), followed by excretion through an outer membrane channel (TolC). The AcrB-TolC interaction is mediated by a periplasmic accessory protein (AcrA) and the transport process is driven by proton motive force (32). Energy dependent efflux-pumps of the RND family are known to be involved in solvent tolerance (e.g. *n*-hexane or toluene) in several Gram-negative bacteria, in particular *E. coli* and *Pseudomonas* sp. (38).

Interestingly, the hypothetical proteins EbA332 and EbA335 possess signal sequences for periplasmic localisation (12). Though sequence analysis of EbA332 and EbA335 revealed no significant similarity to AcrA and TolC, respectively, the proteins are relatively similar in size (400 and 500 amino acids for AcrA and TolC, respectively, and 450 and 550 aa for EbA332 and EbA335, respectively). In addition, prediction of EbA335 secondary structure elements indicated formation of numerous beta-sheets and turn regions, possibly forming a beta barrel as known for outer membrane porins (49). Considering the specific formation of these proteins in the presence of aromatic solvents, one may speculate that *ebA327*, *ebA332*

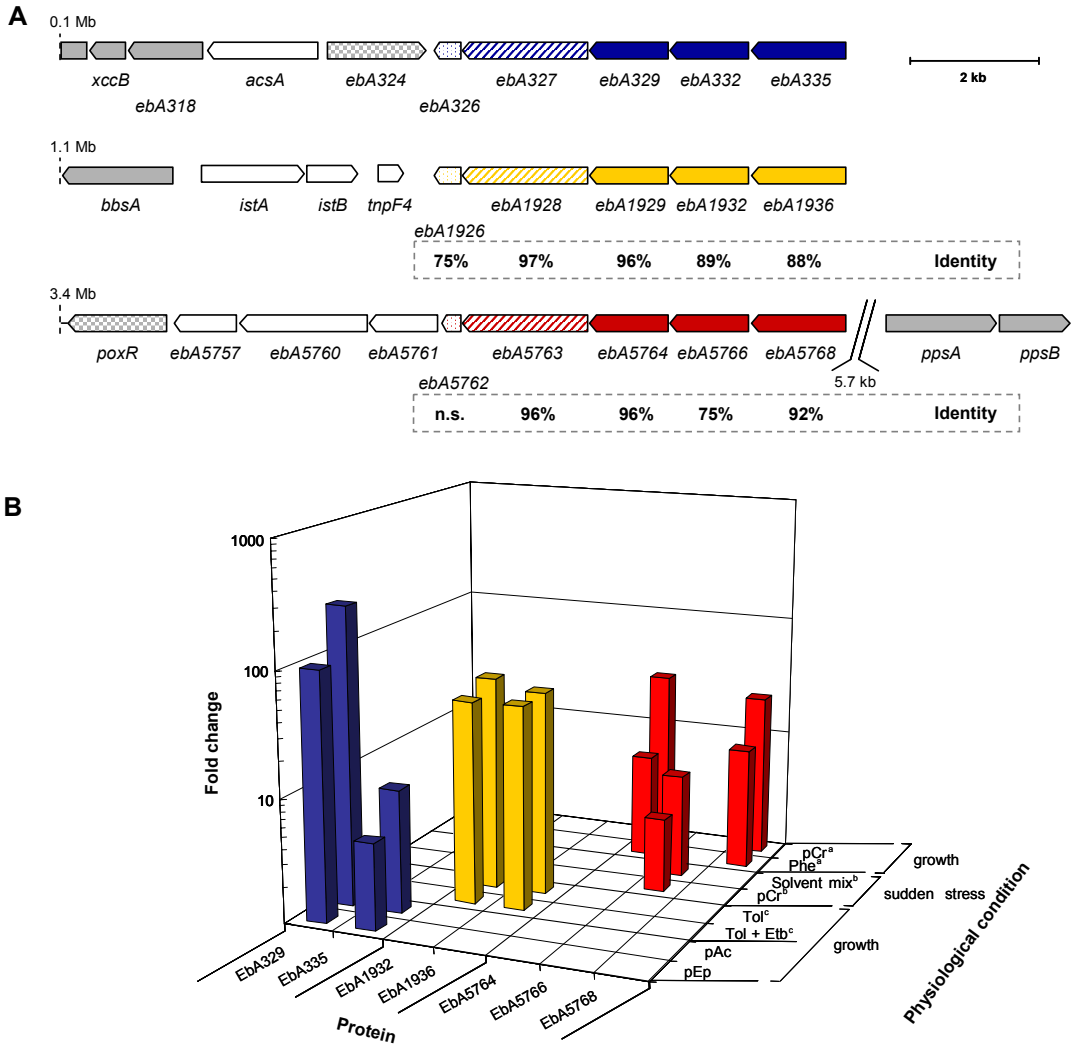


FIG 6. Gene products possibly involved in solvent stress tolerance of "*A. aromaticum*" strain EbN1. (A) Chromosomal gene location is indicated by their nucleotide positions. Genes encoding stress related proteins are highlighted in blue, yellow and red, respectively: hypothetical proteins (filled), predicted efflux pump (hatched) and predicted universal stress protein (dotted). Amino acid sequence identities of gene products (referring to EbA326-335) are indicated below the paralogous genes (n.s., no significant similarity). Genes encoding proteins of aromatic compound catabolism are highlighted in grey (filled) and respective regulatory proteins are checkered. (B) Fold changes in abundance of identified hypothetical proteins (EbA329-335, EbA1932-1936 and EbA5764-5768) during anaerobic growth with different aromatic substrates, as determined by 2D DIGE.

[a] Anaerobic growth with benzoate represents the reference state. No up-regulation during anaerobic growth with phenylalanine, phenylacetate, benzyl alcohol, benzaldehyde, *p*-hydroxybenzoate, *m*-hydroxybenzoate and *o*-aminobenzoate (48).

[b] Anaerobic growth with succinate represents the reference state (45).

[c] Anaerobic growth with benzoate represents the reference state. No induction during anaerobic growth with ethylbenzene (23).

and *ebA335* encode a so far uncharacterized solvent specific efflux system, analogous to the AcrAB-TolC system of *E. coli* and *Pseudomonas* sp. (Fig. S2).

Remarkably, the chromosome of strain EbN1 harbours two additional gene clusters, which display high sequence similarity (76-97% aa sequence identities; Fig. 6A) to *ebA326-335* and exhibit the same gene order (Fig. 6; ref. 34). The first cluster (*ebA1926-1936*) is located adjacent to the catabolic genes of anaerobic toluene degradation and the second (*ebA5762-5768*) near *ppsB* (encoding a subunit of predicted phenylphosphate synthetase) (Fig. 6; ref. 48). In addition, the three paralogous gene clusters are specifically expressed in the presence of different aromatic compounds which correlate well with the genomic context in each case (Fig. 6B). The products of *ebA326-335, ebA1926-1936* and *ebA5762-5768* were specifically formed during anaerobic growth with e.g. *p*-ethylphenol, toluene (23) and phenol/*p*-cresol (48), respectively. Formation of EbA5762-5768 was recently also observed in succinate-utilizing cells of strain EbN1, suddenly stressed with phenolic compounds (45). These analogous regulatory patterns support the predicted solvent related function of the encoded proteins and point to a fine-tuned system of solvent stress tolerance in strain EbN1.

Notably, highly similar gene clusters (42-88 % aa sequence identity; Fig. S3) were detected in the genomes of plant associated *Azoarcus* sp. BH72 (42-80 % aa identity; ref. 22), anaerobic aromatic compound degrading *Thauera aromatica* K172 (80-88 % aa identity; ref. 25) and aerobic aromatic compound degrading and solvent tolerant *P. putida* KT2440 (28). In case of *Azoarcus* sp. BH72 and *T. aromatica* K172, these genes are also located adjacent to genes of aerobic phenol and anaerobic toluene degradation, respectively (Fig. S3), further supporting a general solvent stress related function of these gene clusters.

ACKNOWLEDGEMENTS

We are grateful to D. Lange, internship student Heiko Nacke (both Bremen), C. Karger and A. Sobotta (both Potsdam) for technical assistance. We are indebted to F. Widdel for general support of proteomic studies in our group. This study was supported by the Deutsche Forschungsgemeinschaft and the Max-Planck-Gesellschaft.

REFERENCES

1. **Abril, M. A., C. Michan, K. N. Timmis, and J. L. Ramos.** 1989. Regulator and enzyme specificities of the TOL plasmid-encoded upper pathway for degradation of aromatic hydrocarbons and expansion of the substrate range of the pathway. J Bacteriol **171:**6782-90.
2. **Acuna-Arguelles, M. E., P. Olguin-Lora, and E. Razo-Flores.** 2003. Toxicity and kinetic parameters of the aerobic biodegradation of the phenol and alkylphenols by a mixed culture. Biotechnol Lett **25:**559-64.
3. **Ball, H. A., H. A. Johnson, M. Reinhard, and A. M. Spormann.** 1996. Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J Bacteriol **178:**5755-5761.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248-54.
5. **Brenes, M., C. Romero, A. García, F. J. Hidalgo, and M. V. Ruiz-Méndez.** 2004. Phenolic compounds in olive oils intended for refining: formation of 4-ethylphenol during olive paste storage. J Agric Food Chem **52:**8177-81.
6. **Champion, K. M., K. Zengler, and R. Rabus.** 1999. Anaerobic degradation of ethylbenzene and toluene in denitrifying strain EbN1 proceeds via independent substrate-induced pathways. J Mol Microbiol Biotechnol **1:**157-64.
7. **Clauser, K. R., P. Baker, and A. L. Burlingame.** 1999. Role of accurate mass measurement (+/- 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Anal Chem **71:**2871-82.
8. **Cronan, J. E., and G. L. Waldrop.** 2002. Multi-subunit acetyl-CoA carboxylases. Prog **41:**407-435.
9. **Darby, J. M., D. G. Taylor, and D. J. Hopper.** 1987. Hydroquinone as the ring-fission substrate in the catabolism of 4-ethylphenol and 4-hydroxyacetophenone by *Pseudomonas putida* JD1. J Gen Microbiol **133:**2137-2146.
10. **Dias, L., S. Dias, T. Sancho, H. Stender, A. Querol, M. Malfeito-Ferreira, and V. Loureiro.** 2003. Identification of yeasts isolated from wine-related environments and capable of producing 4-ethylphenol. Food Microbiology **20:**567-574.
11. **Doherty, N. S., B. H. Littman, K. Reilly, A. C. Swindell, J. M. Buss, and N. L. Anderson.** 1998. Analysis of changes in acute-phase plasma proteins in an acute

inflammatory response and in rheumatoid arthritis using two-dimensional gel electrophoresis. Electrophoresis **19:**355-363.

12. **Emanuelsson, O., S. Brunak, G. von Heijne, and H. Nielsen.** 2007. Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protocols **2:**953-71.
13. **Gade, D., J. Thiermann, D. Markowsky, and R. Rabus.** 2003. Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium *Pirellula* sp. strain 1. J Mol Microbiol Biotechnol **5:**240-51.
14. **Gerischer, U.** 2002. Specific and global regulation of genes associated with the degradation of aromatic compounds in bacteria. J Mol Microbiol Biotechnol **4:**111-21.
15. **Heider, J.** 2007. Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. Curr Opin Chem Biol **11:**188-94. Epub 2007 Mar 8.
16. **Helmann, J. D., and M. J. Chamberlin.** 1988. Structure and function of bacterial sigma factors. Annu Rev Biochem **57:**839-72.
17. **Höffken, H. W., M. Duong, T. Friedrich, M. Breuer, B. Hauer, R. Reinhardt, R. Rabus, and J. Heider.** 2006. Crystal structure and enzyme kinetics of the (*S*)-specific 1-phenylethanol dehydrogenase of the denitrifying bacterium strain EbN1. Biochemistry **45:**82-93.
18. **Jeno, P., T. Mini, S. Moes, E. Hintermann, and M. Horst.** 1995. Internal sequences from proteins digested in polyacrylamide gels. Anal Biochem **224:**75-82.
19. **Jones, K. H., P. W. Trudgill, and D. J. Hopper.** 1994. 4-Ethylphenol metabolism by *Aspergillus fumigatus*. Appl Environ Microbiol **60:**1978-83.
20. **Kniemeyer, O., and J. Heider.** 2001. Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. J Biol Chem **276:**21381-21386.
21. **Kniemeyer, O., and J. Heider.** 2001. (*S*)-1-Phenylethanol dehydrogenase of *Azoarcus* sp strain EbN1, an enzyme of anaerobic ethylbenzene catabolism. Arch Microbiol **176:**129-135.
22. **Krause, A., A. Ramakumar, D. Bartels, F. Battistoni, T. Bekel, J. Boch, M. Bohm, F. Friedrich, T. Hurek, L. Krause, B. Linke, A. C. McHardy, A. Sarkar, S. Schneiker, A. A. Syed, R. Thauer, F.-J. Vorholter, S. Weidner, A. Puhler, B. Reinhold-Hurek, O. Kaiser, and A. Goesmann.** 2006. Complete genome of the mutualistic, N_2-fixing grass endophyte *Azoarcus* sp. strain BH72. Nat Biotechnol **24:**1385-91.

23. **Kühner, S., L. Wöhlbrand, I. Fritz, W. Wruck, C. Hultschig, P. Hufnagel, M. Kube, R. Reinhardt, and R. Rabus.** 2005. Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1. J Bacteriol **187:**1493-1503.

24. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of sigma 54 (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol Rev **53:**367-76.

25. **Leuthner, B., C. Leutwein, H. Schulz, P. Horth, W. Haehnel, E. Schiltz, H. Schagger, and J. Heider.** 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica* - a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol Microbiol **28:**615-628.

26. **McIntire, W., D. J. Hopper, and T. P. Singer.** 1985. *p*-Cresol methylhydroxylase. Assay and general properties. Biochemical Journal **228:**325-35.

27. **Mulder, N. J., R. Apweiler, T. K. Attwood, A. Bairoch, A. Bateman, D. Binns, P. Bork, V. Buillard, L. Cerutti, R. Copley, E. Courcelle, U. Das, L. Daugherty, M. Dibley, R. Finn, W. Fleischmann, J. Gough, D. Haft, N. Hulo, S. Hunter, D. Kahn, A. Kanapin, A. Kejariwal, A. Labarga, P. S. Langendijk-Genevaux, D. Lonsdale, R. Lopez, I. Letunic, M. Madera, J. Maslen, C. McAnulla, J. McDowall, J. Mistry, A. Mitchell, A. N. Nikolskaya, S. Orchard, C. Orengo, R. Petryszak, J. D. Selengut, C. J. A. Sigrist, P. D. Thomas, F. Valentin, D. Wilson, C. H. Wu, and C. Yeats.** 2007. New developments in the InterPro database. Nucleic Acids Res **35:**D224-D228.

28. **Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. P. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. C. Lee, E. Holtzapple, D. Scanlan, K. Tran, C. M. Fraser, and et al.** 2003. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ Microbiol **5:**630.

29. **Nyström, T., and F. C. Neidhardt.** 1994. Expression and role of the universal stress protein, UspA, of *Escherichia coli* during growth arrest. Mol Microbiol **11:**537-44.

30. **Oelmüller, U., N. Krüger, A. Steinbüchel, and G. C. Friedrich.** 1990. Isolation of procaryotic RNA and detection of specific mRNA with biotinylated probes. J Microbiol Methods **11:**73-81.

31. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification of realtime RT-PCR. Nucleic Acids Res **29:**e45.

32. **Piddock, L. J. V.** 2006. Multidrug-resistance efflux pumps - not just for resistance. Nat Rev Microbiol **4:**629-36.

33. **Rabus, R., M. Kube, A. Beck, F. Widdel, and R. Reinhardt.** 2002. Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EbN1. Arch Microbiol **178:**506-516.

34. **Rabus, R., M. Kube, J. Heider, A. Beck, K. Heitmann, F. Widdel, and R. Reinhardt.** 2005. The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Arch Microbiol **183:**27-36.

35. **Rabus, R., and F. Widdel.** 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol **163:**96-103.

36. **Rabus, R., H. Wilkes, A. Behrends, A. Armstroff, T. Fischer, A. J. Pierik, and F. Widdel.** 2001. Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. J Bacteriol **183:**1707-15.

37. **Ramakers, C., J. M. Ruijter, R. H. L. Deprez, and A. F. M. Moorman.** 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett **339:**62-66.

38. **Ramos, J. L., E. Duque, M. T. Gallegos, P. Godoy, M. I. Ramos-González, A. Rojas, W. Terán, and A. Segura.** 2002. Mechanisms of solvent tolerance in gram-negative bacteria Annu Rev Microbiol **56:**743-768.

39. **Reeve, C. D., M. A. Carver, and D. J. Hopper.** 1989. The purification and characterization of 4-ethylphenol methylenehydroxylase, a flavocytochrome from *Pseudomonas putida* JD1. Biochem J **263:**431-7.

40. **Reeve, C. D., M. A. Carver, and D. J. Hopper.** 1990. Stereochemical aspects of the oxidation of 4-ethylphenol by the bacterial enzyme 4-ethylphenol methylenehydroxylase. Biochem J **269:**815-9.

41. **Seifert, W. K., and W. G. Howells.** 1969. Interfacially active acids in a California crude oil. Anal Chem **41:**554-562.

42. **Spormann, A. M., and F. Widdel.** 2000. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation **11:**85-105.

43. **Szaleniec, M., C. Hagel, M. Menke, P. Nowak, M. Witko, and J. Heider.** 2007. Kinetics and mechanism of oxygen-independent hydrocarbon hydroxylation by ethylbenzene dehydrogenase. Biochemistry **46:**7637-46.

44. **Tanner, A., and D. J. Hopper.** 2000. Conversion of 4-hydroxyacetophenone into 4-phenyl acetate by a flavin adenine dinucleotide-containing Baeyer-Villiger-type monooxygenase. J Bacteriol **182:**6565-6569.

45. **Trautwein, K., S. Kühner, L. Wöhlbrand, T. Halder, K. Kuchta, A. Steinbüchel, and R. Rabus.** 2008. Solvent stress response of denitrifying "*Aromatoleum aromaticum*" strain EbN1. Appl Environ Microbiol**:**doi:10.1128/AEM.02381-07.

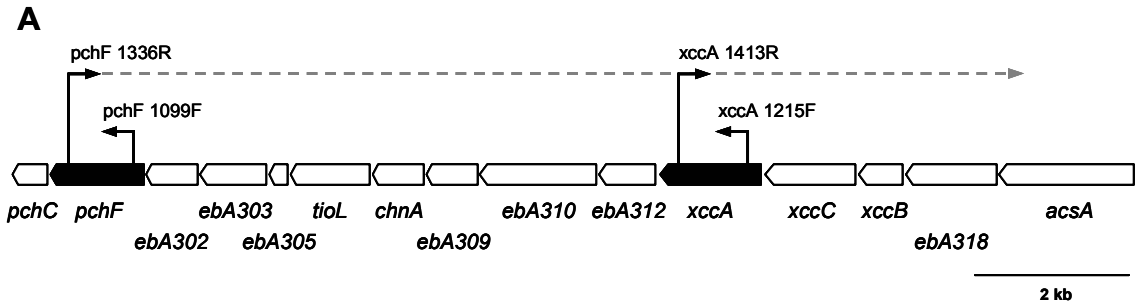
46. **van Beek, S., and F. G. Priest.** 2000. Decarboxylation of substituted cinnamic acids by lactic acid bacteria isolated during malt whisky fermentation. Appl Environ Microbiol **66:**5322-8.

47. **Widdel, F., and R. Rabus.** 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol **12:**259-276.

48. **Wöhlbrand, L., B. Kallerhoff, D. Lange, P. Hufnagel, J. Thiermann, R. Reinhardt, and R. Rabus.** 2007. Functional proteomic view of metabolic regulation in "*Aromatoleum aromaticum*" strain EbN1. Proteomics **7:**2222-39.

49. **Yu, E. W., J. R. Aires, and H. Nikaido.** 2003. AcrB multidrug efflux pump of *Escherichia coli*: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J Bacteriol **185:**5657-64.

SUPPLEMENTARY MATERIAL



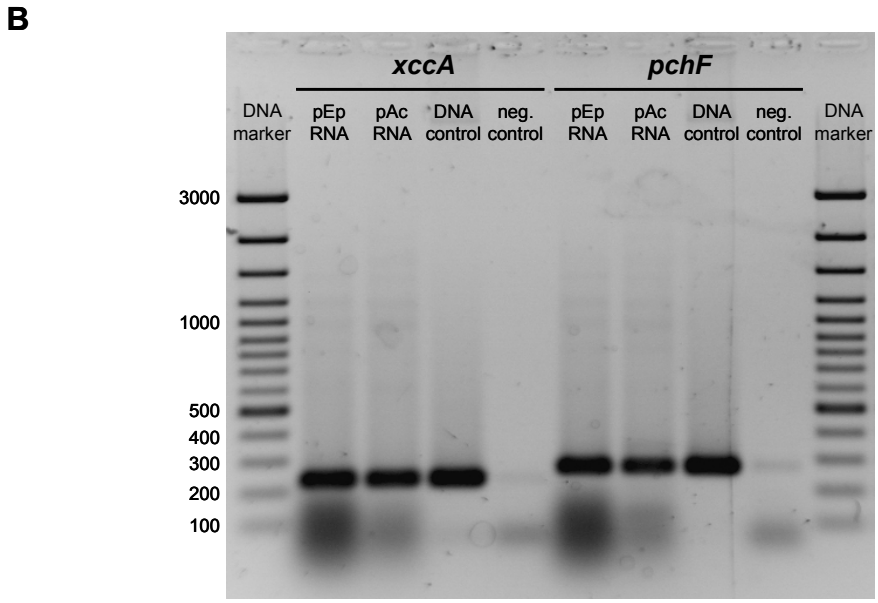


FIG. S1: Reverse transcription PCR targeting genes of the *p*-ethylphenol gene cluster. Location of applied primers are indicated in the scale model of the *p*-ethylphenol genecluster (A). The dotted grey arrow indicates direction of cDNA synthesis. Targeted genes are highlighted in black. Total RNA prepared from cells anaerobically grown with *p*-ethylphenol (pEp) and *p*-hydroxyacetophenone (pAc) and reverse transcribed using the *pchF* antisense primer pchF 1336R. Generated cDNA was used in PCR experiments using *xccA* and *pchF* specific primer pairs (B). DNA and no template controls were applied. Expected product sizes for *xccA* and *pchF* amplification are 200 bp and 240 bp, respectively.

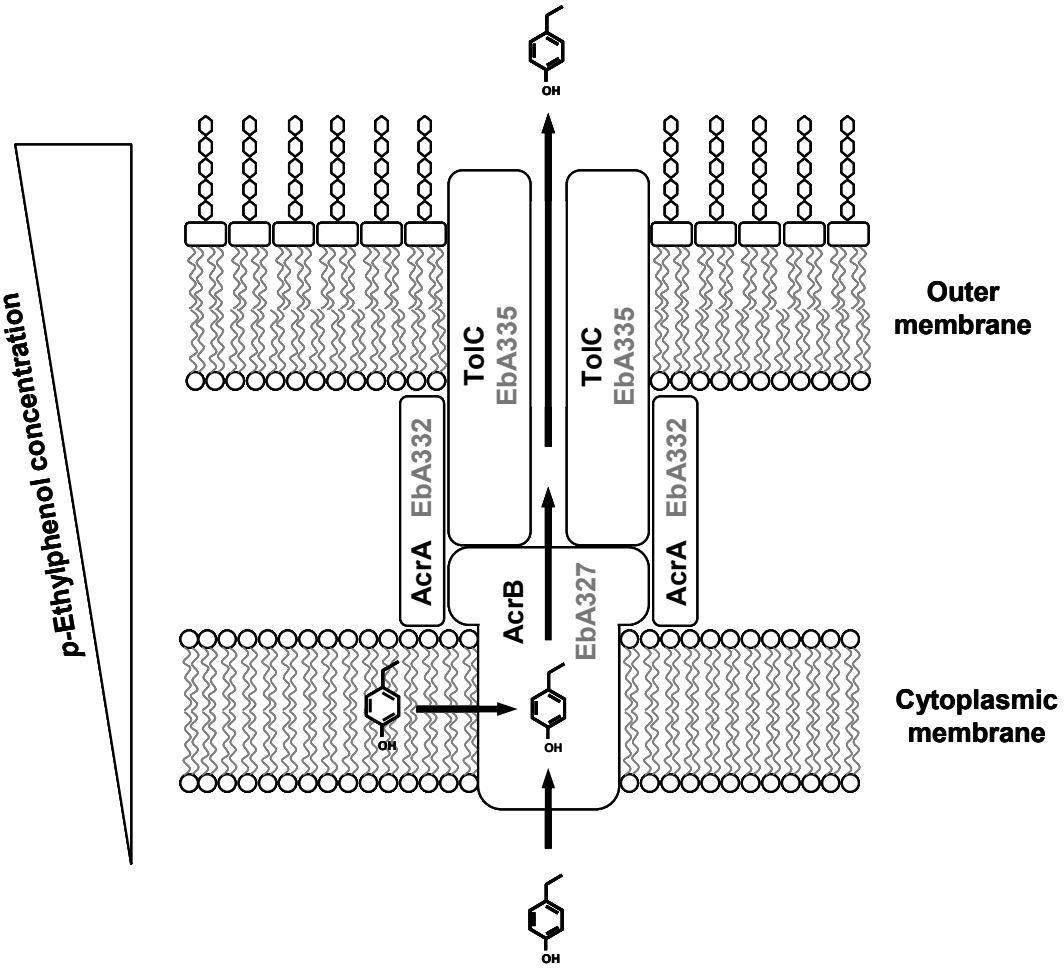


Fig. S2: Model of RND-efflux pump involved in multidrug resistance of *E.coli* (respective Proteins are indicated in black). A similar mechanism may be employed by an efflux-system of strain EbN1 for solvent stress resistance (proposed proteins are indicated in grey). It is assumed, that the transport protein (AcrB/EbA327) captures ist substrate (here exemplified for *p*-ethylphenol) either from within the phospholipid bilayer of the cytoplasmic membrane or from the cytoplasm. Subsequent transport to the extracellular medium proceeds through TolC (EbA335), which forms a channel in the outer membrane. The cooperation between the AcrB (EbA327) and TolC (EbA335) is mediated by the periplasmic accessory protein AcrA (EbA332; modified from Yu et al. 2003, Piddock 2006).

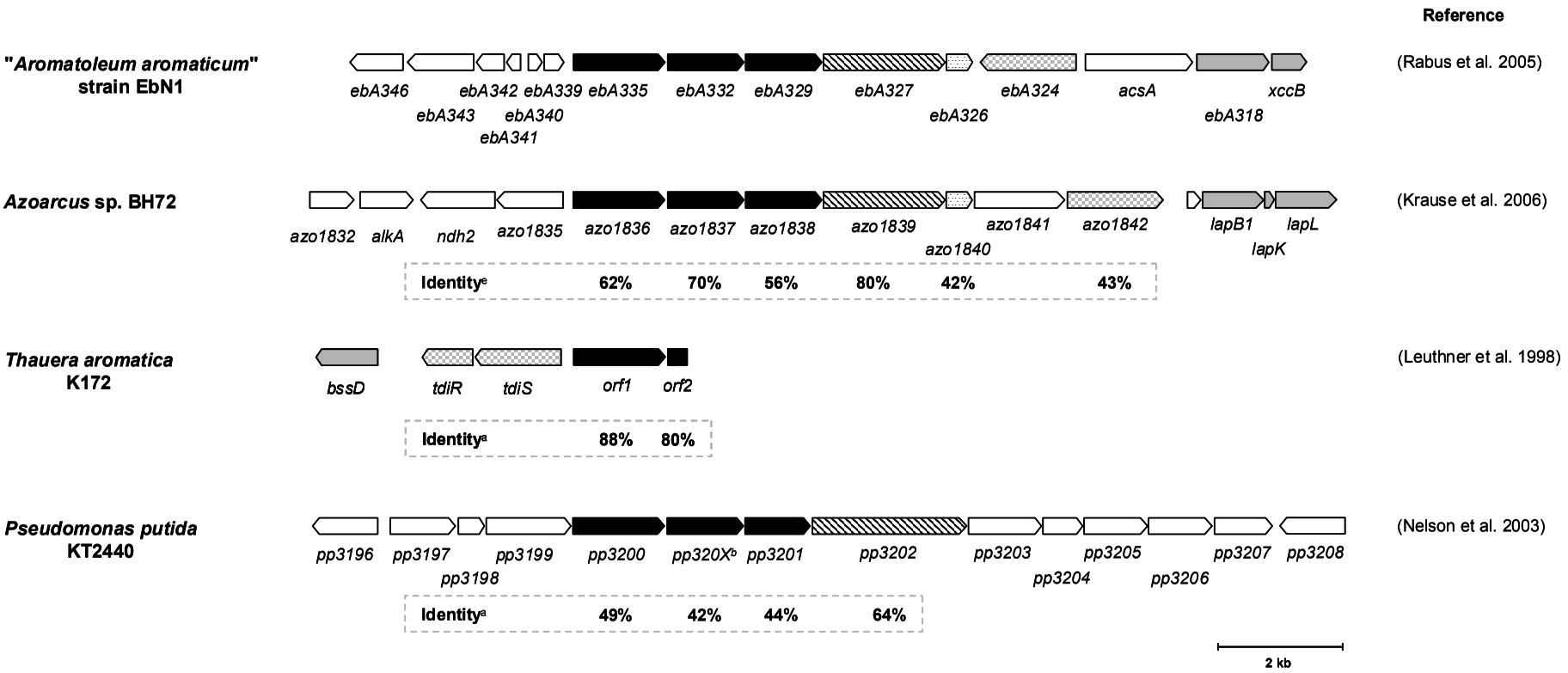


Fig. S3: Comparison of gene clusters encoding solvent related proteins in strain EbN1, phylogenetically plant associated *Azoarcus* sp. BH72, denitrifying anaerobic aromatic compound degrading *Thauera aromatica* and aerobic aromatic compound degrading *Pseudomonas putida* KT2440. Respective genes are highlighted in black: hypothetical proteins (filled), probable efflux pump (hatched) and predicted universal stress protein (dotted). Genes encoding enzymes of aromatic compound catabolism are marked in grey (filled); related regulatory proteins are checkered. Gene abbreviations are as follows: *acsA*, acetoacetyl-CoA synthetase (EbN1); *alkA*, DNA-3-methyladenine glycosylase II (BH72); *bssD*, benzylsuccinate synthase activating enzyme (K172); *lapB1*, catechol 2,3-dioxygenase (BH72); *lapK*, phenol 2-monooxygenase (BH72); *lapL*, phenol 2-monooxygenase p1 component (BH72); *ndh2*, probable NADH dehydrogenase (BH72); *tdiR*, regulator compound of toluene related two-component system (K172); *tdiS*, sensor compound of toluene related two-component system (K172); *xccB*, biotin carboxyl carrier protein (EbN1). Amino acid identities with gene products of strain EbN1 are indicated under respective genes.

[a] amino acid sequence identity; [b] this open reading frame has not been annotated in the genome (Nelson et al. 2003). However, BLAST of EbA332 against the whole genome sequence of *P. putida* KT2440 detected a homologue in between *pp3200* and *pp3201* (nucleotides 3631104 to 3632343).

4

Development of a Genetic System for Denitrifying "*Aromatoleum aromaticum*" Strain EbN1

Lars Wöhlbrand[a] and Ralf Rabus[a, b]*

Manuscript submitted to Journal of Molecular Microbiology and Biotechnology

[a] Max Planck Institute for Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany

[b] Institute of Chemistry and Biology of the Marine Environment (ICBM), University Oldenburg, Carl-von-Ossietzky-Str. 9-11, D-26111 Oldenburg, Germany

* Corresponding author

Abstract

Members of the betaproteobacterial "*Aromatoleum*"/*Azoarcus*/*Thauera* cluster contribute to the biodegradation of aromatic and other recalcitrant compounds in anoxic soils and sediments. The metabolically versatile "*Aromatoleum aromaticum*" strain EbN1 represents a model organism for this cluster, having already been studied on the physiological, genomic, proteomic and biochemical level. Here we report the development of a genetic system for "*A. aromaticum*" strain EbN1 enabling unmarked deletion mutagenesis by heterologous recombination and subsequent complementation. The antibiotic sensitivity of strain EbN1 was characterized and optimal conditions for highly efficient cultivation on solid medium were established. A procedure for introducing foreign DNA into strain EbN1 by conjugation was developed. The effectiveness of the genetic system was demonstrated by unmarked in frame deletion of *ebdC2*, encoding the γ-subunit of the paralogous ethylbenzene dehydrogenase.

Introduction

Aromatic compounds are ubiquitous and abundant compounds in nature and some of them are of environmental concern due to their toxicity and large scale industrial use. Since aromatic compounds often accumulate in anoxic zones of soils and sediments, their anaerobic biodegradation is of ecophysiological and applied interest. In the absence of molecular oxygen, biochemically intriguing reaction mechanisms are employed, which are fundamentally different from their aerobic counterparts [Heider, 2007; Widdel and Rabus, 2001]. Within the last two decades, a variety of anaerobic aromatic compound degrading bacteria were isolated, mainly denitrifiers affiliating with the *Azoarcus*/*Thauera* cluster of the Betaproteobacteria [Widdel and Rabus, 2001]. Indeed, these anaerobic degraders form a distinct cluster, which will be described as new genus "*Aromatoleum*" [Rabus et al., unpublished data].

"*Aromatoleum aromaticum*" strain EbN1 is a metabolically versatile representative of this new group of degraders with the unique ability to degrade toluene and ethylbenzene under anoxic conditions [Rabus and Widdel, 1995]. Besides these alkylbenzenes, strain EbN1 degrades a wide variety of polar aromatic (including phenol, *p*-cresol and *p*-ethylphenol) and aliphatic compounds (including acetone) under anoxic and some also under oxic conditions [Rabus and Widdel, 1995; Wöhlbrand et al., 2007].

The complete genome sequence of strain EbN1 is the first to be determined for an anaerobic hydrocarbon degrader and for a member of the "*Aromatoleum*"/*Azoarcus*/*Thauera* cluster [Rabus et al., 2005]. Genes encoding the first enzyme of anaerobic ethylbenzene degradation, ethylbenzene dehydrogenase (*ebdABCD*), are encoded in an operon-like structure at approx. 1.4 Mb on the chromosome of strain EbN1 [Rabus et al., 2005]. Interestingly, the chromosome harbours a paralogous *ebdABCD2* gene set at approx. 3.4 Mb [Rabus et al., 2005]. The EbdABCD proteins are specifically formed during anaerobic growth with ethylbenzene, which has not been observed for their paralogs [Kühner et al., 2005]. Biochemical studies [Kniemeyer and Heider, 2001; Szaleniec et al., 2007] and the crystal structure [Kloer et al., 2006] of ethylbenzene dehydrogenase revealed that two electrons are abstracted from ethylbenzene to reduce the molybdenum cofactor of the α-subunit. The latter is reoxidized by transferreing the electrons to the heme *b* of the γ-subunit which finally donates them to a so far unknown carrier, most likely cytochrome *c*.

The genetic accessibility of the anaerobic degraders *Azoarcus evansii* [Gescher et al., 2002; Rost et al., 2002] and *Azoarcus* sp. strain T [Achong et al., 2001] as well as *Thauera*

aromatica strain T1 [Coschigano, 2002] has already been demonstrated. Manipulative genetics with "*A. aromaticum*" strain EbN1 has not been possible until now due to (i) its inefficient and non-robust growth on standard solid medium, (ii) unidentified antibiotic resistance markers and (iii) the lack of protocols for DNA transfer into strain EbN1. In this study we describe the development of a genetic system for strain EbN1 exemplified by unmarked deletion of *ebdC2*. Its product, EbdC2, encodes the γ-subunit of a paralogous ethylbenzene dehydrogenase, the physiological function of which is presently unknown.

Results and Discussion

Growth on Solid Medium and Characterization of Antibiotic Resistance

A prerequisite for genetic experiments is the isolation of genetically modified single colonies on solid growth medium. So far, no or only poor growth of strain EbN1 on solid medium was observed (Fig. 1A) [Rabus and Widdel, 1995].

Initial experiments revealed the inability of strain EbN1 to grow on "standard" solid media like LB- or BHI-agar and on solid medium described for the phylogenetically related *Azoarcus* sp. BH72 [Reinhold-Hurek et al., 1993], necessitating the development of a solid medium meeting the specific growth requirements of strain EbN1.

Optimal growth of strain EbN1 was obtained, when the defined mineral medium (solidified with 1.5 % [w/v] agar) was phosphate (20 mM) buffered and contained acetate, pyruvate (5 mM each) and benzoate (4 mM) as a mixture of organic carbon sources. This solid medium also contained 4- to 5-fold increased concentrations of ammonia and nitrate (from 5 mM NH_4^+ and 7 mM NO_3^- to 30 mM each; as compared to the original liquid mineral medium) to avoid limitation of the reduced nitrogen and electron acceptor (in case of anaerobic cultivation). Applying this newly developed medium, visible (1-2 mm in diameter), light brown colonies were obtained after 5-7 days under anoxic conditions (9-10 days under oxic conditions) or 7-9 days, when antibiotics were added (Fig. 1B). A high plating efficiency

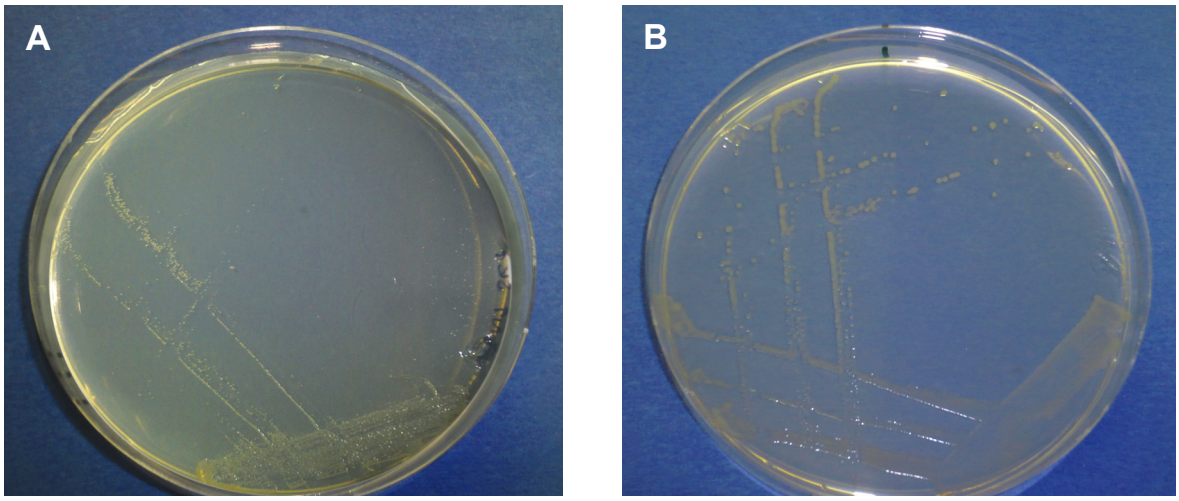


Fig. 1. Anaerobic growth of "*A. aromaticum*" strain EbN1 on solid medium. Incubation of strain EbN1 on the solidified, originally described mineral medium [Rabus and Widdel, 1995] yielded only a few small colonies after 14 days of incubation and only within the first dilution-streak (A). The newly developed solid mineral medium allowed good growth of light brown colonies up to the last dilution-streak after 5-7 days of incubation (B).

was evident from 1.5 (±0.2) × 10^8 CFU/ml obtained on agar plates upon inoculation with liquid cultures of strain EbN1 harboring 1.1 × 10^9 cells/ml.

While lower concentrations of ammonia and nitrate (<30 mM both) in the solid medium lead to smaller colonies even after a prolonged incubation, a further increase beyond 30 mM did not enhance growth any further. Also, increased concentrations of vitamins, trace elements or selenium and tungsten (2-fold each) did not improve growth on solid medium. Yeast extract, tryptone or peptone did neither support growth of strain EbN1 on solid medium nor impair growth when a utilizable organic substrate was provided.

Growth of strain EbN1 (wild type) in liquid and solid mineral medium was completely inhibited by ampicillin (50 µg/ml), chloramphenicol (20 µg/ml), gentamycin (50 µg/ml), kanamycin (50 µg/ml), puromycin (20 µg/ml), rifampicin (400 µg/ml), streptomycin (50 µg/ml) and tetracycline (20 µg/ml), enabling application of a broad range of antibiotic resistance markers to select for genetic variants.

Physiological Characteristics of the Streptomycin Resistant Mutant Strain EbN1-SR7.

To enable counter selection of genetically modified strain EbN1 from donor strains (e.g. *E. coli*) a streptomycin resistant mutant was generated, which is designated strain EbN1-SR7. Physiological characterization revealed its ability to grow with all known substrates under oxic and anoxic conditions (Table 1) without significantly prolonged incubation times. Comparison of anaerobic growth with benzoate revealed a slightly lower maximum optical density of strain EbN1-SR7 (in the presence of streptomycin) as compared to the wild type (Fig. 2). Moreover, the doubling time of strain EbN1-SR7 during linear growth was slightly lower than that of the wild type (3.3 h and 3.0 h, respectively), though this is an approximate value due to the short exponential growth phase as seen in a semi-logarithmic plot of the OD vs. time (not shown). The lower growth yields and doubling times are most probably due to the presence of the antibiotic. In addition to the physiological experiments, the soluble proteomes of wild type strain EbN1 and mutant strain EbN1-SR7 during anaerobic growth with benzoate was analyzed by means of silver-stained 2DE gels (isoelectric focusing pH 3-10). Comparison of the respective protein patterns revealed only minor differences: less than 10 protein spots (out of > 1000) differed between the two strains, most likely related to the streptomycin resistance (data not shown). Overall, strain EbN1-SR7 apparently does not differ from the wild type with respect to the main physiological properties, allowing its use for genetic studies.

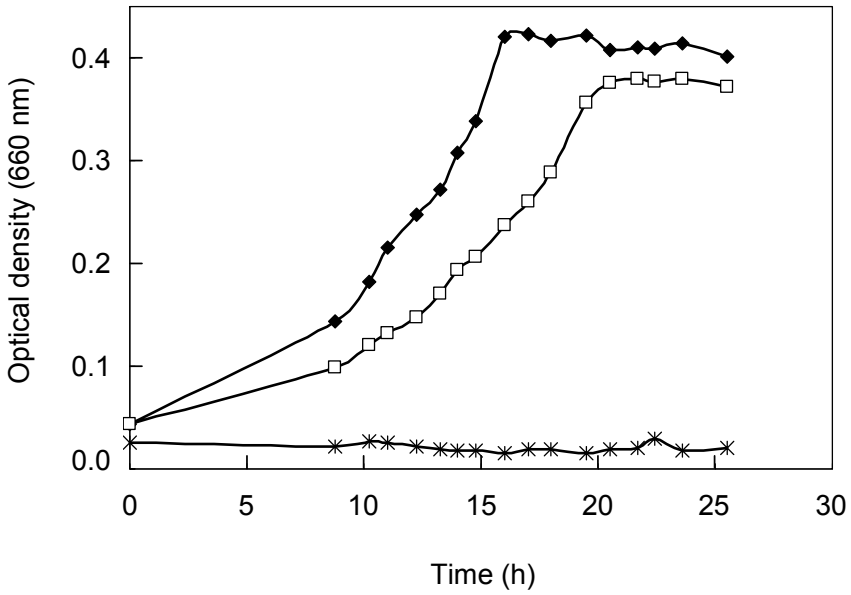


Fig. 2. Time course of anaerobic growth of "*A. aromatoleum*" strains EbN1 wild type (filled diamonds) and the streptomycin-resistent strain EbN1-SR7 (open squares) with benzoate (sterile control, asterisk).

To determine stability of the mutation conferring streptomycin resistance, strain EbN1-SR7 was subcultured in streptomycin-free liquid medium over 5 passages and subsequently plated on streptomycin-containing as well as streptomycin-free solid medium. Determination of CFU revealed, that >75 % of the bacteria retained the resistance in the absence of the antibiotic, demonstrating a high stability of this attribute.

Conjugational Transfer of Plasmid DNA to Strain EbN1-SR7.

Construction of plasmid vectors in an *E. coli* background necessitates protocols to transfer the vector-construct into strain EbN1-SR7. The broad-host-range vector pBBR1-MCS4, carrying an ampicillin resistance [Kovach et al., 1995], was used to establish conjugational plasmid transfer from *E. coli* S17-1 to strain EbN1-SR7. The plasmid is not self transmissible, but can be transferred utilizing the *tra* functions of RP4 that are integrated in the chromosome of *E. coli* S17-1 [Simon et al., 1983]. The conjugation conditions were optimized applying different donor-recipient ratios and varying mating times. Ampicilin-resistant colonies of strain EbN1-SR7 occurred after 7-9 days, when mating was performed over night. Shorter mating times (<6 h) yielded only few ampicillin resistant colonies of strain EbN1-SR7 (<10 CFU/ml), if any. A transformation frequency of 1.3×10^{-5} was obtained

applying a 1:3 donor-recipient ratio, which was significantly lower applying a 1:1 ratio (0.9×10^{-6}). Transformation frequencies within the same range were reported for the proteobacteria *Allochromatium vinosum* (3.6×10^{-4}) and *Bartonella henselae* (2×10^{-5}) using *E. coli* S17-1 as donor during IncP plasmid transfer [Dehio and Meyer, 1997; Pattaragulwanit and Dahl, 1995].

The presence of pBBR1-MCS4 in transconjugants was verified by PCR and isolation of the plasmid DNA. The isolated plasmid revealed restriction patterns identical to those isolated from *E. coli* (data not shown) and could be efficiently transferred back to *E. coli* via transformation, indicating the absence of deletions or rearrangements of the plasmid in strain EbN1-SR7.

Plasmid Stability in Strain EbN1-SR7

The stability of plasmid pBBR-MCS4 in strain EbN1-SR7 was analyzed by anaerobic growth of transconjugants with a mixture of acetate, benzoate and pyruvate in liquid cultures without selection pressure (i.e. in the absence of ampicillin). The respective cultures were transferred over 10 passages (approx. 40 generations). Comparison of CFU/ml on selective and non-selective media revealed, that >80 % of the population retained the transferred plasmid (data not shown), demonstrating a high stability of the plasmid in strain EbN1-SR7.

Generation of an in Frame Deletion Mutant: Strain EbN1-SR7 ΔebdC2

Deletion mutagenesis by homologous recombination was based on the suicide vector pK19mobsacB, carrying a kanamycin resistance and the *sacB* gene. The latter confers sucrose sensitivity, which should facilitate fast detection of double crossover events [Schäfer et al., 1994]. The host range of the plasmid is restricted to *E. coli* and closely related species of the genera *Salmonella* and *Serratia*. In accordance, conjugational transfer of the plasmid pK19mobsacB from *E. coli* S17-1 to strain EbN1-SR7 did not yield kanamycin resistant clones, while transfer of the plasmid pBBR-MCS4 in a control experiment yielded ampicillin resistant clones.

To generate an unmarked Δ*ebdC2* deletion mutant, the adjacent genes *ebdB2* and *ebdD2* were amplified by PCR and cloned into the pK19mobsacB vector in an *E. coli* S17-1 host background. Instead of a complete *ebdC2* gene, the construct contained only its start and stop codon, separated by an XbaI restriction site (Fig. 3A and B). To avoid polar effects in the deletion mutant, the intergenic regions to *ebdC2* were maintained (for details see experimental procedures). The resulting deletion - construct (plasmid pK19ebdB2D2) was transferred to

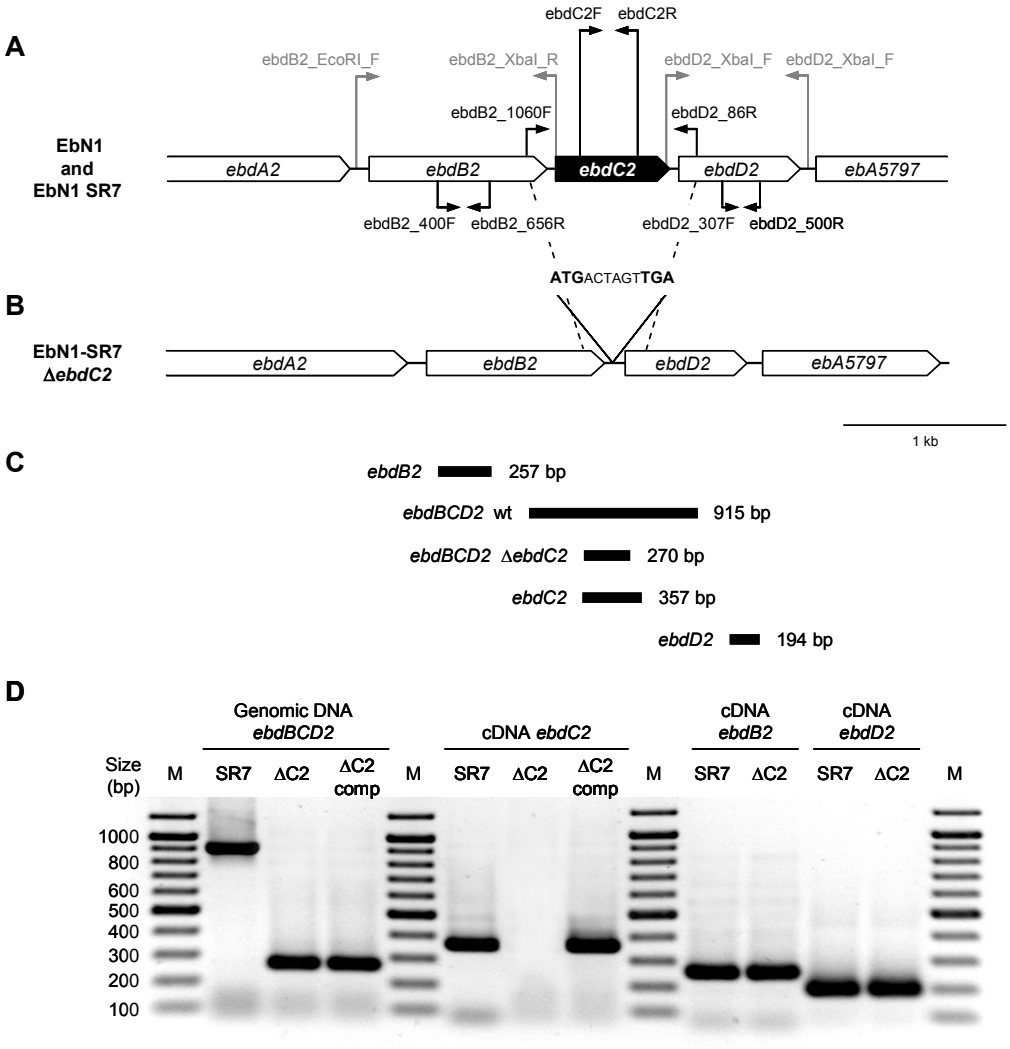


Fig. 3. Schematic depiction of the chromosomal organisation of genes encoding the paralogous ethylbenzene dehydrogenase (*ebdABCD2*) in "*A. aromatoleum*" strains EbN1, EbN1-SR7 (both A) and EbN1-SR7 Δ*ebdC2* (B). Arrows indicate the position of the applied primer pairs. Corresponding nucleotide sequences and amplicon sizes are provided in Table 2. Primers for the construction of the deletion-vector are indicated in grey. The remnants of *ebdC2* in the deletion mutant, i.e. its start and stop codons separated by an XbaI restriction site, are indicated by the insert. (C) The amplicon sizes of primer pairs used for characterization of the mutant are indicated at the respective positions below the gene chart. Comprehensive information on nucleotide sequences and amplicon sizes is given in Table 2. (D) Electropherogram of PCR products obtained from "*A. aromaticum*" strains EbN1-SR7 (abbreviated as SR7), EbN1-SR7 Δ*ebdC2* (abbreviated as ΔC2) and EbN1-SR7 Δ*ebdC2*comp (abbreviated as ΔC2comp). The *ebdC2* specific primers ebdC2F and ebdC2R were applied to confirm *ebdC2* deletion in the genome. The deletion was confirmed by application of primers located in the adjacent genes, ebdB2_1060F and ebdD2_86R, the product of which includes *ebdC2*. Transcription was analyzed using cDNA generated from total RNA prepared from cells anaerobically grown with *p*-cresol to demonstrate expression of *ebdB2* and *ebdD2*. Therefore, the primers ebdB2_400F and ebdB2_656R as well as ebdD2_307F and ebdD2_500R were applied. Respective antisense primers were used for cDNA synthesis. In a control experiment, the same RNA-preparation was used as template, without reverse transcription, yielding no PCR-product (not shown).

strain EbN1-SR7 by conjugation and kanamycin-resistant clones were obtained. PCR based analysis of respective clones demonstrated the integration of the plasmid pK19ebdB2D2 into the chromosome of strain EbN1-SR7 (single cross-over), yielding two distinct products when applying *ebdC2* spanning primers (ebdB2_1060F and ebdD2_86R; Table 2 and Fig. 3A). Subsequent cultivation of clones harboring the single cross-over in kanamycin-free liquid medium allowed recombination without selection pressure. Subsequently, plating on solid sucrose-containing medium was performed to select for double cross-over events; cells containing the *sacB* genes are not viable under these conditions.

PCR analysis, using primers spanning *ebdC2*, located in *ebdB2* and *ebdD2* (ebdB2_1060F and ebdD2_86R, respectively), was applied to identify Δ*ebdC2* mutants. Colony screening revealed the rare occurrence of a double cross-over (only <4 % of screened clones). Sequence analysis of the PCR products confirmed the deletion of *ebdC2* (Fig. 3D), with the start and stop codons being its only remnants. Accordingly, it was impossible to obtain a PCR product applying *ebdC2* specific primers or a combination of an *ebdC2* primer with an appropriate primer located in *ebdB2* or *ebdD2* (Fig. 3 and Table 2).

Development of a Complementation System to Allow in trans *Expression of* ebdC2

To demonstrate that a mutant phenotype is due to gene deletion or disruption, rather than to secondary gene mutation, the respective gene has to be expressed *in trans*. The broad-host-range plasmid pBBR-MCS4 [Kovach et al., 1995] was used to construct an expression vector containing *ebdC2* (MCS4-*ebdC2*), since it is relatively small (5656 bp) and is stably maintained in strain EbN1-SR7 (see above). Conjugational transfer of the plasmid MCS4-*ebdC2* into strain EbN1-SR7 Δ*ebdC2* lead to a large number of ampicillin-resistant transconjugants. Nearly all screened clones harbored the expression vector, which was confirmed by (i) PCR analysis using M13 primers, (ii) sequence analysis of the obtained PCR product and (iii) restriction pattern analysis of the recovered plasmid (data not shown).

Characterization of the ΔebdC2 *Mutant*

To analyze possible polar effects of the *ebdC2* deletion, transcriptional analysis of the flanking genes *ebdB2* (upstream) and *ebdD2* (downstream) was performed. Expression of both genes was detected during anaerobic growth of mutant strain EbN1-SR7 Δ*ebdC2* with *p*-cresol (Fig. 3D), but not with benzoate (Fig. 4), as previously indicated by proteomic studies [Wöhlbrand et al., 2007]. The same expression pattern was observed in strain EbN1-SR7, suggesting no impairment of gene expression (Figs. 3D and 4). In accordance with

ebdB2 and *ebdD2* expression, transcription of *ebdC2* in strain EbN1-SR7 was only detected during anaerobic growth with *p*-cresol, but not with benzoate (Figs. 3D and 4). The Δ*ebdC2* mutant did not express *ebdC2* under either of the two growth conditions, but the complemented mutant expressed *ebdC2* during anaerobic growth with *p*-cresol and benzoate, demonstrating its constitutive expression (Figs. 3D and 4).

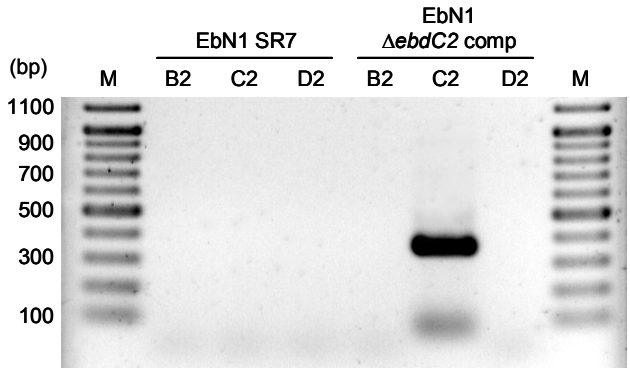


Fig. 4. Transcription of *ebdB2* (B2), *ebdC2* (C2) and *ebdD2* (D2) in "*A. aromaticum*" strains EbN1-SR7 and EbN1-SR7 Δ*ebdC2*comp. Total RNA of cells grown anaerobically with benzoate was used for cDNA synthesis. In a control experiment, the same RNA-preparation was used as template, without reverse transcription, yielding no PCR-product (not shown). Respective primers are listed in Table 2. M, 100 bp size-marker.

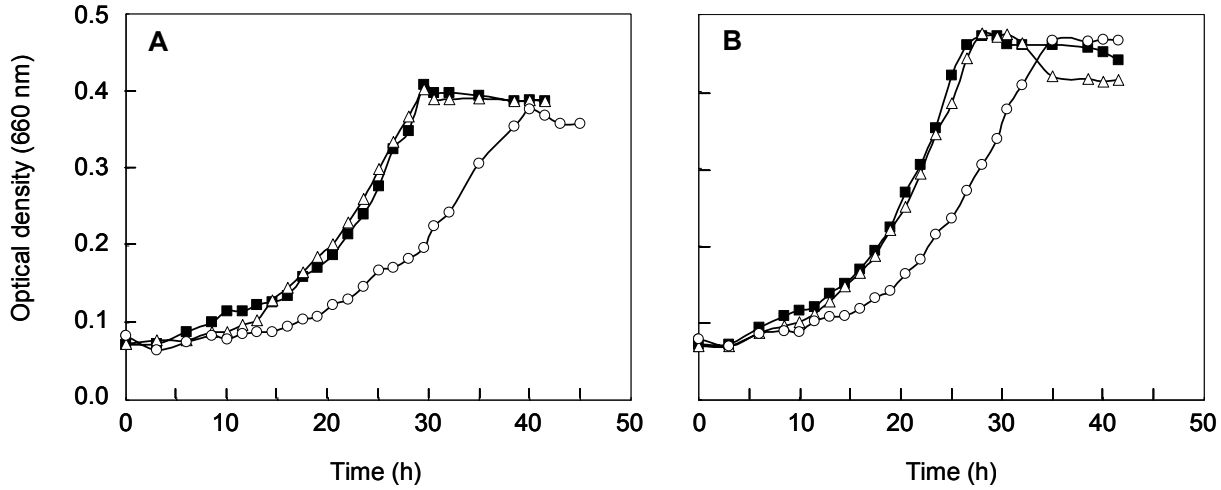


Fig. 5. Time courses of anaerobic growth of "*A. aromaticum*" strains EbN1-SR7 (filled squares), EbN1-SR7 Δ*ebdC2* (open triangles) and EbN1-SR7 Δ*ebdC2*comp (open circles) with ethylbenzene (A) and *p*-cresol (B).

Physiological Characterization of Strain EbN1-SR7 ΔebdC2

Interestingly, the deletion of the γ-subunit of the paralogous ethylbenzene dehydrogenase (*ebdC2*) did not give rise to a distinct phenotype. The generated Δ*ebdC2* mutant was able to grow with all known substrates of wild type strain EbN1 under oxic and anoxic conditions (Table 1) [Rabus and Widdel, 1995; Wöhlbrand et al., 2007]. Furthermore, growth with all substrates was not significantly impaired as exemplified for anaerobic growth with ethylbenzene and *p*-cresol (Fig. 5). The doubling times of mutant strains EbN1-SR7 and EbN1-SR7 Δ*ebdC2* were almost identical (e.g 5.18 h and 5.10 h in case of ethylbenzene). Accordingly, the complemented mutant did not reveal a distinct phenotype, although the growth rate was reduced (Fig. 5). This impairment is most likely due to the presence of the additional antibiotic (ampicillin), as observed for strain EbN1-SR7 exposed to streptomycin (see above). It should be noted, that growth of the complemented mutant was more impaired with ethylbenzene than with *p*-cresol. This physiological effect may be due to the higher solvent stress exerted by ethylbenzene (Trautwein et al. 2008).

Previous studies revealed specifically increased abundances of proteins encoded in the paralogous ethylbenzene dehydrogenase gene cluster during anaerobic growth with *p*-cresol and phenol [Wöhlbrand et al., 2007], but not with ethylbenezene [Kühner et al., 2005]. The high degree of sequence identity of ethylbenzene dehydrogenase and its paralog (52 to 83 %) [Rabus, 2005] suggest that a potential substrate of EbdABC2 should be chemically similar to ethylbenzene. Indeed, *p*-ethylphenol was recently discovered as new substrate for anaerobic growth of strain EbN1. However, a very recent study suggested a *p*-cresol methylenehydroxylase-like protein rather than paralogous EbdABC2 to catalyze the initial dehydrogenation of *p*-ethylphenol [Wöhlbrand et al. unpublished]. Agreeing with this observation, anaerobic growth of strain EbN1-SR7 Δ*ebdC2* with *p*-ethylphenol was not impaired (Table 1). To discover the true substrate of the paralogous enzyme, comprehensive substrate tests were conducted. However, strain EbN1 did not grow with any of the tested diverse aromatic compounds (Table 1) that were selected due to their different degrees of structural similarity to ethylbenzene: ethyltoluenes, ethylanilines, styrene, heterocyclic ethylpyridine, the heterocyclic cyclopentadienes ethylfuran and ethylthiophene, xylenes, fluor-toluenes and also propylphenols.

Overall, the absent effect of the *ebdC2* deletion on the physiology of strain EbN1-SR7 Δ*ebdC2* under any tested growth condition disagrees with an involvement of paralogous ethylbenzene dehydrogenase in the known catabolic pathways of strain EbN1. Even though proteins encoded in the same gene cluster were abundantly formed [Wöhlbrand et al., 2007],

Table 1. Growth substrates of "*A. aromaticum*" strains EbN1 (wild type), EbN1-SR7, EbN1-SR7 Δ*ebdC2* and complemented EbN1-SR7 Δ*ebdC2*comp under oxic and anoxic (i.e. nitrate-reducing) conditions. Concentrations of the tested compounds are given in parenthesis. Concentrations of soluble compounds are given in mM. Concentrations in % (v/v) refer to dilutions of poorly water soluble compounds in heptamethylnonane as inert carrier phase. + O_2 oxic conditions; – O_2 anoxic conditions; + growth; – no growth; n.d. not determined.

Tested compounds	"*Aromatoleum aromaticum*" strain			
	EbN1 (wild type)	EbN1-SR7	EbN1-SR7 Δ*ebdC2*	EbN1-SR7 Δ*ebdC2*comp
	– O_2 / + O_2	– O_2 / + O_2	– O_2 / + O_2	– O_2 / + O_2
Alkylbenzenes				
Toluene (1 %)	+ / –[b]	+ / –	+ / –	+ / –
Ethylbenzene (2 %)	+ / –[b]	+ / –	+ / –	+ / –
Styrene (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
o-, *m*-, *p*-Fluortoluene (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
o-, *m*-, *p*-Xylene (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
o-, *m*-, *p*-Ethyltoluene (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
Heterocyclic ethylbenzene derivatives				
o-, *m*-, *p*-Ethylaniline (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
o-, *m*-, *p*-Ethylpyridine (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
2-Ethylfuran (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
2-Ethylthiophene (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
Phenolic compounds				
Phenol (0.5)	+ / –[c]	+ / –	+ / –	+ / –
p-Chlorphenol (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
p-Cresol (2)	+ / –[c]	+ / –	+ / –	+ / –
p-Ethylphenol (0.5 %)	+ / –[d]	+ / –	+ / –	+ / –
o-, m-Ethylphenol (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
o-, m-, p-Propylphenol (>0.1 %)[a]	– / n.d.	n.d.	n.d.	n.d.
Alcohols, aldehydes and ketones				
Benzyl alcohol (2)	+ / –[b]	+ / –	+ / –	+ / –
Ethanol (2)	+ / +[b]	+ / +	+ / +	+ / +
2-Butanol (5)	+ / n.d.[b]	+ / n.d.	+ / n.d.	+ / n.d.
2-Propanol (5)	+ / n.d.[b]	+ / n.d.	+ / n.d.	+ / n.d.
Benzaldehyde (2)	+ / –[b]	+ / –	+ / –	+ / –
Acetophenone (2)	+ / +[b]	+ / +	+ / +	+ / +
p-Hydroxyacetophenone (2)	+ / n.d.[d]	+ / n.d.	+ / n.d.	+ / n.d.
Acetone (2)	+ / n.d.[b]	+ / n.d.	+ / n.d.	+ / n.d.
2-Butanone (2)	+ / n.d.[b]	+ / n.d.	+ / n.d.	+ / n.d.
Carboxylic acids				
o-Aminobenzoate (2)	+ / –[e]	+ / –	+ / –	+ / –
Benzoate (4)	+ / +[b]	+ / +	+ / +	+ / +
p-Hydroxybenzoate (4)	+ / –[e]	+ / –	+ / –	+ / –
m-Hydroxybenzoate (4)	+ / +[e]	+ / +	+ / +	+ / +
Phenylacetate (4)	+ / +[b]	+ / +	+ / +	+ / +
Gentisate (2)	– / +[e]	– / +	– / +	– / +
Acetate (5)	+ / +[b]	+ / +	+ / +	+ / +
Propionate (5)	+ / +[b]	+ / +	+ / +	+ / +
Pyruvate (5)	+ / +[b]	+ / +	+ / +	+ / +

[a] Growth was tested with concentrations of 0.1 %, 0.5 % and 1 % (v/v) in HMN.
[b] Taken from Rabus and Widdel 1995.
[c] Taken from Rabus et al. unpublished.
[d] Taken from Wöhlbrand et al. unpublished.
[e] Taken from Wöhlbrand et al. 2007.

and *ebdB2*, *ebdC2* and *ebdD2* transcribed during anaerobic growth with *p*-cresol (Fig 3D), the deletion of *ebdC2* did not have an effect on anaerobic growth with *p*-cresol. Therefore the catabolic function of the paralogous ethylbenzene dehydrogenase, if any, remains unclear at present.

Concluding remarks

The present study demonstrated the genetic accessibility of "*A. aromaticum*" strain EbN1. The established protocols enable introduction of foreign DNA into strain EbN1 via conjugation, generation of unmarked deletion mutants, and expression of proteins from extrachromosomal elements. These genetic tools together with the availability of the complete genome sequence of strain EbN1 [Rabus et al., 2005] open new avenues for molecular studies of the physiology of this bacterium. Indeed, previous proteomic studies with strain EbN1 [Kühner et al., 2005; Wöhlbrand et al., 2007] revealed the specific and abundant formation of proteins with to date unknown functions under various defined growth conditions. A genetic approach promises to verify the actual involvement of these proteins in the respective metabolic pathways and to provide clues about their functions. In addition, the relatively high solvent tolerance of strain EbN1 [Trautwein et al., 2008] and the ability to express proteins from extrachromosomal elements provides a solid basis for conducting biochemical studies of enzymes involved in anaerobic aromatic compound degradation also originating from other, related organisms (e.g. *Thauera* spp.), which may be difficult to perform in an *E. coli* background.

Experimental procedures

Bacterial Strains and Plasmids

Plasmids, "*A. aromaticum*" strain EbN1 (wild type and mutants) and *E. coli* strains used in this study are described in Table 3. Preparation of chemically competent *E. coli* S17-1 cells was performed according to the method described by Inoue et al. [1990]. Chemicals used were of analytical grade.

Culturing Conditions and Growth Media

E. coli strains were cultivated as described [Sambrook and Russell, 2001]. Liquid cultures of "*A. aromaticum*" strain EbN1 were performed in mineral medium as reported previously [Rabus and Widdel, 1995] applying a mixture of acetate (5 mM), benzoate (4 mM) and pyruvate (5 mM) as carbon source, unless stated otherwise.

Solid growth medium for strain EbN1 contained per liter: 0.23 g $NaSO_4$, 1.0 g NaCl, 2.54 g $NaNO_3$, 1.6 g NH_4Cl, 15.0 g agar. After autoclaving KH_2PO_4 (4 mM), K_2HPO_4 (16 mM), $CaCl_2$ (0.2 mM), $MgCl_2$ (1.6 mM), vitamins, EDTA-chelated mixture of trace elements, and selenate and tungsten solution [Widdel and Bak, 1992] were added from sterile stock solutions. The pH was adjusted to 7.2 and acetate (5 mM), benzoate (4 mM) and pyruvate (5 mM) were added as carbon sources. Incubation was carried out at 28 °C in anaerobic jars (Ochs, Bovenden-Lenglern, Germany) under an N_2-atmosphere.

Antibiotics were used in agar plates or liquid cultures at the following concentrations (unless indicated otherwise): ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; streptomycin, 50 µg/ml.

Substrate test experiments were performed as previously described [Rabus and Widdel, 1995]. Anaerobic growth experiments with ethylbenzene and *p*-cresol were performed in 500 ml flat-bottles containing 400 ml anoxic medium as described [Rabus and Widdel, 1995]. Essentially, substrate-adapted cultures were used as inoculum and sampling (1 ml) was carried out under strictly anoxic conditions [Widdel and Bak, 1992]. Optical density was monitored at 660 nm (UV-1202, Shimadzu, Duisburg, Germany). The end of incubation was indicated by depletion of nitrate and nitrite as determined with Merckoquant test stripes (Merck, Darmstadt, Germany).

Table 2: Sequences of oligonucleotide primers derived from the genome sequence of "*A. aromaticum*" strain EbN1 used in this study. Integrated restriction sites are underlined.

Primer	Target gene	Sequence (5´→ 3´)	Product length (bp)
***ebdC2* Deletion construct (pK19mobsacB)**			
ebdB2_EcoRI_F	*ebdB2*	AAGAATTCACTGTCACTGGAGGTAAACAT	1096
ebdB2_XbaI_R		AATCTAGACATTTGCGTCTCCGATGTCAT	
ebdD2_XbaI_F	*ebdD2*	AATCTAGATGAGGATGACAGATGAACAGC	688
ebdD2_XbaI_R		AATCTAGAGACCGGGTACCGCCCCGCCTA	
Control of *ebdC2* deletion			
ebdC2F	*ebdC2*	AAGATCATGGGGCCAACAAAAGTC	357
ebdC2R		CCAGGGAGCGGCGGAAAATGA	
ebdB2_1060_F	*ebB2C2D2*	TTGCATGCGGAGCGGGAGAAGTCG	270[a] / 915[b]
ebdD2_86_R		CGTCGCTGGCGGGATAGGCAAACA	
***ebdC2* Complementation construct (pBBR1-MCS4)**			
ebdC2_ApaI_F	*ebdC2*	AGGGCCCGCGGGTTCACCAACGATCCCG	706
ebdC2_SpeI_R		AACTAGTCTGTCATCCTCACTTTTGAAT	
Control of *ebdB2* and *ebdD2* transcription			
ebdB2_400F	*ebdB2*	CAGGGCGGCGGGCAATGG	257
ebdB2_656R		TCGAGGCGCGGAAAACACA	
ebdD2_307_F	*ebD2*	TCGGCCGTTGATCAAAAGAAAT	194
ebdD2_500_R		CCCTCGGCTGCCACATAGTC	

[a] Referring to mutant strain EbN1-SR7 Δ*ebdC2*.
[b] Referring to strain EbN1 wild type.

Table 3. Strains and plasmids used in this study

Strains or plasmids	Genotype, markers and further characteristics	Source or reference
Strains		
E. coli S17-1	*thi, pro, hsdR, recA* with RP4-2[Tc::Mu-Km::Tn7]	[Simon et al., 1983]
"*A. aromaticum*" strain EbN1		
EbN1	Wild type	[Rabus and Widdel, 1995]
EbN1-SR7	Str^r	This study
EbN1-SR7-Δ*ebdC2*	Str^r , Δ*ebdC2*	This study
EbN1-SR7-Δ*ebdC2*comp	Str^r , Δ*ebdC2*, pBBR1-MCS4 *ebdC2*	This study
Plasmids		
pK19mobsacB	Km^r, *sacB* modified from *B. subtilis*, *lacZ*α	[Schäfer et al., 1994]
pK19ebdB2	Km^r, *sacB* modified from *B. subtilis*, *lacZ*α *ebdB2*	This work
pK19ebdB2D2	Km^r, *sacB* modified from *B. subtilis*, *lacZ*α *ebdB2, ebdD2*	This work
pBBR1-MCS4	Ap^r, *mob, lacZ*α	[Kovach et al., 1995]
pBBR1-MCS4 *ebdC2*	Ap^r, *mob*, *lacZ*α, *ebdC2*	This study

Determination of Antibiotic Sensitivity of Strain EbN1

To determine endogenous antibiotic resistances, strain EbN1 was grown in liquid and on solid medium containing ampicillin (50 µg/ml), chloramphenicol (20 µg/ml), gentamycin (50 µg/ml), kanamycin (50 µg/ml), puromycin (20 µg/ml), rifampicin (400 µg/ml), streptomycin (50 µg/ml) or tetracycline (20 µg/ml), respectively. Stock solutions of antibiotics were prepared according to standard protocols [Sambrook and Russell, 2001] and stored at -20 °C.

Determination of Plating Efficiency

The cell numbers of fully grown liquid cultures of strain EbN1 were determined by means of the MPN method. Full growth in liquid media was correlated to complete depletion of nitrate and nitrite. Two independent cultures were used to inoculate MPN-series of a 10-fold serial dilution (up to 10^{-11}) with four replicates performed per dilution step.

Plating efficiency was determined by plating 10-fold serial dilutions of the same cultures used for the MPN series in quadruple and subsequent determination of colony forming units (CFU).

*Isolation of Streptomycin Resistant "*A. aromaticum*" Strain EbN1-SR7*

Spontaneous streptomycin resistant mutants were obtained by growing strain EbN1 in liquid media containing 10 to 100 µg/ml streptomycin and subsequently isolating single colonies from solid medium. Selected mutants were physiologically characterized by growth experiments with all known organic substrates under oxic and nitrate-reducing conditions using 22 ml hungate tubes (Table 1) [Rabus and Widdel, 1995]. Time courses of anaerobic growth with benzoate (4 mM) were determined using 400 ml cultures inoculated with 20 ml of fully grown, benzoate-adapted pre-cultures. Wild type strain EbN1 was used as reference. Finally, dilution PCR of whole cells was used for sequencing the 16S rDNA [Kühner et al., 2005].

DNA techniques

Genomic DNA of strain EbN1 was isolated with phenol:chloroform:isoamylalcohol (PCI) according to standard methods [Sambrook and Russell, 2001]. Plasmid DNA was isolated from *E. coli* and strain EbN1 applying alkalic lysis as described [Sambrook and Russell, 2001].

Primers for amplification of genomic DNA from strain EbN1 were manually designed from the whole genome sequence (Accession No. CR555306) and are listed in Table 2. PCR amplification was performed using *Taq* polymerase (Eppendorf, Hamburg, Germany) under standard conditions. Restriction enzymes, T4-DNA-ligase and DNA-size marker (100 bp Plus) were obtained from Fermentas GmbH (St. Leon-Rot, Germany) and used according to manufacturers instructions.

Preparation of total RNA from substrate-adapted cells and RT-PCR were carried out as previously described [Kühner et al., 2005].

Ligation and Transformation of Plasmid Constructs

PCR products containing restriction sites for cloning were purified with PCI and subsequent ethanol precipitation [Sambrook and Russell, 2001]. Both, purified PCR-product and plasmid were digested with appropriate restriction enzymes according to manufacturer's instructions and purified again. For ligation, digested PCR-product and plasmid were mixed in a 3:1 molecular ratio and PCR-H_2O added to a final volume of 8 µl. This mixture was denatured for 10 min at 70 °C and chilled on ice. Subsequently, T4-ligase buffer (1×), ATP (25 µM) and T4-ligase (2.5 u) were added, mixed carefully and incubated at 12 °C for at least 5 h (or over night). Prior to transformation, the ligation mixture was denatured at 65 °C (10 min) and chilled on ice.

Transfomation of 2.5 or 5 µl of the ligation reaction into chemical competent *E. coli* S17-1 was performed as described [Inoue et al., 1990]. Single colonies grown on selective solid medium were picked and screened by PCR for correct insert size, applying M13 primers. Plasmids from positive clones were purified and used as template for sequencing of the insert to verify its identity.

Conjugational Plasmid Transfer

Strains of *E. coli* S17-1 containing the appropriate plasmid served as donors in agar-plate matings. Overnight cultures of the *E. coli* donor and the EbN1-SR7 recipient strain (24 h incubation) were mixed in a 1:1 or 1:3 ratio (total volume 1 ml), respectively, and harvested by centrifugation using a table top centrifuge (10 min, 13000 rpm). The pellet was washed with 1 ml liquid mineral medium (containing 5 mM pyruvate) and resuspended in 20 µl of liquid mineral medium. The bacterial suspension was spread as single drop on solid mineral medium (containing 10 g/l tryptone) and incubated at 28 °C over night under oxic conditions. Subsequently, bacteria were resuspended in 1 ml liquid mineral medium (containing 5 mM

pyruvate), transferred to a sterile 2 ml reaction tube and incubated at 28 °C for 3 h. Finally, 10-times serial dilutions with liquid mineral medium were plated on solid mineral medium containing the appropriate antibiotics and incubated as described above.

Identification of Positive Clones of Strain EbN1

Colonies obtained on selective solid medium were picked and transferred to 22 ml hungate tubes containing 2.5 ml liquid mineral medium (5 mM acetate, 5 mM pyruvate and 4 mM benzoate) supplemented with the appropriate antibiotics. The headspace was flushed with N_2:CO_2 (90:10; v/v), sealed with a butylene stopper and incubated at 28 °C. For PCR based genotypic verification, 1 ml of grown culture was withdrawn, transferred to a 1.5 ml reaction tube and harvested. The cell pellet was resuspended in 20 µl PCR-H_2O and 2 µl thereof used as template for PCR analysis (e.g. using ebdB2_1060_F and ebdD2_86_R primers, Table 2).

Construction of an in Frame ebdC2 *Deletion Mutant*

To avoid impairment of gene transcription downstream of *ebdC2*, a modified copy of the chromosomal *ebd2*-operon was constructed in the suicide vector pK19mobsacB. The construct contained *ebdB2*, *ebdD2* and only the start and stop codon of *ebdC2* separated by an XbaI restriction site (Fig. 6).

In a first step, *ebdB2* was amplified from genomic DNA of strain EbN1with primers containing an EcoRI (ebdB2_EcoRI_F) and a XbaI (ebdB2_XbaI_R) restriction site (Table 2). Directly upstream of the XbaI site, the reverse primer contained the start codon of *ebdC2* and the upstream intergenic region to the stop codon of *ebdB2*. The obtained 1096 bp PCR-product was purified using PCI and ethanol precipitation. The latter and the plasmid pK19mobsacB were digested with EcoRI and XbaI, purified and ligated (see above). The ligation mix was transformed into *E. coli* S 17-1 and kanamycin resistant colonies were screened for correct inserts using *ebdB2* and M13 primers. The created plasmid containing *ebdB2* was designated as plasmid pK19ebdB2.

In a second step, *ebdD2* was amplified from genomic DNA of strain EbN1 with a primerpair containing the XbaI restriction sites (ebdD2_XbaI_F and ebdD2_XbaI_R; Table 2). The forward primer (ebdD2_XbaI_F) contained directly downstream of the restriction site the stop codon of *ebdC2* and the intergenic region connecting to *ebdD2*. The resulting 688 bp PCR product was digested with XbaI, purified and ligated into the XbaI digested plasmid pK19ebdB2 and transformed into *E. coli* S 17-1. Resulting kanamycin-

resistant colonies were screened for the correct insert using M13 primers. The correct orientation of the *ebdD2* insert was verified with primers spanning the ligation site (ebdB2_1060_F and ebdD2_86_R; Fig. 3A). The obtained plasmid pK19ebdB2D2 (Fig. 6) was sequenced to ensure correct sequence of the structural genes *ebdB2* and *ebdD2* as well as the remainder of *ebdC2* (i.e. the start and stop codon).

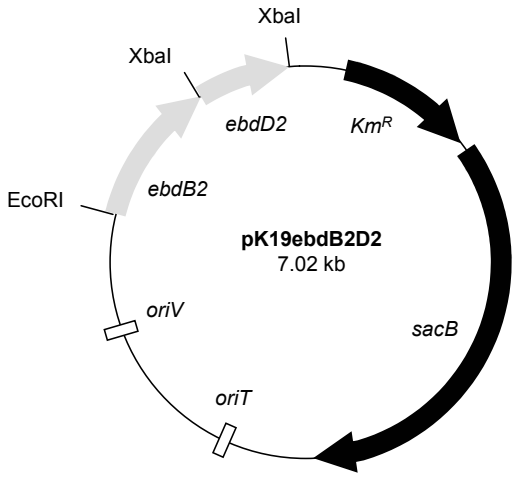


Fig. 6. Schematic depiction of the constructed *ebdC2* deletion-vector pK19ebdB2D2. The plasmid derived genes encoding the kanamycin resistance (Km^R) and the *sacB* genes are indicated in black. Genes encoding subunits of the paralogous ethylbenzene dehydrogenase (*ebdB2* and *ebdD2*) are marked in grey. Restriction sites used for cloning are indicated [adapted from Schäfer et al. 1994].

After conjugational transfer of the plasmid pK19ebdB2D2 from *E. coli* S17-1 to strain EbN1-SR7 (see above), aliquots of the cell suspension were plated on solid mineral medium containing kanamycin and streptomycin to select for mutants of strain EbN1-SR7, which integrated the plasmid into the chromosome (single cross over). Obtained clones were screened for the single cross over by PCR, using ebdB2_1060_F and ebdD2_86_R primers, spanning the *ebdC2* gene (Fig. 3A). Positive transconjugants revealing a large (915 bp, "wild type") and a small (270 bp, construct) amplicon were transferred to liquid medium containing only streptomycin to allow a second cross over event. Since cells with the plasmid pK19ebdB2D2 integrated into the chromosome are unable to grow in the presence of sucrose (due to the *sacB* gene), fully grown cultures were plated on solid medium containing streptomycin and 7.5 % (w/v) sucrose to select for clones, which have lost the integrated plasmid. Clones growing on sucrose plates were screened for the Δ*ebdC2* genotype using ebdB2_1060_F and ebdD2_86_R primers as well as *ebdC2* specific primers (Table 2). The

correct deletion of *ebdC2* was confirmed by sequencing the amplicon of the *ebdC2*-spanning primers (see also Fig. 3).

Construction of Complementation Plasmid

A complementation plasmid was derived from the broad-host-range vector pBBR-MCS4 [Kovach et al., 1995]. The *ebdC2* gene was amplified from genomic DNA using primers containing an ApaI (ebdC2_ApaI_F) and a SpeI (ebdC2_SpeI_R) restriction site, respectively (Table 2). Besides the coding sequence, the amplicon included 50 bp upstream of the start-codon, to include the ribosomal-binding-site of *ebdC2*. Ligation, transformation and identification of positive clones was performed as described above. The construct was confirmed by PCR and sequencing using M13 primers and subsequently transferred to EbN1-SR7 Δ*ebdC2* via conjugation (see above). Transcription of the plasmid derived *ebdC2* was confirmed by RT-PCR (Fig. 3D and 4).

Acknowledgements

We thank D. Lange and S. Lahme (Bremen) for technical assistance and I. Werner and O. Grundmann (Bremen) for valuable technical discussions. This work was supported by the Deutsche Forschungsgemeinschaft and the Max-Planck Gesellschaft.

References

Achong GR, Rodriguez AM, Spormann AM: Benzylsuccinate synthase of *Azoarcus* sp. strain T: cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization. J Bacteriol 2001; 183:6763-6770.

Coschigano PW: Construction and characterization of insertion/deletion mutations of the *tutF*, *tutD*, and *tutG* genes of *Thauera aromatica* strain T1. FEMS Microbiol Lett 2002; 217:37-42.

Dehio C, Meyer M: Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn5 in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. J Bacteriol 1997; 179:538-540.

Gescher J, Zaar A, Mohamed M, Schagger H, Fuchs G: Genes coding for a new pathway of aerobic benzoate metabolism in *Azoarcus evansii*. J Bacteriol 2002; 184:6301-6315.

Heider J: Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. Curr Opin Chem Biol 2007; 11:188-194.

Inoue H, Nojima H, Okayama H: High efficiency transformation of *Escherichia coli* with plasmids. Gene 1990; 96:23-28.

Kloer DP, Hagel C, Heider J, Schulz GE: Crystal structure of ethylbenzene dehydrogenase from *Aromatoleum aromaticum*. Structure 2006; 14:1377-1388.

Kniemeyer O, Heider J: Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. J Biol Chem 2001; 276:21381-21386.

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, 2nd, Peterson KM: Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 1995; 166:175-176.

Kühner S, Wöhlbrand L, Fritz I, Wruck W, Hultschig C, Hufnagel P, Kube M, Reinhardt R, Rabus R: Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1. J Bacteriol 2005; 187:1493-1503.

Pattaragulwanit K, Dahl C: Developement of a genetic system for a purple sulfur bacterium: conjugative plasmid transfer in *Chromatium vinosum*. Arch Microbiol 1995; 164:217-222.

Rabus R, Widdel F: Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol 1995; 163:96-103.

Rabus R: Functional genomics of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Appl Microbiol Biotechnol 2005; 68:580-587.

Rabus R, Kube M, Heider J, Beck A, Heitmann K, Widdel F, Reinhardt R: The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. Arch Microbiol 2005; 183:27-36.

Reinhold-Hurek B, Hurek T, Claeyssens M, van Montagu M: Cloning, expression in *Escherichia coli*, and characterization of cellulytic enzymes of *Azoarcus* sp., a root-Invading diazotroph. J Bacteriol 1993; 175:7056-7065.

Rost R, Haas S, Hammer E, Herrmann H, Burchhardt G: Molecular analysis of aerobic phenylacetate degradation in *Azoarcus evansii*. Mol Genet Genomics 2002; 267:656-663.

Sambrook J, Russell DW: Molecular clonig: a laboratory manual. ed 3rd, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory press, 2001

Schäfer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A: Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 1994; 145:69-73.

Simon R, Priefer U, Puhler A: A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. Nat Biotechnol 1983:784 - 791.

Szaleniec M, Hagel C, Menke M, Nowak P, Witko M, Heider J: Kinetics and mechanism of oxygen-independent hydrocarbon hydroxylation by ethylbenzene dehydrogenase. Biochemistry 2007; 46:7637-7646.

Trautwein K, Kühner S, Wöhlbrand L, Halder T, Kuchta K, Steinbüchel A, Rabus R: Solvent stress response of denitrifying "*Aromatoleum aromaticum*" strain EbN1. Appl Environ Microbiol 2008: 74(8):2267-74.

Widdel F, Bak F: Gram-negative mesophilic sulfate-reducing bacteria. in Balows A, Trüper, HG., Dworkin, M., Harder, W., Schleifer, KH. (ed): The Prokaryotes. New York: Springer-Verlag, 1992, vol 4, 2278-3352.

Widdel F, Rabus R: Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 2001; 12:259-276.

Wöhlbrand L, Kallerhoff B, Lange D, Hufnagel P, Thiermann J, Reinhardt R, Rabus R: Functional proteomic view of metabolic regulation in "*Aromatoleum aromaticum*" strain EbN1. Proteomics 2007; 7:2222-2239.

5

Aromatoleum gen. nov., a novel genus accommodating the phylogenetic lineage including *Azoarcus evansii* and related species, and proposal of *A. aromaticum* sp. nov., *A. petroleum* sp. nov., *A. bremensis* sp. nov., *A. toluolicum* sp. nov., and *A. diolicum* sp. nov.

Ralf Rabus,[1,2]* Lars Wöhlbrand,[2] Daniela Lange,[2] Markus Meyer,[3] Barbara Reinhold-Hurek,[4] Friedrich Widdel[2] and Peter Kämpfer[5]

Manuscript in preparation

[1]Institut für Chemie und Biologie des Meeres (ICBM), Universität Oldenburg, Carl-von-Ossietzky Str. 9-11, D-26111 Oldenburg, Germany

[2]Max-Planck-Institut für Marine Mikrobiologie, Celsiusstraße 1, D-28359 Bremen, Germany

[3]Bruker Daltonik GmbH, Fahrenheitstr. 4, D-28359 Bremen, Germany

[4]Laboratorium für Allgemeine Mikrobiologie, Universität Bremen, PO Box 330440, D-28334 Bremen, Germany

[5]Institut für Angewandte Mikrobiologie, Justus Liebig Universität, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany

* Corrsponding author

ABSTRACT

Comparative 16S rRNA gene sequence analysis indicates that two distinct sublineages exist within the genus *Azoarcus*: the *Azoarcus evansii* lineage, which comprises *Azoarcus evansii* (type strain KB740^T = DSM 6898^T), *A. buckelii* (type strain U120^T = DSM 14744^T), *A. anaerobius* (type strain LuFRes1^T = DSM 12081^T), *A. tolulyticus* (type strain Tol-4^T = ATCC 51758^T), *A. toluvorans* (type strain Td21^T = ATCC 700604^T = DSM 15124^T) and *A. toluclasticus* (type strain MF63^T = ATCC 700605^T), and the *Azoarcus indigens* lineage, which comprises *A. indigens* (type strain VB32^T = ATCC 51398^T = LMG 9092^T), *A. communis* (type strain SWub3^T = ATCC 51397^T = LMG 9095^T) and *Azoarcus* sp. strain BH72. This phylogenetic discrimination is supported by physiological differences. Members of the *A. evansii* lineage have remarkable anaerobic degradation capacities encompassing a large variety of alkylbenzenes, polar aromatic compounds and monoterpenes, and involving novel biochemical reactions in many cases. In contrast, members of the *A. indigens* lineage are plant associated and lack these anaerobic degradation capacities. It is proposed that species of the *A. evansii* lineage should be classified in a novel genus, *Aromatoleum* gen. nov. Finally, based on the literature and new growth, DNA–DNA hybridization and proteomic data, the following six new species are proposed: *A. aromaticum* sp. nov. (type strain EbN1^T = DSM 19018^T and strain pCyN1 = DSM 19016), *A. petroleum* sp. nov. (type strain ToN1^T = DSM 19019^T), *A.bremensis* sp. nov. (type strain PbN1^T = DSM 19017^T), *A. toluolicum* sp. nov. (type strain T^T = DSM 19020^T) and *A. diolicum* sp. nov. (type strain 22Lin^T = DSM 15408^T).

INTRODUCTION

A key role of bacteria in terrestrial and aquatic environments is the remineralization of natural and anthropogenic organic compounds to CO_2. Correspondingly, bacteria harbour a broad spectrum of degradation reactions. The exceptional chemical stability of alkylbenzenes and polar aromatic compounds represents a special challenge for their biodegradation. Under oxic conditions, oxygenases are used, which employ highly reactive oxygen species derived from molecular oxygen (Harayama *et al*., 1992). However, in many habitats, such as aquatic sediments, deeper soil layers and groundwater aquifers, anoxic conditions prevail, demanding oxygen-independent degradation pathways.

Isolation of pure cultures with respective anaerobic degradation capacities was instrumental for elucidation of novel pathways (Heider & Fuchs, 1997; Harwood *et al*., 1999; Widdel & Rabus, 2001). Denitrifying isolates were preferentially used for biochemical studies, since they grow relatively fast and yield considerable cell mass. The majority of these denitrifying strains can be grouped into two phylogenetic clusters within the *Betaproteobacteria*. One cluster corresponds to the genus *Thauera* (Macy *et al*., 1993) and includes besides the well studied *Thauera aromatica* K172^T (Anders *et al*., 1995) other aromatic compound degraders, namely *T. aminoaromatica* S2^T (Mechichi *et al*., 2002), *T. chlorobenzoica* 3CB-1^T (Song *et al*., 2001), *T. mechernichensis* TL1^T (Scholten *et al*., 1999) and *T. phenylacetica* B4P^T (Mechichi *et al*., 2002), and the monoterpene degraders *T. linaloolentis* 47Lol^T and *T. terpenica* 58Eu^T (Foss & Harder, 1998). The other cluster consists of the *Azoarcus* genus, which was originally described to contain mainly N_2-fixing endophytes or root surface colonizers of Kallar grass, such as *A. indigens* VB32^T and *A. communis* SWub3^T (Reinhold-Hurek *et al*., 1993). The first taxonomically described anaerobic degrader of aromatic compounds from this second cluster is *A. evansii* KB740^T (Anders *et al*., 1995) and the biochemical differences to *A. indigens* VB32^T and *A. communis* SWub3^T were already obvious. In subsequent studies, the anaerobic aromatic compound degraders *A. buckelii* U120^T (Mechichi *et al*., 2002), *A. anaerobius* LuFRes1^T (Springer *et al*., 1998), *A. tolulyticus* Tol-4^T (Zhou *et al*., 1995), *A. toluvorans* Td-21^T and *A. toluclasticus* MF63^T (Song *et al*., 1999) were described and taxonomically allocated into the genus *Azoarcus*. This group of "degraders" is equally close related to the plant-associated *Azoarcus* spp. and the *Thauera* spp., and it has been noted before that the broad metabolic versatility of this group requires

the description of a new genus (Reinhold-Hurek & Hurek, 2000). Phenotypic separation of the cluster of degraders from the plant-associated *Azoarcus* spp. was further corroborated in the present study by the absence of nitrogenase activity and *nifH* genes in most of the degraders and the inability of *A. indigens* VB32^T, *A. communis* SWub3^T and *Azoarcus* sp. BH72 to degrade aromatic compounds under anoxic conditions.

Therefore, we propose to consolidate these findings by allocating this cluster of denitrifying degraders to a novel genus, *Aromatoleum* gen. nov. In addition to the six already described species of this new genus, we propose here five new species from various aquatic and terrestrial habitats (Table 1). Most of the *Aromatoleum* spp. can be metabolically differentiated by the anaerobic utilization of specific substrates (Table 2) that include alkylbenzenes (e.g. toluene), monoterpenes (e.g. limonene) and polar aromatic compounds (e.g. 2-aminobenzoate). In summary, the genus *Aromatoleum* displays a thus far unknown degradative versatility. Biochemical investigations of the individual pathways lead to the discovery of a broad spectrum of novel reactions from anaerobic as well as aerobic pathways (Table 3; for overview see Boll *et al*., 2002; Heider & Fuchs, 1997; Heider *et al*., 1999; Hylemon & Harder, 1999; Spormann *et al*., 1999; Widdel & Rabus, 2002; Rabus, 2005).

Table 1. Habitats and geographical distribution of isolates from the new genus *Aromatoleum*

Species	Strain	Substrate used for isolation	Habitat	Geographical location	Reference
A. aromaticum	EbN1^T	Ethylbenzene	Ditches, River Weser	Bremen (Germany)	Rabus & Widdel, 1995
A. aromaticum	pCyN1	*p*-Cymene	Ditches, River Weser	Bremen (Germany)	Harms *et al*., 1999
A. bremensis	PbN1^T	Propylbenzene	Ditches, River Weser	Bremen (Germany)	Rabus & Widdel, 1995
A. petroleum	ToN1^T	Toluene	Ditches, River Weser	Bremen (Germany)	Rabus & Widdel, 1995
Aromatoleum sp.	EB1	Ethylbenzene	Refinery treatment pond	San Francisco Bay (USA)	Ball *et al*., 1996
A. toluolicum	T^T	Toluene	Petroleum contaminated aquifer	Switzerland	Kuhn et al., 1988; Dolfing *et al*., 1990
A. diolicum	22Lin^T	Cyclohexane-1,2-diol	Activated sludge	Lintel (Germany)	Harder, 1997
A. evansii	KB740^T	2-Aminobenzoate	Creek sediment	USA	Braun & Gibson, 1984; Anders *et al*., 1995
A. buckelii	U120^T	*o*-Cresol	Oxic soil	Ulm (Germany)	Tschech & Fuchs, 1987; Mechichi *et al*., 2002
A. anaerobius	LuFRes1^T	Resorcinol	Activated sludge	Tübingen (Germany)	Gorny *et al*., 1992; Springer *et al*., 1998
A. tolulyticus	Tol-4^T	Toluene	Petroleum contaminated aquifer sediment	Northern Michigan (USA)	Zhou *et al*., 1995
A. toluvorans	Td21^T	Toluene	Soil	Clinton County, Michigan (USA)	Zhou *et al*., 1995
A. toluclasticus	MF63^T	Toluene	Shallow aquifer sediment	Moffett Field, California (USA)	Song *et al*., 1999

METHODS

Bacterial strains. Bacterial strains from the proposed *Aromatoleum* genus used in this study are listed in Table 1. The denitrifying strains EbN1^T, ToN1^T, PbN1^T, mXyN1^T and pCyN1 have been subcultured in our laboratory since their isolation (Rabus & Widdel, 1995; Harms *et al*., 1999). The strains T^T (= DSM 9506^T), 22Lin^T (= DSM 15408^T), KB740^T (= DSM 6898^T), U120^T (= DSM 14744^T), LuFRes1^T (= DSM 12081^T) and Td21^T (= DSM 15124^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). The strains Tol-4^T (= ATCC 51758) and MF63^T (= ATCC 700605) were obtained from the American Type Culture Collection (= ATCC; Manassas, VA, USA). *Azoarcus indigens* VB32^T (= ATCC 51398^T), *A. communis* SWub3^T (= ATCC 51397^T) and *Azoarcus* sp. BH72 were provided by the laboratory of B. Reinhold-Hurek.

Cultivation conditions and substrate utilization tests. Growth under denitrifying conditions was tested in defined, ascorbate-reduced (4 mM) medium with nitrate (7 mM) as electron acceptor (Rabus & Widdel, 1995). Nitrate-free medium (containing 5·6 mM NH_4Cl) was used when growth was tested under oxic conditions (Rabus & Widdel, 1995). Anaerobic cultivation was carried out at 28 °C in anoxically sealed glass tubes, and hydrocarbons and other poorly water soluble organic compounds were added as dilute solutions in inert 2,2,4,4,6,8,8-heptamethylnonane as previously described (Rabus *et al*., 1993; Rabus & Widdel, 1995). Soluble organic substrates were added from sterile stock solutions (Widdel & Bak, 1992). Three parallel cultures were used for each individual substrate test. All growth tests for an individual strain were inoculated from the same culture grown with benzoate (4 mM) under nitrate-reducing conditions, except for *A. indigens* VB32^T, *A. communis* SWub3^T and *Azoarcus* sp. BH72 (5 mM malate under oxic conditions). Growth was monitored by measuring the optical density at 660 nm over a period of at least 4 weeks. Some tests for carbon sources used for aerobic growth were carried out using Biotype 100 strips (API Biomerieux) as described previously (Reinhold-Hurek & Hurek, 2000) with readings after 7 days. Mass cultivation (400 ml or 2 l volume) for FAME-analyses, DNA/DNA hybridization or mass spectrometric protein profiling was carried out with benzoate (4 mM) as substrate, except for *T. phenylacetica* B4P^T, *T. chlorobenzoica* 3CB-1^T, *T. linaloolentis* 47Lol^T, *T. terpenica* 58Eu^T (each 10 mM acetate), *T. pivalivorans* Piv1^T (4 mM pivalate) and strain pCyN2 (5 % [v/v] *p*-cymene in carrier phase). Cells were harvested by centrifugation

(10000 ***g***, 30 min, 4 °C), washed with Tris/HCl buffer (100 mM, pH7·5, containing 5 mM $MgCl_2.6H_2O$), frozen in liquid nitrogen and stored at -80 °C until further analysis.

Test for active nitrogenase. The presence of active nitrogenase was tested by the acetylene reduction assay essentially as described before (Reinhold-Hurek *et al*., 1987). Inoculum cultures were grown in nitrogen-limited medium (2 mM NH_4Cl and 3 mM $NaNO_3$) with acetate (10 mM) to an optical density of 0·2 at 660 nm. Two different cultivation approaches were used to determine the capacity of nitrogen fixation: (i) Cultures were grown in nitrogen-free semi-solid medium (standard mineral medium with 0·2 % [w/v] agar, 20 mM K-phosphate buffer, pH 7·0, and 10 mM $NaHCO_3$) with acetate (10 mM) as organic substrate and oxygen as electron acceptor (Fig. 1B). Tests were carried out in 15 ml tubes sealed with rubber stoppers. Each tube contained 5 ml semi-solid medium to establish microaerophilic growth conditions. Cultures were incubated for 5 days at 28 °C prior to replacing 10 % (v/v) of the gaseous phase by acetylene. (ii) Alternatively, cultures were grown in nitrogen-free medium (pH 7·2), containing (l^{-1} deionized water) 1·12 g KH_2PO_4, 5·6 g K_2HPO_4, 0·8 g NH_4Cl, 0·23 g Na_2SO_4, 0·324 g $MgCl_2.6H_2O$, 0·0294 g $CaCl_2.2H_2O$, 0·7 % (w/v) agar, vitamins, EDTA-chelated mixture of trace elements, and selenite and tungstate solution (Widdel & Bak, 1992). As carbon sources a mixture of the following compounds was applied: acetate, succinate (each 3 mM), benzoate, *p*-hydroxybenzoate and phenylacetate (4 mM). Tests were carried out in 22 ml tubes, containing 7 ml of medium. These cultures were incubated for 3 months at 28 °C prior to replacing 10 % (v/v) of the gaseous phase with acetylene (Fig. 1C). In both cases, cultures were inoculated with 1 % (v/v) of the respective preculture, controls contained no inoculum and formed ethylene was determined about 30 h after addition of acetylene.

The formation of ethylene from acetylene reduction was measured using a gas chromatograph (GC-14A; Shimadzu, Duisburg, Germany) equipped with a HP-PLOT/U column (30 m length, 0·533 mm diameter; Agilent-Technologies, Palo Alto, CA, USA) at a temperature of 55 °C. H_2 was used as carrier gas at a flow rate of 0·05 ml s^{-1}. The injector temperature was 130 °C and the flame ionization detector (run at 150 °C) was provided with H_2 and synthetic air at flow rates of 0·05 ml s^{-1} and 2·6 ml s^{-1}, respectively. Samples (0·1 ml) were applied with a gas tight syringe.

Test for *nifH* genes. To screen for the presence of the *nifH* gene coding for the Fe-containing subunit of nitrogenases, degenerated *nifH* primers were used (nifH1: ADN GCC ATC ATY TCN CC and nifH2: TGY GAY CCN AAR GCN GA; Zehr & McReynolds, 1989). PCR was carried out at a 50 µl scale using *Taq* Polymerase (Sigma, Taufkirchen, Germany). Whole cells (2 µl cell suspension) were directly used as template. Thermocycling was performed under standard conditions and PCR-products were analyzed by agarose gel electrophoresis. To verify the amplification of *nifH* genes, obtained PCR-products were sequenced (Table 5).

FAME analysis. Fatty acids were analysed according to the procedures described by Kämpfer & Kroppenstedt (1996).

DNA base ratio and DNA/DNA-hybridization. For determination of G+C content DNA was isolated by chromatography on hydroxyapatite as described by others (Cashion *et al*., 1977). The G+C content of the DNA was determined by HPLC according to Mesbah *et al*. (1989). DNA–DNA hybridization experiments were performed with the strains shown in Table 1 using the method described by Ziemke *et al.* (1998), except that for nick translation, 2 µg of DNA was labeled during a 3 h incubation at 15 °C.

Construction of phylogenetic tree. The 16S rRNA genes were analyzed as described by Kämpfer *et al.* (2003). Phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004) and also the software package Mega (Molecular Evolutionary Genetics Analysis) version 2.1 (distance options according to the Kimura-2 model) and clustering with the neighbor-joining method and maximum parsimony was performed by using Bootstrap values based on 1000 replications.

MALDI-MS-based strain-specific proteomic fingerprints. To limit cultivation dependent differences, all four analyzed strains (ToN1^T, KB740^T, EbN1^T and pCyN1) were grown in the same medium with benzoate as organic substrate and harvested during mid-linear growth. Bacterial cells (10 mg) were suspended in 300 µl of Milli-Q water and then quickly mixed with 900 µl of ethanol (70 % final conc.). After centrifugation (12000 ***g***, 5 min, 4 °C) and removal of the supernatant, cells were extracted with 50 µl formic acid, mixed with 50 µl acetonitrile and then centrifuged again. From the supernatant 8 technical replicates (1 µl each) were spotted onto stainless steel targets (MTP 384 format, Bruker Daltonics, Bremen, Germany). After drying, 1·5 µl of matrix solution (saturated α-cyano-4-hydroxy-cinnamic

acid, HCCA in 50 % acetonitrile, 2·5 % TFA) were overlaid and dried. Spectra were generated with an autoflex III MALDI-TOF system (Bruker Daltonics) using the following settings: linear mode with a laser repetition of 200 Hz, the pulsed ion extraction was set to 210 ns, the digitizer was operated with 0·5 GHz sampling rate. The spectra from 25 different positions (40 laser shots each) were averaged to a sum spectrum (1000 laser shots in total) for each technical replicate.

Calculation of score values and Principal Component Analysis for proteomic fingerprints. Analyses were based on two consecutive experiments. Firstly, the BioTyper 1.0 software (Bruker Daltonics) was used to generate library spectra for each bacterial strain consisting of 16 technical replicates in each case. Secondly, three independent cultures (biological replicates) for each analyzed strain were processed as described above (8 technical replicates for each culture) to generate the sample spectra.

For score calculation the software compares peak lists (max. 100 peaks) of unknown sample spectra with known library spectra lists. Each peak in the library spectrum with a frequency of 100 % is assigned the maximal score value 100, whereas peaks with lower frequency yield a score value of 100 multiplied by their frequency (e.g. 20 % frequency = 100 × 0·2 = 20). The sum of all score values for a library spectrum results in a maximum score for this entry (not > 10000). Each sample spectrum gets a relative score (*actual score/maximum score*) which reflects how many percent of peaks of the library spectrum match with peaks of the sample spectrum.

For determining the number of sample peaks (relative peak number) matching to those of the library spectra, the mass accuracy is taken into account. When matching peaks fit into an inner mass window (200 ppm mass tolerance), they are directly summed up. However, when they only fit in an outer mass window (500 ppm mass tolerance) their summed number is halfed. Based on the matches of peaks to the inner and outer mass window a relative peak number is calculated according to the following formula:

$$\text{rel. peak number} = [\text{peak number}_{\text{inner mass window}} + (0{\cdot}5 \times \text{peak number}_{\text{outer mass window}})] / \text{total peak number}_{\text{unknown spectrum}}$$

The final score of each unknown spectrum is then calculated as follows:

$$\text{final score (max. 1000)} = \text{rel. score} \times \text{rel. peak number} \times \text{I-corr.} \times 1000$$

I-corr. = applied to weight the intensity-symmetry distribution of matching peaks

The Principal Component Analysis was used as an unsupervised tool for discrimination of spectra (Jolliffe, 2002). This method reduces the high number of dependent variables to a few main dependencies. The Principal Components (PCs) are orthogonal, have no opposite correlation and represent the eigenvectors of the correlation matrix of the spectra. Subsequent cluster analysis allows differentiating individual species as distinct clusters. Here we used hierarchical clustering with an Euclidian distance measurement, the linkage algorithm was based on average.

Visualization of spectra and expression differences was done by ClinProTools 2.1 software (Bruker Daltonics)

RESULTS AND DISCUSSION

Members of *Aromatoleum* gen. nov. and their habitats

The new genus *Aromatoleum* proposed in this study encompasses a betaproteobacterial cluster of thirteen described isolates (Table 1). Seven of them (strains EbN1^T, ToN1^T, PbN1^T, pCyN1, EB1, T^T and 22Lin^T) have not been taxonomically described to date, while the other six have been related to the genus *Azoarcus*: *Azoarcus evansii* KB740^T (here circumscribed as *Aromatoleum evansii*), *Azoarcus buckelii* U120^T (here circumscribed as *Aromatoleum buckelii*), *Azoarcus anaerobius* LuFRes1^T (here circumscribed as *Aromatoleum anaerobius*), *Azoarcus tolulyticus* Tol-4^T (here circumscribed as *Aromatoleum tolulyticus*), *Azoarcus toluvorans* Td21^T (here circumscribed as *Aromatoleum toluvorans*) and *Azoarcus toluclasticus* MF63^T (here circumscribed as *Aromatoleum toluclasticus*).

Most of the *Aromatoleum* species have been isolated under nitrate-reducing conditions with alkylbenzenes or polar aromatic compounds from aquatic habitats such as river or aquifer sediments (Table 1). Some species, e.g. *A. tolulyticus* Tol-4^T, were obtained from petroleum contaminated sites pointing to their potential role in bioremediation. Song *et al*. (1999) isolated 21 strains with toluene or phenol as organic substrate and nitrate as electron acceptor from various locations in the USA and Brazil. All of these strains affiliate with the *Aromatoleum* lineage, corroborating its geographic extension.

Physiological properties of *Aromatoleum* spp.

The most intriguing property of *Aromatoleum* spp. is the wide range of recalcitrant organic compounds, which they are capable of degrading anaerobically (Table 2). The newly described *Aromatoleum* spp. strains EbN1^T, pCyN1, ToN1^T, PbN1^T, 22Lin^T and T^T can be nutritionally distinguished by their specificities towards individual alkylbenzene substrates used for anaerobic growth. Strains EbN1^T, ToN1^T, pCyN1 and T^T all share the capacity to grow anaerobically with toluene, but differ with respect to additional alkylbenzene substrates (Table 2). E.g., strain EbN1^T also utilizes ethylbenzene and strain pCyN1 *p*-ethyltoluene, *p*-cymene and several monoterpenes. Interestingly, none of these strains is capable of degrading alkylbenzenes under oxic conditions, which is to date only described for toluene-degrading *Thauera* sp. stain DNT-1 (Shinoda *et al*., 2004). Strain 22Lin^T is the only one

Table 2. General and catabolic properties of isolates from the new genus *Aromatoleum*

Properties*	*Aromatoleum*													*Azoarcus*		
	***A. aromaticum* EbN1^T**	***A. aromaticum* pCyN1**	***A. bremensis* PbN1^T**	***A. petroleum* ToN1^T**	***Aromatoleum* sp. EB1**	***A. tolulicum* T^T**	***A. diolicum* 22Lin^T**	***A. evansii* KB740^T**	***A. buckelii U120^T***	***A. anaerobius* LuFRes1^T**	***A. tolulyticus* Tol-4^T**	***A. toluvorans* Td21^T**	***A. toluclasticus* MF63^T**	***A. indigens* VB32^T**	***A. communis* SWub3^T**	***Azoarcus* sp. BH72**
General properties																
Temperature optimum [°C]	31	32	ND	ND	30	30	30	37	28	30	ND	ND	ND	40	37	40
G+C content (mol%)	65·0	61·0	66·8	66·0	66·0	66·1[§]	66·7	67·5	66·0	66·5	66·9	68·6	66·8	66·6	62·4	67·6
Aerobic growth	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Denitrification	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-[§]	-[§]
Fermentative[†]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	ND	ND
N_2-fixation[‡]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	+/-[§]	-[§]	-[§]	-[§]	+[§]	+[§]	ND	+	+	+
Motility	-	-	+	+	ND	+	+	+/-	+/-	+	+	+	ND	+	+	+
Aromatic compounds (anaerobic)																
Toluene (2 %)	+	+	-	+	-	+	-	-	-	-[§]	+	+	+	-[§]	-[§]	-[§]
Ethylbenzene (2 %)	+	-	+	-	+	-[§]	-[§]	-[§]	-	-[§]	-	-[§]	ND	-[§]	-[§]	-[§]
m-Xylene (2 %)	-	-	-	-	-	+[§]	-[§]	-[§]	-[§]	-[§]	-	-[§]	ND	-[§]	-[§]	-[§]
p-Ethyltoluene (2 %)	-	+	-[§]	-[§]	ND	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
Propylbenzene (2 %)	-	-	+	-	ND	-[§]	-	-[§]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
Benzyl alcohol (0·5, 2)	+	+	+	+	ND	-	-[§]	+	-	-[§]	-[§]	+[§]	ND	-[§]	-[§]	-[§]
Benzaldehyde (0·5, 2)	+	+	+	+	+	+	+[§]	+	-	-[§]	+[§]	+[§]	ND	-[§]	-[§]	-[§]

1-Phenylethanol (0·5, 2)	+	-§	+	-	+	-§	-§	-§	-§	-§	-§	-§	ND	-§	-§	-§
Acetophenone (1 %)	+	-	+	-	+	-§	-§	-§	-§	-§	-§	-§	ND	-§	-§	-§
p-Hydroxyacetophenone (2)	+§	-§	-§	-§	ND	-§	-§	-§	-§	-§	-§	-§	ND	-§	-§	-§
(*R*)-1-Phenylpropanol (0·5, 2)	-	-§	+	-	ND	-§	-§	-§	-§	-§	-§	-§	ND	-§	-§	-§
(*S*)-1-Phenylpropanol (0·5, 2)	-	-§	+	-	ND	-§	-§	-§	-§	-§	-§	-§	ND	-§	-§	-§
Propiophenone (1 %)	-	-§	+	-	+	-§	-§	-§	-§	-§	-§	-§	ND	-§	-§	-§
Phenylacetate (1, 4)	+	+	+	+	+	+§	+	+	+	+	+	+	+	-§	-§	-§
Benzoate (1, 4)	+	+	+	+	ND	+	+	+	+	+	+	+	+	-§	-§	-§
2-Aminobenzoate (1, 4)	+§	+§	+§	+§	ND	+§	-§	+	+	+§	+	+§	ND	-§	-§	-§
3-Hydroxybenzoate (2)	+§	+§	+§	+§	ND	+§	+§	+§	+§	+§	+§	+§	ND	-§	-§	-§
4-Hydroxybenzoate (1, 4)	+§	+§	+§	+§	+	+	+§	+	+	+	ND	+	ND	-§	-§	-§
Cinnamate (2)	+§	+§	+§	+§	ND	-§	-§	+§	+§	+§	+§	+§	ND	-§	-§	-§
Hydrocinnamate (2)	+§	+§	+§	+§	ND	-§	-§	+§	+§	+§	-§	+§	ND	-§	-§	-§
p-Cumarate (2)	+§	+§	+§	+§	ND	+§	+§	+§	+§	+§	+§	+§	ND	-§	-§	-§
3-(4-Hydroxyphenyl)propionate (2)	+§	+§	+§	+§	ND	+§	+§	+§	+§	+§	+§	+§	ND	-§	-§	-§
Phenol (0·5, 2)	+§	-	+	+	ND	+§	+§	-	+	+	+	+	+	-§	-§	-§
o-Cresol (0·5, 2)	-	-	-§	-§	ND	-	-§	-	+	-	-§	-§	ND	-§	-§	-§
m-Cresol (0·5, 2)	-	-	-§	-§	ND	+§	-§	-	-	-	-§	-§	ND	-§	-§	-§
p-Cresol (0·5, 2)	+	+	-	+	ND	+	+§	+	+	+	+§	+§	ND	-§	-§	-§
p-Ethylphenol (0·5 %)	+§	-§	-§	-§	ND	-§	-§	-§	-§	-§	-§	-§	ND	-§	-§	-§
Resorcinol (1)	-§	-§	-§	-§	ND	-§	-§	-§	-	+	-§	-§	ND	-§	-§	-§
Tyrosine (0·5, 2)	-	+	+§	+§	ND	+§	+§	-§	+	+	+§	-§	ND	-§	-§	-§
Phenylalanine (0·5, 2)	+	+	+§	+§	ND	+§	+§	+	+	+	-	+	-	-§	-§	-§
Aromatic compounds (aerobic)																
Toluene (2 %)	-	-§	-§	-	-	-	-§	-§	-§	-§	+	+	+	-§	-§	-§
Benzoate (1, 4)	+	+§	+	+	+	+§	+§	+	+	-§	+	+	+	-§	+§	+§
Phenylacetate (1)	+	+§	+	+	ND	+§	+§	+	+	-§	+	+	ND	-§	+§	+§
3-Hydroxybenzoate (2)	+§	+§	+§	-§	ND	+§	+§	+§	+§	+§	+§	+§	ND	+§	+§	+§
Gentisate (2)	+§	-§	-§	+§	ND	-§	-§	-§	-§	-§	-§	-§	ND	+§	+§	+§
Phenylalanine (2)	+§	+§	+§	+§	ND	+§	+§	+§	+§	+§	+§	+§	ND	+§	+§	+§
Alcenoic compounds (anaerobic)																

p-Cymene (2, 5 %)	-	+	-	-	ND	-[§]	-	-[§]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
α-Phellandrene (1, 2 %)	-[§]	+	-[§]	-[§]	ND	-[§]	-	-[§]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
α-Terpinene (1, 2 %)	-[§]	+	-[§]	+[§]	ND	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
Limonene (1, 2 %)	-[§]	+	-[§]	+[§]	ND	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
Cyclohexane carboxylate (0·5, 2)	+[§]	+	-[§]	+[§]	ND	+	+	+	+	+	+[§]	+[§]	ND	-[§]	-[§]	-[§]
Cyclohexanol (1, 2 %)	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-	-[§]	-	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
Cyclohexane-1,2-diol (1, 5)	-[§]	-[§]	-[§]	-[§]	ND	-[§]	+	-[§]	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
Other substrates (anaerobic)																
Glucose (1, 5)	-	-	-[§]	+[§]	+	+[§]	+	+	-	-	+	-	ND	-[§]	-	-
Fructose (1, 5)	-	-	-[§]	+[§]	ND	+[§]	+[§]	+[§]	-	-	+[§]	-[§]	ND	-[§]	-	-
Adipate (1, 5)	-[§]	-[§]	-[§]	-[§]	ND	-[§]	+	+	-	-	+	+	+	-[§]	+	+
Malate (1, 5)	+	+	+[§]	+[§]	ND	+[§]	+	+	+	-	+	+[§]	ND	+[§]	+	+
Succinate (1, 5)	+	+	+[§]	+[§]	ND	+[§]	+	+	+	+	+	+	ND	+[§]	+	+
Lactate (5, 10)	+	+	+[§]	+[§]	+	+[§]	+	+[§]	+	+	+	+	+	+[§]	+	+
Pyruvate (1, 5)	+	+	+	+	+	+	+[§]	+[§]	+	+	+	+	+	-[§]	+	+
Acetate (1, 5)	+	+	+	+	+	+[§]	+	+	+	+	+	+	+	-[§]	+	+
Acetone (0·5, 2)	+	+	+	+	+	-[§]	+[§]	+	-[§]	-[§]	-[§]	-[§]	ND	-[§]	-[§]	-[§]
Ethanol (1, 5)	+	+	-[§]	+[§]	ND	+[§]	-[§]	-	+	+	+	+[§]	ND	-[§]	+	+

***** Data was taken from: *Aromatoleum aromaticum* EbN1[T], *Aromatoleum bremensis* PbN1[T] and *Aromatoleum petroleum* ToN1[T] (Rabus *et al*., 1995); *Aromatoleum aromaticum* pCyN1 (Harms *et al*., 1999); *Aromatoleum toluolicum* T[T] (Dolfing *et al*., 1990); *Aromatoleum diolicum* 22Lin[T] (Harder, 1997); *Aromatoleum evansii* KB740[T](Anders *et al*., 1995); *Aromatoleum buckelii* U120[T] (Mechichi *et al*., 2002); *Aromatoleum anaerobius* LuFRes1[T] (Gorny *et al*., 1992; Springer *et al*., 1998); *Aromatoleum tolulyticus* Tol-4[T] (Fries *et al*., 1994; Zhou *et al*., 1995); *Aromatoleum toluvorans* Td21[T] (Fries *et al*., 1994; Song *et al*., 1999); *Aromatoleum toluclasticus* MF63[T] (Song *et al*., 1999); *Azoarcus indigens* VB32[T], *Azoarcus communis* SWub3[T], *Azoarcus* sp. BH72 (Reinhold-Hurek *et al*., 1993; Reinhold-Hurek & Hurek, 2000). Concentrations are given in [% (v/v)] or in [mM].

† Fermentative growth was tested in with lactate (5 mM) as sole source of organic carbon and ammonium (6 mM) as nitrogen source.

‡ See also Fig. 1.

§ Determined in this study.

ND, no data available

Among these strains growing anaerobically with cyclohexane-1,2-diol. Anaerobic 2-aminobenzoate and phenol degradation are well studied in *A. evansii* KB740^T and *A. buckelii* U120^T, but are also known from *A. aromaticum* EbN1^T. However, most *Aromatoleum* spp. have some – albeit limited – capacities to utilize carbohydrates under oxic conditions, in contrast to members of *Azoarcus* spp.

Many of the degradative capacities are performed in the absence of molecular oxygen and involve novel biochemical reactions (Table 3). The most widespread initial reaction in the anaerobic degradation of alkylbenzenes (e.g. toluene, xylenes) known to date is their radical driven addition to fumarate yielding the corresponding arylsuccinates. Ethylbenzene on the other hand is anaerobically hydroxylated to (*S*)-1-phenylethanol by denitrifiers. Benzoate is activated to benzoyl-CoA (the central intermediate of anaerobic aromatic compound degradation), followed by reductive dearomatization and hydrolytic ring cleavage. New reaction principles have also been recognized for anaerobic degradation of phenylacetate and resorcinol. Many novel enzymes have been purified and characterized from these pathways, e.g. phenylglyoxylate:NAD$^+$ oxidoreductase from *A. evansii* KB740^T (Hirsch *et al*., 1998), ethylbenzene dehydrogenase from *A. aromaticum* EbN1^T (Kniemeyer & Heider, 2001), or benzylsuccinate synthase from *A. toluolicum* T^T (Beller & Spormann, 1997). In addition, coding genes were discovered, e.g. *ebd* genes for ethylbenzene dehydrogenase of *Aromatoleum sp.* strain EB1 (Johnson *et al*., 2001). Applying whole genome shotgun sequencing in conjunction with proteomics the complete gene set for anaerobic ethylbenzene and toluene degradation were recently determined in *A. aromaticum* EbN1^T (Rabus *et al*., 2002; Kube *et al*., 2004; Kühner *et al*., 2005).

Some members of the *Aromatoleum* group have previously been shown to grow anaerobically with crude oil as the only source of organic substrates. *A. aromaticum* EbN1^T utilizes toluene and ethylbenzene also directly from crude oil (Rabus & Widdel, 1996). Furthermore, enrichments with crude oil as the only source of organic carbon and nitrate as electron acceptor were dominated by bacteria affiliating with the *Aromatoleum*/*Azoarcus*/*Thauera* group of *Betaproteobacteria* (Rabus *et al*., 1999). The ability to utilize hydrocarbons during anaerobic growth with crude oil has implications for both, bioremediation efforts at contaminated sites (Pelz *et al*., 2001; Reusser *et al*., 2002) and the biogeochemistry of oil fields and reservoirs (Head *et al*., 2003).

Table 3. Novel biochemical reactions discovered among members of the new genus *Aromatoleum*

Reaction	Organism	Reference
Anaerobic		
Toluene → (*R*)-Benzylsuccinate	*A. toluolicum* T^T *A. aromaticum* $EbN1^T$ *A. tolulyticus* $Tol\text{-}4^T$	Beller *et al*., 1997 Rabus & Heider, 1998 Migaud *et al*., 1996
o-Xylene → *o*-Methylbenzylsuccinate	*A. toluolicum* T^T	Beller *et al*., 1997
m-Xylene → *m*-Methylbenzylsuccinate	*A. toluolicum* T^T	Krieger *et al*., 1999
Ethylbenzene → (*S*)-1-Phenylethanol	*A. aromaticum* $EbN1^T$ *Aromatoleum* sp. EB1	Rabus & Heider, 1998 Ball *et al*., 1996
Propylbenzene → 1-Phenylpropanol	*A. aromaticum* $EbN1^T$	Kniemeyer & Heider, 2001
Resorcinol → Hydroxyhydroquinone → 2-Hydroxy-1,4-benzoquinone	*A. anaerobius* $LuFRes1^T$	Phillip & Schink, 1998
2-Aminobenzoate → 2-Aminobenzoyl-CoA	*A. evansii* $KB740^T$	Altenschmidt *et al*., 1991
Phenylglyoxylate → Benzoyl-CoA + CO_2	*A. evansii* $KB740^T$	Hirsch *et al*., 1998
Benzoate → Benzoyl-CoA	*A. evansii* $KB740^T$	Altenschmidt *et al*., 1991
Benzoyl-CoA → Cyclohexa-1,5-diene-1-carbonyl-CoA	*A. evansii* $KB740^T$	Ebenau-Jehle *et al*., 2003
Aerobic		
Phenylacetate → Phenylacetyl-CoA	*A. evansii* $KB740^T$	Mohamed *et al*., 2002
Phenylacetate → Homogentisate	*A. evansii* $KB740^T$	Mohamed *et al*., 2002
2-Aminobenzoyl-CoA → 5-Oxo-2-aminocyclohexadienyl-CoA	*A. evansii* $KB740^T$	Hartmann *et al*., 1999
Benzoate → Benzoyl-CoA → β-Keto-adipyl-CoA	*A. evansii* $KB740^T$	Zaar *et al*., 2001, 2004; Gescher *et al*., 2005

Most members of the *Aromatoleum* lineage are apparently incapable of N_2-fixation, as evident from the frequent absence a *nifH* gene (Fig. 1A) and lack of ethylene formation in soft agar cultures (Fig. 1B and C). However, it should be noted that VFe and FeFe nitrogenases display 10- to 100-fold lower activities for acetylene formation than MoFe nitrogenases (Eady *et al*., 1996). Thus, it cannot be excluded that some of the investigated strains may actually possess one of these two alternative nitrogenases. However, the respective genes should be amplified by the primer set used here (Tan *et al.*, 2003). Nevertheless, anaerobic degradation of aromatic compounds/hydrocarbons, aerobic utilization of carbohydrates and plant-associated life style, respectively, are suitable phenotypic characteristics that could be applied to differentiate the genera *Aromatoleum* and *Azoarcus* (Table 6).

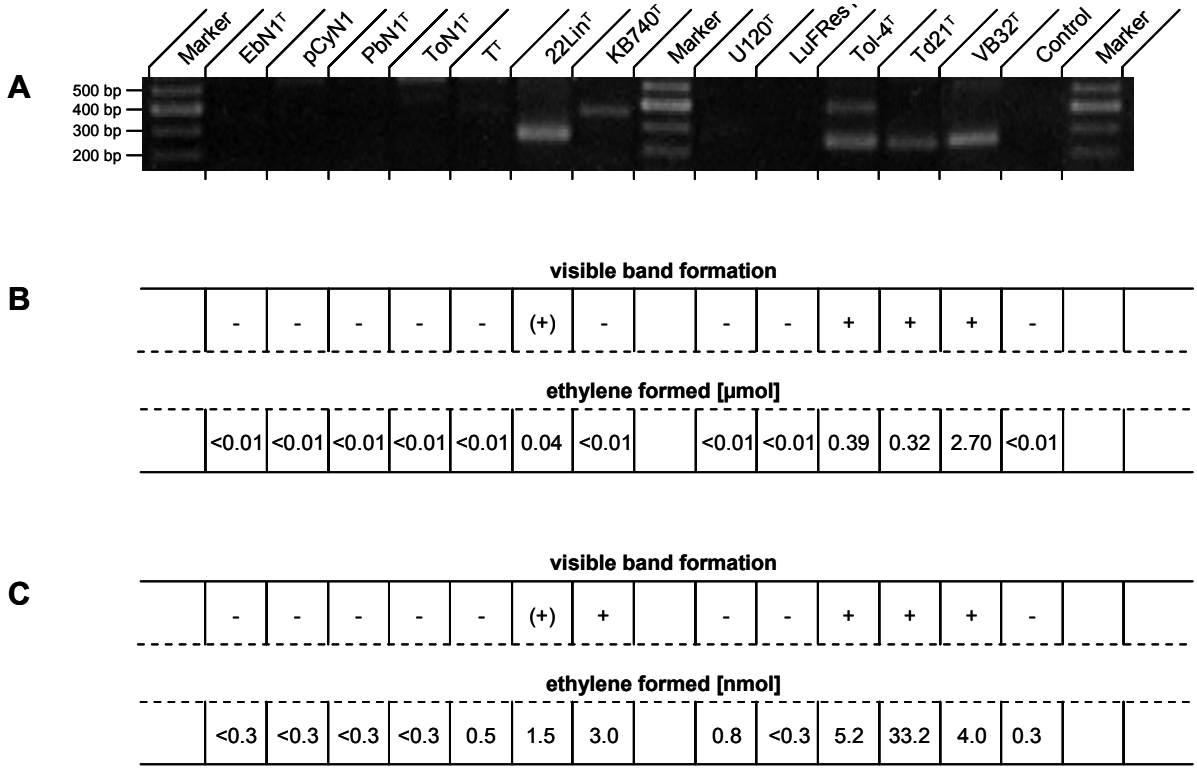


Fig. 1. Distribution of the capacity to fix N_2 among members of the *Aromatoleum* genus. (a) Amplification of *nifH* genes. Formation of bands and reduction of acetylene to ethylene during microaerophilic growth in semi-solid medium with (b) 0·2 % (w/v) agar, acetate as growth substrate and short-term incubation (5 days), or (c) 0·7 % (w/v) agar, a mixture of growth substrates and long-term incubation (3 months).

The complete genome sequence of *Aromatoleum aromaticum* EbN1^T

The complete genome sequence of *A. aromaticum* EbN1^T was only recently determined (Rabus *et al*., 2005). It consists of a circular chromosome (4·3 Mb) and two plasmids (207 and 224 kb), which contain a total of 4603 predicted coding regions (4133 on the chromosome). Gene clusters for 10 anaerobic and 4 aerobic pathways of aromatic compound degradation encode more than 150 proteins, emphazising the importance of these degradation capacities for the metabolism of *A. aromaticum* EbN1^T. The unusually high frequency of mobile genetic elements (e.g. more than 200 transposases) indicate a high plasticity of the genome, which could be advantageous for lateral transfer of catabolic capacities. The latter process may have contributed to shaping the overall metabolic diversity within the *Aromatoleum* lineage. In addition, the presence of a complete denitrification system and several terminal oxidases for high and low oxygen concentrations point to a pronounced respiratory flexibility. The multitude of predicted regulatory proteins probably could enable *A. aromaticum* EbN1^T to finely tune its metabolic equipment in response to fluctuating availability of nutrients. Agreeing with this assumption, recent proteomic investigations revealed a high degree of substrate-specific regulation of degradation pathways in *A. aromaticum* EbN1^T (Kühner *et al*., 2005; Wöhlbrand *et al*., 2007).

Genome comparison of *Aromatoleum aromaticum* EbN1^T and *Azoarcus* sp. strain BH72

Recently, also the complete genome of *Azoarcus* sp. strain BH72 was determined (Krause *et al*., 2006) and comparison with *A. aromaticum* EbN1^T corroborated the ecophysiological difference between the N_2-fixing grass endophytes of the *Azoarcus* lineage and the soil inhabiting degradation specialists of the *Aromatoleum* lineage. (i) Despite their phylogenetic relatedness, both genomes display a surprisingly low degree of synteny. (ii) The genome of strain BH72 contains several gene clusters for putative surface components possibly involved in the endophytic lifestyle. These genes are either completely missing in strain EbN1^T or more highly related to genes of plant-associated or pathogenic bateria. (iii) In contrast to strain EbN1^T, a complete denitrification system is absent in strain BH72. (iv) The genome of strain BH72 contains only very few phage or transposon related genes, which could reflect the

relatively stable, low-stress microenvironment in the plant, not requiring the endophyte to exhibit a high degree of genetic adaptability.

The *Azoarcus* and *Thauera* groups

Azoarcus spp. were originally isolated from Kallar grass collected in Pakistan (Reinhold *et al.*, 1986; Reinhold-Hurek *et al*., 1993). Species of *Azoarcus sensu stricto* sp. strain BH72 and *A. indigens* were recognized as endophytes of roots of Kallar grass and rice species (Reinhold-Hurek *et al*., 1993; Engelhard *et al.*, 2000). Strain BH72 even provides reduced nitrogen from N_2-fixation to its host plant, thriving in return on simple plant metabolites such as malate (Hurek *et al.*, 2002). *A. communis,* however, harbours a root surface-associated strain SWub3^T as well as strains from oil-contaminated sludge such as strain S2 (Reinhold-Hurek *et al.*, 1993). The type species is *Azoarcus indigens* VB32^T. Thus far, N_2-fixing, plant-associated *Azoarcus* spp. have not been associated with the degradation of recalcitrant compounds. Key properties are rather colonization of plant tissue and mechanisms of plant-microbe interactions. Here, growth studies demonstrated that the plant-associated *Azoarcus* spp. in fact lack the capacity for anaerobic utilization of aromatic or terpenoic compounds (Table 2).

The type strain of the *Thauera* genus, *T. selenatis* AX^T, was the first organism demonstrated to use selenate as electron acceptor for anaerobic respiration (Macy *et al*., 1993). In addition, the *Thauera* genus contains several denitrifying bacteria that display remarkable degradative capacities as observed with the *Aromatoleum* spp.. *T. aromatica* K172^T (Anders *et al*., 1995) is well studied with respect to anaerobic degradation of various aromatic compounds such as toluene, phenol, phenylalanine, 2-aminobenzoate and benzoate; except for toluene, these compounds are also utilized under oxic conditions (e.g. Heider *et al*., 1998). *T. phenylacetica* B4P^T, *T. aminoaromatica* S2^T (Mechichi *et al*., 2002), *T. mechernichensis* TL1^T (Scholten *et al*., 1999) and *T. chlorobenzoica* 3CB-1^T (Song *et al*., 2001) grow anaerobically and aerobically with a variety of substituted benzoates. The characteristic property of *T. linaloolentis* 47Lol^T and *T. terpenica* 58Eu^T is the anaerobic utilization of oxygen-containing monoterpenes, such as menthol (Foss & Harder, 1998). Strain mXyN1^T can grow anaerobically with *m*-xylene in addition to toluene (Rabus & Widdel, 1995) and utilizes both alkylbenzenes also from crude oil (Rabus & Widdel, 1996). Strain pCyN2^T grows anaerobically with *p*-cymene and several monoterpenes, but not with toluene (Harms *et al*.,

1999). Phylogenetic analyses suggest that strains mXyN1^T and pCyN2^T represent new *Thauera* species, with their closest relatives being *T. terpenica* 58Eu^T and *T. aromatica* K172^T, respectively (Fig. 2).

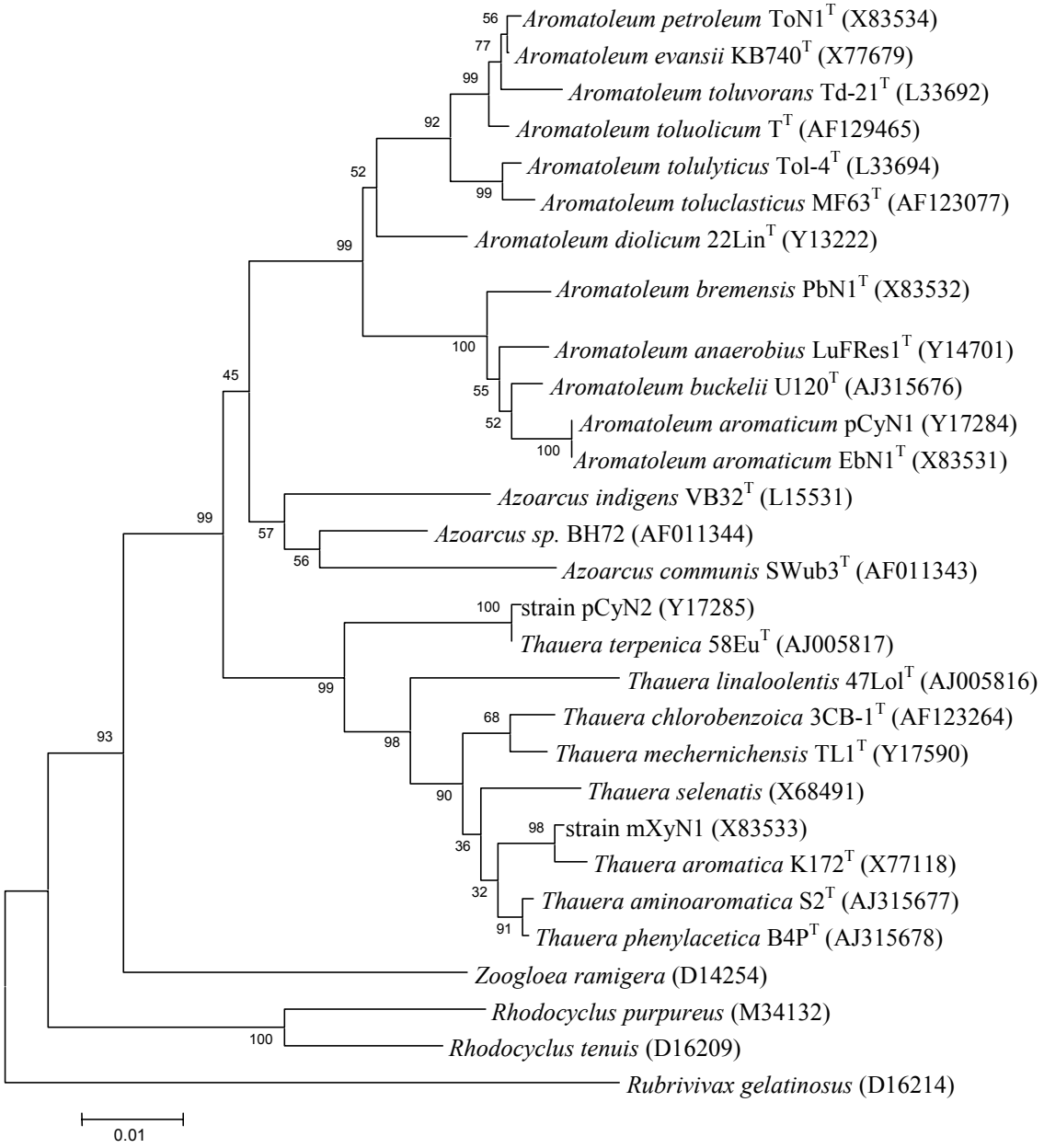


Fig. 2. Phylogenetic tree (neighbor-joining) revealing the clustering of the genera *Aromatoleum*, *Azoarcus* and *Thauera* within the *Rhodocyclus* group of *Betaproteobacteria*.

DNA base ratios and DNA/DNA-hybridization groups

The rather narrow range of the G+C-content (61·0 to 68·6 mol%; Table 2) among the *Aromatoleum* spp. is indicative of a close genetic relationship. DNA/DNA-hybridization experiments demonstrate that strains EbN1^T and pCyN1 represent the same species (DNA-DNA-similarity 78·7 %, reciprocal 103·5 %), agreeing with their close branching in the phylogenetic tree (Fig. 2). Notably, they have markedly different substrate ranges, with only strain pCyN1 anaerobically degrading monoterpenes. In contrast, strains ToN1^T and KB740^T represent different species, despite their close branching in the phylogenetic tree (Fig. 2).

Phylogenetic relationship

The phylogenetic tree (Fig. 2) reveals that the genera *Aromatoleum*, *Azoarcus* and *Thauera* form three distinct clusters within the *Rhodocyclus* group of *Betaproteobacteria*. Close genetic relationship between the two ethylbenzene-degrading strains EbN1^T and EB1 is indicated by the high degree of sequence similarity of the respective ethylbenzene dehydrogenase subunits (Johnson *et al*., 2001; Rabus *et al*., 2002). FAME analysis revealed overall very similar fatty acid compositions among the various tested species from the *Aromatoleum*, *Azoarcus* and *Thauera* genera, with 16:1ω7c and 16:0 representing generally the most abundant fatty acids (Table 4), agreeing with previous studies (Song *et al*., 1999, 2001). Thus, phylogenetic clustering among the studied bacteria may reflect to a large extent their metabolic specificities.

The 16S-rRNA gene based phylogenetic tree (Fig. 2) revealed very close relationships within two strain pairs (ToN1^T and KB740^T, EbN1^T and pCyN1), even though these strains differ markedly with respect to their degradative capacities (Table 2). Comparative peptide profiling by mass spectrometry was performed to further differentiate these two incidents of closely related strains. The concept of identifying microorganisms by MALDI-MS relies on the reproducible measurement of stably formed proteins (Lay, 2001), which are mostly ribosomal (Arnold & Reilly, 1999; Fenselau & Dimerev, 2001). In the present study, each analyzed culture was correctly identified as first hit when matching the respective sample spectra with the library spectra. In general, a minimum score value of 100 reflects a very close relationship or identity of the tested (unknown) organisms to the library entry. The score values for each of the four analyzed strains matched against its correct library entry (self-matching) and

Table 4. Relative amounts of cellular fatty acids (FAME) of members of the genera *Aromatoleum*, *Azoarcus* and *Thauera*

	Aromatoleum											*Azoarcus*	*Thauera*									
FAME *	**EbN1**	**pCyN1**	**PbN1**	**ToN1**	**T**	**22Lin**	**KB740**	**U120**	**LuFRes1**	**Tol-4**	**Td21**	**VB32**	**K172**	**TL1**	**S2**	**B4P**	**3CP-1**	**47Lol**	**58Eu**	**Piv1**	**pCyN2**	**mXyN1**
8:0 3OH																	0·0				0·02	
10:0	0·5	0·6	0·6	0·4	0·8	0·8	0·4	1·0	0·5	0·4	0·4	0·3	0·5	0·3		0·3	0·5	0·4	0·3	0·4	0·6	0·4
10:0 3OH	5·8	5·0	6·0	5·4	5·7	4·2	5·5	8·2	5·2	5·9	4·8	4·5	5·2	3·3	3·1	2·8	5·4	5·5	4·2	4·7	2·1	6·0
unknown 11·799											0·2											
12:0	5·7	6·0	5·8	5·6	5·7	4·6	5·8	6·0	5·2	6·2	4·4	5·7	5·4	4·9	5·0	3·6	5·2	5·5	4·3	4·9	6·1	5·9
12:0 3OH														1·8	1·9	1·5					0·4	0·5
14:0	0·6		0·4	0·4	0·3	0·7	0·6	0·4	1·9	0·4	0·6		0·4		1·2	1·4	0·4	0·3	0·4	0·4		
Mixture†	52·0	53·3	48·0	47·2	52·9	57·2	49·4	53·5	47·2	53·4	54·4	41·6	41·6	44·0	40·7	56·2	43·7	45·7	55·6	48·8	54·7	41·5
16:1 ω5c	0·6	0·7	0·8	1·0	1·9	1·2	0·5	1·9	0·8	0·8	0·7		0·8	0·8	1·0	1·2	0·7	0·8	1·0	1·5	0·7	0·7
16:0	25·8	22·6	26·4	31·1	21·7	22·5	32·2	14·5	32·9	25·2	28·3	35·9	30·0	23·1	39·6	20·6	29·2	25·3	24·0	21·6	23·3	31·3
17:0 cyclo												1·0								0·3		
18:1 ω7c	9·2	11·8	12·0	8·7	11·3	6·5	5·8	14·5	2·4	7·7	6·2	10·3	15·8	21·9	7·6	9·8	14·4	16·2	10·1	17·1	12·2	13·6
18:1 ω5c																				0·3		
18:0												0·6	0·3				0·3	0·4				
Mixture‡						2·4			4·0							2·5						
18:0				0·2																		
Total	**97·0**	**100·0**	**99·5**	**99·9**	**93·4**	**100·0**	**98·3**	**99·5**	**98·2**	**100·0**	**98·4**	**96·3**	**98·4**	**99·4**	**99·4**	**99·0**	**99·6**	**99·3**	**99·3**	**99·7**	**100·0**	**99·5**

* Relative amounts of FAME are given in [%].

† 16:1 ω7c and 15 iso 2OH could not be separated under the applied analytic conditions. However, in analogy to previous studies (Song *et al*., 1999, 2001) it is most likely 16:1 ω7c.

‡ The unknown 18·846, 19:1 ω6c and 19:0 cyclo ω10c could not be separated under the applied analytic conditions.

ranged from 150 to >300. The second hit in the result list always represented the most closely related strain (based on 16S rRNA gene). The differences in score values between first and second hit were ~50 for strains EbN1^T and pCyN1, and ~130 for strains ToN1^T and KB740^T. Unsupervised multivariate analysis (Principal Component Analysis) of mass spectra clearly allowed discriminating the two strain pairs (Fig. 3A): the variation within three independent cultivations and 8 technical replicates is much smaller than the variation between the strains. The reproducibility of biological and technical replicates is shown in Fig. 3 B and C. The high resolution of MALDI-MS-based protein profiling of whole cells allows differentiation on the strain level. In case of strains EbN1^T and pCyN1 the resolved differences in protein profiles were obviously too sensitive for the current species definition.

Table 5. Sequence similarities of amplification products from tested strains to known *nifH* genes

Strain	Similarity [%]	Homolog	Organism
A. diolicum 22 Lin^T	91*	*nifH*	*Sinorhizobium* sp. CFNEI 54
	83†	*nifH*	*Aromatoleum tolulyticus* Tol-4^T
A. tolulyticus Tol-4^T	89*	*nifH*	*Methylocystis echinoides*
	92*	*nifH*	*Aromatoleum tolulyticus* Tol-4^T
	80†		*Ideonella* sp. Long 7
	80†	*nifH*	*Aromatoleum tolulyticus* Tol-4^T
A. toluvorans Td21^T	88*	*nifH*	*Methylocystis* sp. LWS
	94†	*nifH*	*Derxia gummosa*
A. indigens VB32^T	95*	"Mo-Nitrogenase"	*Azoarcus* sp. BH72
	95*	*nifH*	*Azoarcus indigens* VB32^T
	82†	*nifH*	*Azoarcus indigens* VB32^T
	81†	"Mo-Nitrogenase"	*Azoarcus* sp. BH72

* Sequencing with forward-primer
† Sequencing with reverse-primer

Table 6. Phenotypic properties that are suitable for differentiation of the genera *Aromatoleum* und *Azoarcus*.

Property	*Aromatoleum*	*Azoarcus*
Anaerobic degradation of aromatic compounds and hydrocarbons	+	-
Endophytic life style	-	+

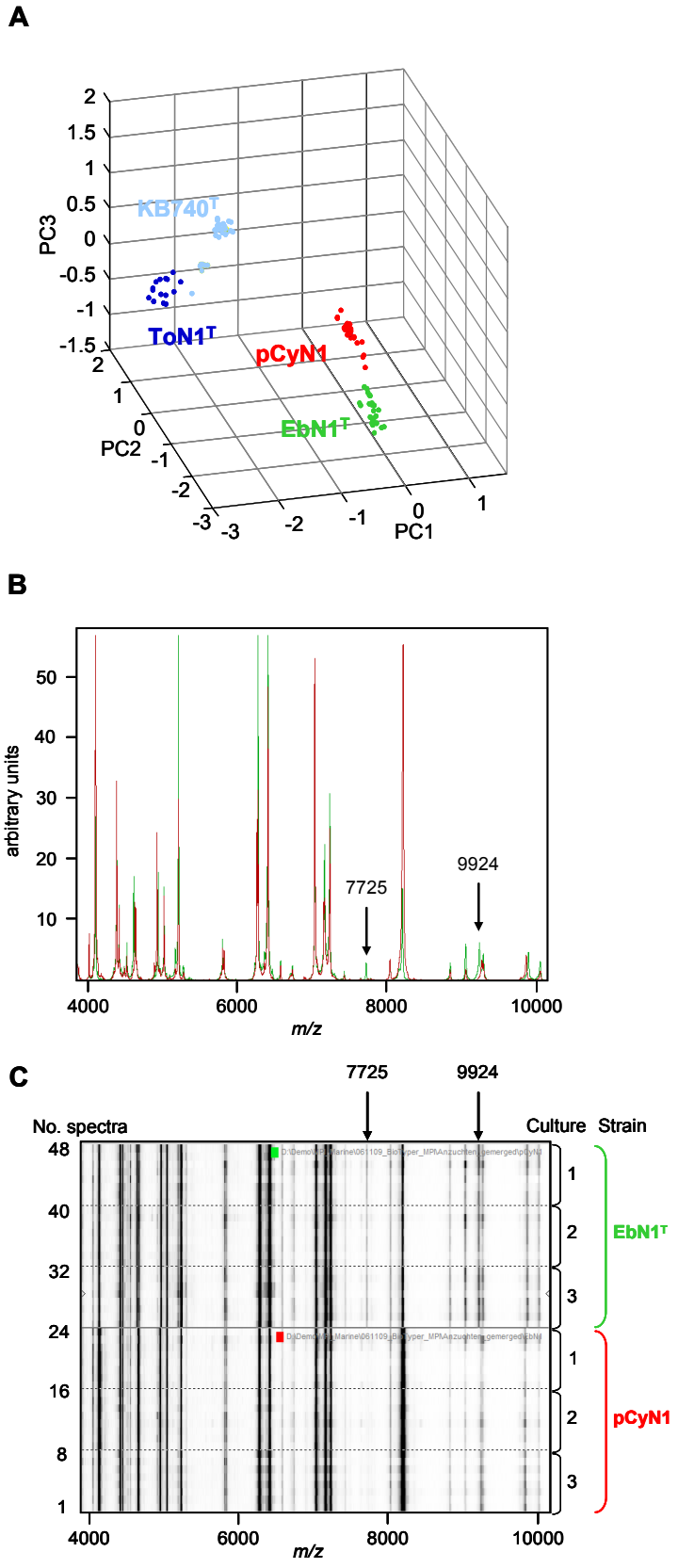


Fig. 3. MALDI-MS-based differentiation of closely related *Aromatoleum* strains. (a) Principal Component Analysis of MALDI-MS spectra from the *Aromatoleum* strains EbN1^T, pCyN1, ToN1^T and KB740^T. For each organism 24 spectra were analyzed, which were obtained from 3 independent cultures and 8 technical replicates. (b) Averaged MALDI-MS spectra based on 24 single measurements from strains EbN1^T (green) and pCyN1 (red). Two remarkable *m/z* ion differences (7725 and 9924), which are only present in strain EbN1^T, are marked by arrows. (c) Gel view representation of MALDI-MS spectra, reflecting the intensity of *m/z* ions with a gray scale.

Description of *Aromatoleum* gen. nov.

Ar.o.ma'to.le.um L. n., *Aromatoleum*, indicating that the members of this genus grow with a multitude of aromatic compounds and crude oil components. Cells are rod-shaped and often motile. They are facultative aerobes that use nitrate as alternative electron acceptor under anoxic conditions. During denitrification nitrite may be accumulated intermediately. They oxidize organic carbon substrates completely to CO_2. Their outstanding physiological property is the anaerobic degradation of alkylbenzenes or monoterpenes. Various polar aromatic compounds may be utilized under anoxic and oxic conditions. Low molecular weight acids (e.g. lactate or malate) and alcohols (e.g. ethanol) also serve as growth substrate. Their G+C-content ranges from 61·0 to 68·6 mol%. *Aromatoleum* belongs to the *Rhodocyclus* group of *Betaproteobacteria*, with *Azoarcus* and *Thauera* as the most closely related genera.

Description of *Aromatoleum aromaticum* sp. nov.

ar.o.ma'ti.cum. M. L. adj. *aromaticum*, indicating that the organism can grow with multiple aromatic compounds.

A. aromaticum has rod-shaped cells (0·6-1·0 × 1·2-2·5 μm) and is non-motile. The temperature range for growth is from 5 to 40 °C, with an optimum at 31 to 32 °C. The pH range for growth is between 6·1 and 9·0, with an optimum around 7·4. The type strain EbN1^T was isolated with ethylbenzene under nitrate-reducing conditions; shortest doubling times with ethylbenzene are 11 h. *A. aromaticum* EbN1^T is the only known denitrifier that degrades toluene in addition to ethylbenzene. Both alkylbenzenes can also be utilized from crude oil under nitrate-reducing conditions. They cannot be degraded aerobically. During denitrification nitrite is intermediately accumulated. Anaerobic growth of strain EbN1^T also occurs with benzyl alcohol, (*R*)- and (*S*)-1-phenylethanol, 2-phenylethanol, benzaldehyde, acetophenone, 4-hydroxyacetophenone, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate, phenylacetate, cinnamate, hydrocinnamate, *p*-cumarate, 3-(4-hydroxyphenyl)propionate, phenol, *p*-cresol, *p*-ethylphenol, phenylalanine, acetone, 2-butanone, succinate, malate, ropionate, butyrate, valerate, caproate, lactate, pyruvate, acetate, ethanol, propanol, isopropanol, 1-butanol and 2-butanol. Compounds that are not utilized by strain EbN1^T: *n*-alkanes, alicyclic compounds, monoterpenes, higher fatty acids and carbohydrates. Complete substrate oxidation to CO_2 proceeds via the TCA-cycle. A second strain of this species, strain pCyN1, was isolated with *p*-cymene under nitrate-

reducing conditions; shortest doubling times are around 12 h. *p*-Cymene can also be utilized under aerobic conditions. Moreover, the alkylbenzenes toluene and *p*-ethyltoluene, and the monoterpenes α-phellandrene, α-terpinene, γ-terpinene, limonene, isolimonene, sabinene, α-pinene and *p*-menth-1-ene support anaerobic growth of strain pCyN1. Further anaerobic growth substrates of strain pCyN1 are: *p*-cresol, benzyl alcohol, 3-phenyl-1-propanol, *p*-isopropylbenzyl alcohol, benzaldehyde, *p*-ethylbenzaldehyde, *p*-isopropylbenzaldehyde, benzoate, phenylacetate, 3-phenylpropionate, *p*-ethylbenzoate, *p*-propylbenzoate, *p*-isopropylbenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate, cinnamate, hydrocinnamate, *p*-cumarate, 3-(4-hydroxyphenyl)propionate, mandelate, phenylalanine, tyrosine, tryptophane and several low molecular weight alcohols and acids. Habitat: *A. aromaticum* strains EbN1^T and pCyN1 were isolated from ditches and Weser sediment (Bremen). The G+C contents are 65·0 and 61·0 mol%, respectively. The closest relative is *A. buckelii* strain U120^T. *A. aromatoleum* contains the type strain EbN1^T (= DSM 19018^T) and the strain pCyN1 (= DSM 19016).

Description of *Aromatoleum petroleum* sp. nov.

pe'tro.le.um M. L. adj. *petroleum*, indicating that the organism can grow with crude oil.

Cells are rod-shaped (0·6-0·8 × 1-2 µm) and motile. *A. petroleum* was isolated with toluene under nitrate-reducing conditions. Toluene can also be utilized from crude oil under denitrifying conditions, but not aerobically. Anaerobic growth also occurs with phenol, *p*-cresol, benzyl alcohol, benzaldehyde, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate, cinnamate, hydrocinnamate, *p*-cumarate, 3-(4-hydroxyphenyl)propionate, acetone, 2-butanone, benzoate, phenylacetate, phenylalanine, pyruvate and acetate. Habitat: *A. petroleum* was isolated from ditches and Weser sediment (Bremen). The G+C content is 66·0 mol%. The closest relative is *A. evansii* KB740^T. The type strain is ToN1^T (= DSM 19019^T).

Description of *Aromatoleum bremensis* sp. nov.

bre.men'sis N. L. m *bremensis*, indicating that samples for initial enrichments originated from Bremen.

Cells are rod-shaped (1 × 1·5-2·5 μm) and motile. *A. bremensis* was isolated with propylbenzene under nitrate-reducing conditions. Further substrates for anaerobic growth are: ethylbenzene, phenol, benzyl alcohol, 1-phenylethanol, 1-phenyl-1-propanol, benzaldehyde, acetophenone, propiophenone, phenylacetate, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2-aminobenzoate, cinnamate, hydrocinnamate, *p*-cumarate, 3-(4-hydroxyphenyl)propionate, acetone, 2-butanone, acetate and pyruvate. Habitat: *A. bremensis* was isolated from ditches and Weser sediment (Bremen). The G+C content is 66·8 mol%. The closest relative is *A. anaerobius* LuFRes^T. The type strain is PbN1^T (= DSM 19017^T).

Description of *Aromatoleum toluolicum* sp. nov.

tol.u.o'li.cum M. L. adj. *toluolicum*, indicating that the organism can grow with toluene.

Cells are rod-shaped (0·5-0·7 × 1·5-3·5 μm) and motile. *A. toluolicum* was isolated with toluene under nitrate-reducing conditions. Further anaerobic growth substrates are: *m*-xylene, benzaldehyde, phenylacetate, benzoate, 2-aminobenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, *p*-cumarate, 3-(4-hydroxyphenyl)propionate, phenol, *m*-cresol, *p*-cresol, tyrosine, phenylalanine and cyclohexane carboxylate. Habitat: *A. toluolicum* was isolated from a toluene-degrading laboratory aquifer column. The G+C content is 66·1 mol%. The closest relative is *A. evansii* KB740^T. The type strain is T^T (= DSM 19020^T).

Description of *Aromatoleum diolicum* sp. nov.

di.o'li.cum M. L. adj. *diolicum*, indicating that the organism can grow with alicyclic diols, e.g. cyclohexane-1,2-diol.

Cells are oval to rod-shaped (1·3-2·2 × 0·7-1 μm) and motile. The temperature range for growth is between 16 and 38 °C, with an optimum around 30 °C. *A. diolicum* was isolated with cyclohexane-1,2-diol under nitrate-reducing conditions. Shortest doubling times during anaerobic growth with cyclohexane-1,2-diol were 5 h. Further anaerobic growth substrates are: 2-hydroxycyclohexanone, cyclohexane-1,2-dione, cyclohexanecarboxylate, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, *p*-cumarate, 3-(4-hydroxyphenyl)propionate,

phenylacetate, glucose, adipate, lactate and acetate. Habitat: *A. diolicum* was isolated from activated sludge from a waste-water plant. The G+C content is 66·7 mol%. The closest relatives are *A. toluolicum* T^T and *A. tolulyticus* Tol-4^T. The type strain is 22Lin^T (= DSM 15408^T).

Transfer of aromatic compound-degrading *Azoarcus* spp. to *Aromatoleum* gen. nov.

The aromatic compound-degrading denitrifiers *Azoarcus evansii* $KB740^T$, *A. buckelii* $U120^T$, *A. anaerobius* $LuFRes1^T$, *A. tolulyticus* Tol-4^T, *A. toluvorans* $Td21^T$ and *A. toluclasticus* $MF63^T$ are all members of the same phylogenetic clusters as the above described *Aromatoleum* spp. Moreover, they share the capacity to degrade aromatic compounds under nitrate-reducing conditions. Transfer from the *Azoarcus* genus to the *Aromatoleum* genus should reflect this prominent metabolic difference to the plant-associated species of *Azoarcus sensu stricto*.

Circumscription of *Azoarcus evansii* as *Aromatoleum evansii* comb. nov.

Aromatoleum evansii was isolated with 2-aminobenzoate under nitrate-reducing conditions (Braun & Gibson, 1984) and formerly described as *Azoarcus evansii* (Anders *et al*., 1995). Details on its physiological characteristics were previously reported (Anders *et al*., 1995). The type strain is $KB740^T$ (= DSM 6898^T).

Circumscription of *Azoarcus buckelii* as *Aromatoleum buckelii* comb. nov.

Aromatoleum buckelii was isolated with phenol under nitrate-reducing conditions (Tschech & Fuchs, 1984) and formerly described as *Azoarcus buckelii* (Mechichi *et al*., 2002). Details on its physiological characteristics and original description were previously reported (Mechichi *et al*., 2002). The type strain is $U120^T$ (= DSM 14744^T).

Circumscription of *Azoarcus anaerobius* as *Aromatoleum anaerobius* comb. nov.

Aromatoleum anaerobius was isolated with phenol under nitrate-reducing conditions (Gorny *et al*., 1992) and formerly described as *Azoarcus anaerobius* (Springer *et al*., 1998). Details on its physiological characteristics and original description were previously reported (Springer *et al*., 1998). The type strain is LuFRes1^T (= DSM 12081^T).

Circumscription of *Azoarcus tolulyticus* as *Aromatoleum tolulyticus* comb. nov.

Aromatoleum tolulyticus was isolated from toluene-degrading, nitrate-reducing enrichment cultures (Fries *et al*., 1994) and formerly described as *Azoarcus tolulyticus* (Zhou *et al*., 1995). Details on its physiological characteristics and original description were previously reported (Zhou *et al*., 1995). The type strain is Tol-4^T (= ATCC 51758^T).

Circumscription of *Azoarcus toluvorans* as *Aromatoleum toluvorans* comb. nov.

Aromatoleum toluvorans was isolated from toluene-degrading, nitrate-reducing enrichment cultures (Fries *et al*., 1994) and formerly described as *Azoarcus toluvorans* (Song *et al*., 1999). Details on its physiological characteristics and original description were previously reported (Song *et al*., 1999). The type strain is Td21^T (= ATCC 700604^T = DSM 15124^T).

Circumscription of *Azoarcus toluclasticus* as *Aromatoleum toluclasticus* comb. nov.

Aromatoleum toluclasticus was isolated from toluene-degrading nitrate-reducing enrichment cultures (Fries *et al*., 1994) and formerly described as *Azoarcus toluclasticus* (Song *et al*., 1999). Details on its physiological characteristics and original description were previously reported (Song *et al*., 1999). The type strain is MF63^T (= ATCC 700605^T).

Acknowledgements

We would like to thank Thomas Meier (Leipzig) for helpful discussions about BioTyper analyses. This study was supported by the Deutsche Forschungsgemeinschaft, the Max-Planck-Society and Bruker Daltonik GmbH.

REFERENCES

Altenschmidt, U., Oswald, B. & Fuchs, G. (1991). Purification and characterization of benzoate-coenzyme A ligase and 2-aminobenzoate-coenzyme A ligases from a denitrifying *Pseudomonas* sp. *J Bacteriol* **173**, 5494-5501.

Anders, H.-J., Kaetzke, A., Kämpfer, P., Ludwig, W. & Fuchs, G. (1995). Taxonomic position of aromatic-degrading denitrifying Pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the beta subclass of the *Proteobacteria*. *Int J Syst Bacteriol* **45**, 327-333.

Arnold, R. J. & Reilley J. P. (1999). Observation of *E. coli* ribosomal proteins and their posttranslational modifications by mass-spectrometry. *Anal Biochem* **269**, 105-112.

Ball, H. A., Johnson, H. A., Reinhard, C. & Spormann, A. M. (1996). Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. *J Bacteriol* **178**, 5755-5761.

Beller, H. R. & Spormann, A. M. (1997). Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. *J Bacteriol* **179**, 670-676.

Beller, H. R. & Spormann, A .M. (1998). Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. *J Bacteriol* **180**, 5454-5457.

Biegert, T., Fuchs, G. & Heider, J. (1996). Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and succinate. *Eur J Biochem* **238**, 661-668.

Boll, C., Fuchs, G. & Heider, J. (2002). Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr Opin Chem Biol* **6**, 604-611.

Braun, K. & Gibson, D. T. (1984). Anaerobic degradation of 2-aminobenzoate (anthranilic acid) by denitrifying bacteria. *Appl Environ Microbiol* **48**, 102-107.

Cashion, P., Holder-Franklin, C. A., McCully, J. & Franklin, C. (1977). A rapid method for the base ratio determination of bacterial DNA. *Anal Biochem* **81**, 461-466.

Dolfing, J., Zeyer, J., Binder-Eicher, P. & Schwarzenbach, R. P. (1990). Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. *Arch Microbiol* **154**, 336-341.

Eady, R. R. (1996). Structure-function relationships of alternative nitrogenases. *Chem Rev* **96**, 3013-3030.

Ebenau-Jehle, C., Boll, C. & Fuchs, G. (2003). 2-Oxoglutarate:$NADP^+$ oxidoreductase in *Azoarcus evansii*: properties and function in electron transfer reactions in aromatic ring reduction. *J Bacteriol* **185**, 6119-6129.

Engelhard, M., Hurek, T. & Reinhold-Hurek, B. (2000) Preferential occurrence of diazotrophic endophytes, *Azoarcus* spp., in wild rice species and land races of *Oryza sativa* in comparison with modern races. *Environ Microbiol* **2**, 131-141.

Fenselau, C. & Dimerev, P. A. (2001). Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrometry Rev* **20**, 157-171.

Foss, S. & Harder, J. (1998). *Thauera linaloolentis* sp. nov. and *Thauera terpenica* sp. nov., isolated on oxygen-containing monoterpenes (linalool, menthol, and eucalyptol) and nitrate. *System Appl Microbiol* **21**, 365-373.

Fries, M. R., Zhou, J., Chee-Sanford, J. & Tiedje, J. C. (1994). Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. *Appl Environ Microbiol* **60**, 2802-2810.

Gescher, J., Eisenreich, W., Worth, J., Bacher, A. & Fuchs, G. (2005). Aerobic benzoyl-CoA catabolic pathway in *Azoarcus evansii*: studies on the non-oxygenolytic ring cleavage enzyme. *Mol Microbiol* **56**, 1586-1600.

Gorny, N., Wahl, G., Brune, A. & Schink B. (1992). A strictly anaerobic nitrate-reducing bacterium growing with resorcinol and other aromatic compounds. *Arch Microbiol* **158**, 48-53.

Harayama, S., Kok, M. & Neidle, E. L. (1992). Functional and evolutionary relationships among diverse oxygenases. *Annu Rev Microbiol* **46**, 565-601.

Harder, J. (1997). Anaerobic degradation of cyclohexane-1,2-diol by a new *Azoarcus* species. *Arch Microbiol* **168**, 199-204.

Harms, G., Rabus, R. & Widdel, F. (1999). Anaerobic oxidation of the aromatic plant hydrocarbon *p*-cymene by newly isolated denitrifying bacteria. *Arch Microbiol* **172**, 303-312.

Hartmann, S., Hultschig, C., Eisenreich, W., Fuchs, G., Bacher, A. & Ghisla, S. (1999). NIH shift in flavin-dependent monooxygenation: mechanistic studies with 2-aminobenzoyl-CoA monooxygenase / reductase. *Proc Natl Acad Sci USA* **96**, 7831-7836.

Harwood, C. S., Burchardt, G., Hermann, H. & Fuchs, G. (1999). Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. *FEMS Microbiol Rev* **22**, 439-458.

Head, I. C., Jones, D. C. & Larter, S. R. (2003). Biological activity in the deep subsurface and the origin of heavy oil. *Nature* **426**, 244-253.

Heider, J. & Fuchs, G. (1997). Anaerobic metabolism of aromatic compounds. *Eur J Biochem* **243**, 577-596.

Heider, J., Boll, C., Breese, K., Breinig, S., Ebenau-Jehle, C., Feil, U., Gad´on, N., Laempe, D., Leuthner, B., Mohamed, C. E., Schneider, S., Burchhardt, G. & Fuchs, G. (1998). Differential induction of enzymes involved in anaerobic metabolism of aromatic compounds in the denitrifying bacterium *Thauera aromatica*. *Arch Microbiol* **170**, 120-131.

Heider, J., Spormann, A. M., Beller, H. R. & Widdel, F. (1999). Anaerobic bacterial metabolism of hydrocarbons. *FEMS Microbiol Rev* **22**, 459-473.

Hirsch, W., Schägger, H. & Fuchs, G. (1998). Phenylglyoxylate:NAD^+-oxidoreductase (CoA benzoylating), a new enzyme of anaerobic phenylalanine metabolism in the denitrifying bacterium *Azoarcus evansii*. *Eur J Biochem* **251**, 907-915.

Hurek, T., Handley, L., Reinhold-Hurek, B. & Piché, Y. (2002). *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. *Mol Plant-Microbe In* **15**, 233-242.

Hylemon, P. B. & Harder, J. (1999). Biotransformation of monoterpenes, bile acids, and other isoprenoids in anaerobic ecosystems. *FEMS Microbiol Rev* **22**, 475-488.

Johnson, H. A., Pelletier, D. A. & Spormann, A. M. (2001). Isolation and characterization of anaerobic ethylbenzene dehydrogenase, a novel Mo-Fe-S Enzyme. *J Bacteriol* **183**, 4536-4542.

Jolliffe, I. T. (2002). Principal Component Analysis. *Spinger, 2nd edition.*

Kämpfer, P. & Kroppenstedt, R. M. (1996). Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* **42**, 989–1005.

Kämpfer, P., Dreyer, U., Neef, A., Dott, W. & Busse, H.-J. (2003). *Chryseobacterium defluvii* sp. nov., isolated from wastewater. *Int J Syst Evol Microbiol* **53**, 93–97.

Kersters, K., De Vos, P., Gillis, M., Swings, J., Vandamme, P. & Stackebrandt, E. (2004). Introduction to the Proteobacteria. In *The prokaryotes: an evolving electronic resource for the microbiological community*. [Online] Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. Springer Science Online, Heidelberg, Germany. www.prokaryotes.com.

Kniemeyer, O. & Heider, J. (2001). Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. *J Biol Chem* **276**, 21381-21386.

Krause, A., Ramakumar, A., Bartels, D., Battistoni, F., Bekel, T., Boch, J., Böhm, M., Friedrich, F., Hurek, T., Krause, L., Linke, B., McHardy, A. C., Sarkar, A., Schneiker, S., Syed, A. A., Thauer, R., Vorhölter, F.-J., Weidner, S., Pühler, A., Reinhold-Hurek, B., Kaiser, O. & Goesmann, A. (2006). Complete genome of the mutualistic, N_2-fixing grass endophyte *Azoarcus* sp. strain BH72. *Nature Biotechnol* **24**, 1385-1391.

Krieger, C. J., Beller, H. R., Reinhard, C. & Spormann, A. M. (1999). Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. *J Bacteriol* **181**, 6403-6410.

Kube, M., Heider, J., Amann, J., Hufnagel, P., Kühner, S., Beck, A., Reinhardt, R. & Rabus, R. (2004). Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1. *Arch Microbiol* **181**, 182-194.

Kuhn, E. P., Zeyer, J., Eicher, P. & Schwarzenbach, R. P. (1988). Anaerobic degradation of alkylated benzenes in denitrifying laboratory aquifer columns. *Appl Environ Microbiol* **54**, 490-496.

Kühner, S., Wöhlbrand, L., Fritz, I., Wruck, W., Hultschig, C., Hufnagel, P., Kube, M., Reinhardt, R. & Rabus R. (2005). Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1. *J Bacteriol* **187**, 1493-1503.

Lay, J. O. Jr. (2001). MALDI-TOF mass spectrometry of bacteria. *Mass Spectrometry Rev* **20**, 172-194.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai , T., Steppi, S., Jobb, G., Förster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., König, A., Liss, T., Lüßmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. & Schleifer, K.-H. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363-1371.

Macy, J. C., Rech, S., Auling, G., Dorsch, C., Stackebrandt, E. & Sly, L. I. (1993). *Thauera selenatis* gen. nov., sp. nov., a member of the beta subclass of *Proteobacteria* with a novel type of anaerobic respiration. *Int J Syst Bacteriol* **43**, 135-142.

Mechichi, T., Stackebrandt, E., Gad'on, N. & Fuchs, G. (2002). Phylogenetic and metabolic diversity of bacteria degrading aromatic compounds under denitrifying conditions, and description of *Thauera phenylacetica* sp. nov., *Thauera aminoaromatica* sp. nov., and *Azoarcus buckelii* sp. nov. *Arch Microbiol* **178**, 26-35.

Mesbah, C., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159-167.

Migaud, C. E., Chee-Sanford, J. C., Tiedje, J. C. & Frost, J. W. (1996). Benzylfumaric, benzylmaleic, and *Z*- and *E*-phenylitaconic acids: synthesis, characterization, and correlation with a metabolite generated by *Azoarcus tolulyticus* Tol-4 during anaerobic toluene degradation. *Appl Environ Microbiol* **62**, 974-978.

Mohamed, C. E.-S., Ismail, W., Heider, J. & Fuchs, G. (2002). Aerobic metabolism of phenylacetic acids in *Azoarcus evansii*. *Arch Microbiol* **178**, 180-192.

Pelz, O., Chatzinotas, A., Andersen, N., Bernasconi, S. M., Hesse, C., Abraham, W.-R. & Zeyer, J. (2001). Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations. *Arch Microbiol* **175**, 270-281.

Philipp, B. & Schink, B. (1998). Evidence of two oxidation reaction steps initiating anaerobic degradation of resorcinol (1,3-dihydroxybenzene) by the denitrifying bacterium *Azoarcus anaerobius*. *J Bacteriol* **180**, 3644-3649.

Rabus, R. (2005). Biodegradation of hydrocarbons under anoxic conditions. In *Petroleum microbiology*, pp. 277- 299. Edited by B. Ollivier & M. Magot. Washington: ASM-Press.

Rabus, R. & Widdel, F. (1995). Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Arch Microbiol* **163**, 96-103.

Rabus, R. & Widdel, F. (1996). Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. *Appl Environ Microbiol* **62**, 1238-1241.

Rabus, R., Nordhaus, R., Ludwig, W. & Widdel, F. (1993). Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. *Appl Environ Microbiol* **59**, 1444-1451.

Rabus, R., Wilkes, H., Schramm, A., Harms, G., Behrends, A., Amann, R. & Widdel, F. (1999). Anaerobic utilization of alkylbenzenes and *n*-alkanes from crude oil in

an enrichment culture of denitrifying bacteria affiliating with the β-subclass of *Proteobacteria*. *Environ Microbiol* **1**, 145-157.

Rabus, R., Kube, M., Beck, A., Widdel, F. & Reinhardt, R. (2002). Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EbN1. *Arch Microbiol* **178**, 506-516.

Rabus, R., Kube, M., Heider, J., Beck, A., Heitmann, K., Widdel, F. & Reinhardt, R. (2005). The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. *Arch Microbiol* **183**, 27-36.

Reinhold, B., Hurek, T., Niemann, E.-G. & Fendrik, I. (1986). Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. *Appl Environ Microbiol* **52**, 520-526.

Reinhold-Hurek, B. & Hurek, T. (2000). Reassessment of the taxonomic structure of the diazotrophic genus *Azoarcus sensu lato* and description of three new genera and new species, *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus fungiphilus* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **50**, 649-659.

Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis, C., Kersters, K., Thielemans, S. & De Ley, J. (1987). *Azospirillum halopraeferens* sp. nov., a nitrogen-fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth). *Int J Syst Bacteriol* **37**, 43-51.

Reinhold-Hurek, B., Hurek, T., Gillis, C., Hoste, B., VanCanneyt, C., Kersters, K. & De Ley, J. (1993). *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. *Int J Syst Bacteriol* **43**, 574-584.

Reusser, D. E., Istok, J. D., Beller, H. R. & Field, J. A. (2002). *In situ* transformation of deuterated toluene and xylene to benzylsuccinic acid analogues in BTEX-contaminated aquifers. *Environ Sci Technol* **36**, 4127-4134.

Scholten, E., Lukow, T., Auling, G., Kroppenstedt, R. C., Rainey, F. A. & Diekmann, H. (1999). *Thauera mechernichensis* sp. nov., an aerobic denitrifier from a leachate treatment plant. *Int J Syst Bacteriol* **49**, 1045-1051.

Shinoda, Y., Sakai, Y., Uenishi, H., Uchihashi, Y., Hiraishi, A., Yukawa, H., Yurimoto, H. & Kato, N. (2004). Aerobic and anaerobic toluene degradation by a newly isolated denitrifying bacterium, *Thauera* sp. strain DNT-1. *Appl Environ Microbiol* **70**, 1385-1392.

Song, B., Young, L. Y. & Palleroni, N. J. (1998). Identification of denitrifier strain T1 as *Thauera aromatica* and proposal for emendation of the genus *Thauera* definition. *Int J Syst Bacteriol* **48**, 889-894.

Song, B., Häggblom, C. C., Zhou, J., Tiedje, J. C. & Palleroni, N. J. (1999). Taxonomic characterization of denitrifying bacteria that degrade aromatic compounds and description of *Azoarcus toluvorans* sp. nov. and *Azoarcus toluclasticus* sp. nov. *Int J Syst Bacteriol* **49**, 1129-1140.

Song, B., Palleroni, N. J., Kerkhof, L. J. & Häggblom, C. C. (2001). Characterization of halobenzoate-degrading denitrifying *Azoarcus* and *Thauera* isolates and description of *Thauera chlorobenzoica* sp. nov. *Int J Syst Evol Microbiol* **51**, 589-602.

Springer, N., Ludwig, W., Philipp, B. & Schink B. (1998). *Azoarcus anaerobius* sp. nov., a resorcinol-degrading, strictly anaerobic, denitrifying bacterium. *Int J Syst Bacteriol* **48**, 953-956.

Spormann, A. M. & Widdel, F. (2000). Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. *Biodegradation* **11**, 85-105.

Tan, Z., Hurek, T. & Reinhold-Hurek, B. (2003). Effect of N-fertilization, plant genotype and environmental conditions on *nifH* gene pools in roots of rice. *Environ Microbiol* **5**, 1009-1015.

Tschech, A. & Fuchs, G. (1987). Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. *Arch Microbiol* **148**, 213-217.

Widdel, F. & Bak, F. (1992). Gram-negative mesophilic sulphate-reducing bacteria. In *The Prokaryotes*, vol. 4, pp. 3352-3378. Edited by A. Balows, C. Dworkin, W. Harder, K. H. Schleifer & H. G. Trüper. New York: Springer-Verlag.

Widdel, F. & Rabus, R. (2001). Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotechnol* **12**, 259-276.

Wöhlbrand, L., Kallerhoff, B., Lange, D., Hufnagel, P., Thiermann, J., Reinhardt, R. & Rabus, R. (2007). Functional proteomic view of metabolic regulation in "*Aromatoleum aromaticum*" strain EbN1. *Proteomics* **7,** 2222-2239.

Zaar, A., Eisenreich, W., Bacher, A. & Fuchs, G. (2001). A novel pathway of aerobic benzoate catabolism in the bacteria *Azoarcus evansii* and *Bacillus stearothermophilus*. *J Biol Chem* **276**, 24997-25004.

Zaar, A., Gescher, J., Eisenreich, W., Bacher, A. & Fuchs, G. (2004). New enzymes involved in aerobic benzoate metabolism in *Azoarcus evansii*. *Mol Microbiol* **54**, 223-238.

Zehr, J. P. & McReynolds, L. A. (1989). Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl Environ Microbiol* **55**, 2522-2526.

Zhou, J., Fries, C. R., Chee-Sanford, J. & Tiedje J. C. (1995). Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyticus* sp. nov. *Int J Syst Bacterial* **45**, 500-506.

Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.

C Weitere Publikationen

Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1.

Simon Kühner, Lars Wöhlbrand, Ingo Fritz, Wasko Wruck, Claus Hultschig, Peter Hufnagel, Michael Kube, Richard Reinhardt und Ralf Rabus

Journal of Bacteriology (2005) **187** (4): 1493-503

Danksagung

An dieser Stelle möchte ich allen Personen danken, ohne die diese Arbeit nicht in der vorliegenden Form hätte entstehen können.

Außerordentlich dankbar bin ich Prof. Ralf Rabus für die Möglichkeit dieses spannende Thema zu bearbeiten. Darüber hinaus möchte ich ihm für die interessanten und inspirierenden Diskussionen sowie das Vertrauen in meine Arbeit danken. Als Doktorvater hat er maßgeblich zum Gelingen dieser Arbeit beigetragen.

Bei Prof. Friedrich Widdel möchte ich mich dafür bedanken, dass er mir die Möglichkeit gab meine Arbeit in seiner Abteilung anzufertigen und die proteomischen Untersuchungen am MPI unterstützt. Auch ein herzliches Dankeschön für die Übernahme des Zweitgutachtens.

Dr. Olav Grundmann und Dipl. Ing. Insa Werner möchte ich für die "gentechnischen Ratschläge" danken, die mir auf dem Weg zu meiner ersten eigenen Mutante sehr geholfen haben.

Mein besonderer Dank gilt auch der Arbeitsgruppe Rabus: Mercí Daniela fürs frühe Aufstehen, Messen, Füttern, Blättchen einlegen und vieles mehr. Kathleen Trautwein sei für die wundervollen Schock-Experimente ("Schätzchen Spezial") und die weit reichenden Diskussionen gedankt. An dieser Stelle möchte ich auch Dr. Dörte Gade danken, die mich in die Geheimnisse der DIGE-Technologie einführte und mir wichtige Ratschläge zuteil werden ließ.

Ich möchte allen jetzigen und frühren Mitarbeitern der Abteilung Mikrobiologie für die angenehme und produktive Arbeitsatmosphäre danken. Besonders meinen lieben Büro-Kollegen Thomas, Hoa und Katja (und Bob) sei für die erfrischende und humorvolle Zeit. Nicht zu vergessen Nadine, Insa, und Mirko…

Mein ganz besonderer Dank gilt Jessi, für ihre Geduld, ihr Verständnis und die geleistete Aufbauarbeit in der Endphase der Doktorarbeit. Ebenso möchte ich meiner Mutter für ihre stetige Unterstützung und positiven Zuspruch in den ganzen Jahren ganz herzlich danken.