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Untersuchungen zur Regulation der anaeroben Verwertung von Alkylbenzolen im denitrifizierenden Stamm EbN1

Untersuchungen zur Regulation der anaeroben Verwertung von Alkylbenzolen im denitrifizierenden Stamm EbN1

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Meiner Familie

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Teil II: Publikationen

Abkürzungen

2DE	Zwei-dimensionale Gelelektrophorese
bp	Basenpaare
BS	Benzylsuccinat
CoA	Co-Enzym A
COG	Cluster of orthologous Groups
DIGE	Difference gel electrophoresis
HPLC	High Performance Liquid Chromatography
K_{MB}	Verteilungskoeffizient Membran/Puffer
K_{OW}	Verteilungskoeffizient *n*-Oktanol/Wasser
log	Logarithmus
MALDI	Matrix-assisted laser desorption/ionisation
MPS	(1-Methylpentyl)succinat
MS	Massenspektrometrie
OD	Optische Dichte
Orf	Open reading frame, offener Leserahmen
PC	Phosphatidylcholin
PE	Phosphatidylethanolamin
PES	(1-Phenylethyl)succinat
PG	Phosphatidylglycerin
PHA	Polyhydroxyalkanoat
PHB	Poly-β-hydroxybutyrat
PL	Phospholipid
RT-PCR	Reverse Transcriptase – Polymerase chain reaction
TOF	Time of flight

Zusammenfassung

Umweltbelastungen durch die chemisch stabilen Kohlenwasserstoffe sind toxikologisch und ökologisch bedenklich. Häufig stellen sich –auf Grund der Aktivität aerober Bakterien– in kurzer Zeit anoxische Bedingungen ein, sodaß dem anaeroben Abbau eine wichtige Bedeutung zukommt. Ein Vertreter dieser Mikroorganismen ist Stamm EbN1, der Toluol und Ethylbenzol unter Nitrat-reduzierenden Bedingungen abbauen kann. Stamm EbN1 gehört zu einer erst seit wenigen Jahren bekannten Gruppe von Betaproteobakterien, die anaerob Kohlenwasserstoffe abbauen können und geographisch weit verbreitet sind. Eine bislang einzigartige Eigenschaft von Stamm EbN1 ist der anaerobe Abbau von Toluol und Ethylbenzol über zwei vollkommen unterschiedliche Reaktionssequenzen.

In der vorliegenden Arbeit wurde die Regulation dieser beiden Abbauwege bzw. der toxikologische Effekt der beiden Alkylbenzole mit einem physiologisch/proteomischen Ansatz untersucht. Dabei wurden hauptsächlich drei Fragestellungen verfolgt: (1) Wie spezifisch ist die substrat-abhängige Regulation der beiden Abbauwege? (2) Welche molekular-physiologischen Effekte sind bei Wachstum mit Alkylbenzolen zu beobachten, wenn diese in sublethalen Konzentrationen vorliegen? (3) Welche Reaktion erfolgt auf Alkylbenzol-Stress, wenn die beiden entsprechenden Abbauwege nicht operativ sind?

(1) Wachstumsexperimente zeigten, daß die Fähigkeit zur anaeroben Alkylbenzol-Verwertung induziert werden muß, d.h. sie setzt erst nach einer Inkubation von 15 – 30 h ein. Die Induktion der Abbauleistungen wurde nicht durch das jeweils andere Alkylbenzol-Substrat unterdrückt. Diese physiologischen Befunde zu den Regulationsmustern wurden nachfolgend auf molekularer Ebene (Proteom, Transkriptom) bestätigt bzw. weiter verfolgt. Die Proteom-Analyse führte zur Identifizierung von etwa 60 % der für die beiden Alkylbenzol-Abbauwege vorhergesagten Proteine. Dabei wurde eine Substrat-abhängige Veränderung der relativen Proteinhäufigkeit von bis zu 281-fach beobachtet. Darüber hinaus konnten den bislang vorhergesagten katabolen Operons weitere Gene ko-regulierter Proteine zugeordnet bzw. ein weiteres, Operon-ähnliches Gencluster mit dem anaeroben Toluol-Abbau in Verbindung gebracht werden. Die Integration bisheriger bioinformatischer Vorhersagen mit den experimentellen Befunden dieser Arbeit führte zu folgendem Regulationsmodell: (i) Die Gene des Toluol-Abbaus werden –koordiniert durch das 2-Komponenten Regulatorsystem TdiSR (hoch spezifisch für Toluol)– reguliert. (ii) Im Gegensatz dazu werden die Operons für den

oberen bzw. unteren Teil des Ethylbenzol-Abbaus sequentiell durch die Regulatorsysteme Tcs2/Tcr2 (Ethylbenzol-spezifisch) bzw. Tcs1/Tcr1 (Acetophenon-spezifisch) reguliert. Interessanterweise scheinen beide Systeme eine relaxierte Spezifität zu besitzen und auf Toluol zu reagieren.

(2) Stamm EbN1 toleriert bei Wachstum mit Toluol bzw. Ethylbenzol als alleinigen C-Quellen maximale Konzentrationen (bezogen auf die Wasserphase) von 740 µM bzw. 315 µM. Unter diesen sublethalen Bedingungen fand Wachstum nur mit Nitrat statt, Nitrit wurde intermediär nur in geringeren Mengen gebildet und konnte nicht als alleiniger Elektronenakzeptor verwendet werden. Die Häufigkeit der Nitrit-Reduktase war hingegen stark erhöht und führte -vermutlich durch die Bildung von Nitroxyl-Ionen- zu oxidativem Stress. Eine Folge davon könnte die beobachtete Bildung von Superoxid-Dismutase, stabileren Isoenzymen und Verringerung der cytoplasmatischen Eisenkonzentration sein. Die erhöhte Häufigkeit von Proteinen, die mit der Bildung von PHAs assoziiert sind, korreliert mit dem 10 % Trockengewichtsanteil von PHB unter den sublethalen Bedingungen. Möglicherweise wird bei beeinträchtigter Denitrifizierung Acetyl-CoA aus der Alkylbenzol-Oxidation statt end-oxidiert in die PHA-Synthese umgeleitet. Unter den sublethalen Bedingungen kam es zu einer Erhöhung des Anteils von Phosphatidylcholin und Methylcyclohexyl-modifiziertem Phosphatidylethanolamin als Lipid-Kopfgruppen zur Stabilisierung der Cytoplasma-Membran.

(3) Bei aktiv Succinat-verwertenden Kulturen von Stamm EbN1 führt die plötzliche Zugabe von 0.98 mM Toluol bzw. 0.37 mM Ethylbenzol zu einer starken Beeiträchtigung von Wachstum und Nitrat-Verbrauch. NO_2^-- und N_2O-Reduktasen sowie PHA-assoziierte Proteine lagen in erhöhten Mengen vor. Unmittelbar nach dem Alkylbenzol-Schock kam es zum vorübergehenden Anstieg von Heat-Shock-Proteinen. Die Bildung von Proteinkomponenten von Exportsystemen für Lösungsmittel wurde nicht beobachtet. Interessanterweise wurden in Kontrollen (Kulturen ohne Alkylbenzol-Schock) Proteine der stationären Phase nicht bei Verringerung der Wachstumsrate, sondern bereits im zweiten Drittel der linearen Wachstumsphase gebildet.

Teil I: Darstellung der Ergebnisse im Gesamtzusammenhang

A Einleitung

1. Vorkommen und allgemeine Eigenschaften von Kohlenwasserstoffen

1.1 Definition und Eigenschaften

Verbindungen, die nur aus Kohlenstoff und Wasserstoff bestehen, sind als Kohlenwasserstoffe definiert. Sie werden in drei Klassen eingeteilt (Abb. 1): Aliphaten wie z.B. *n*-Hexan sind linear oder verzweigt und können Doppel- oder Dreifachbindungen enthalten. Alicyclen unterscheiden sich von Aliphaten durch ringförmig angeordnete Kohlenstoffatome, die aber keine oder nicht vollständig konjugierte Doppelbindungen enthalten (z.B. Cyclohexan) und sich dadurch von den aromatischen Kohlenwasserstoffen mit einem System delokalisierter π-Elektronen (z.B. Benzol) unterscheiden. Bei den für diese Arbeit wichtigen Alkylbenzolen ist der aromatische Ring mit mindestens einer Alkylgruppe substituiert, z.B. Toluol mit einer Methylgruppe und Ethylbenzol mit einer Ethylgruppe (Abb. 1). Diese beiden Verbindungen bilden zusammen mit Benzol und den drei Dimethylbenzol-Isomeren (*ortho*-, *meta*- und *para*-Xylol) die ökonomisch und ökologisch wichtige Gruppe der BTEX.

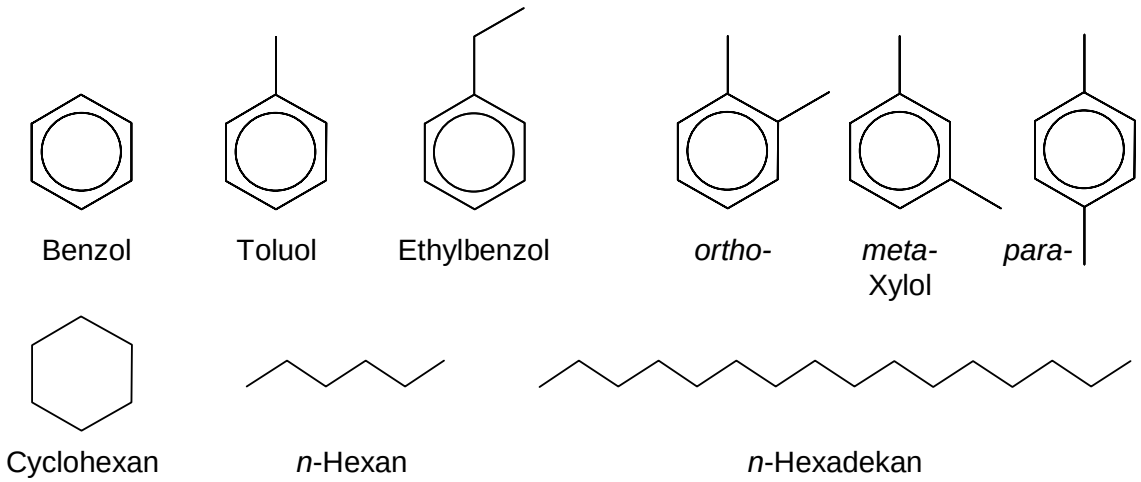

Abb. 1. Beispiele für aromatische (Benzol, Toluol, Ethylbenzol und *o*-, *m*-, *p*-Xylol), alicyclische (Cyclohexan) und aliphatische (*n*-Hexan und *n*-Hexadekan) Kohlenwasserstoffe.

Die kovalente Bindung zwischen Kohlenstoff und Wasserstoff ist aufgrund des geringen Unterschieds der Elektronegativität der Elemente apolar und deshalb reaktionsträge. C,C-Doppelbindungen sind reaktiver, jedoch nicht die Konjugierten des aromatischen Rings.

Dieser letztere Effekt wird Mesomerie-Stabilisierung genannt. Reaktionen, bei denen die Mesomerie gestört oder aufgehoben wird, bedürfen deshalb einer erhöhten Aktivierungsenergie (Tabelle 1).

Die geringe Polarität der Bindungen und der symmetrische Molekülaufbau bestimmen die in apolaren Lösungsmitteln hohe bzw. im extrem polaren Wasser geringe Löslichkeit (Tabelle 1). Entsprechend gibt das Verteilungsgleichgewicht eines Kohlenwasserstoffs zwischen *n*-Oktanol und Wasser (angegeben als Logarithmus K_{OW}) das Verhältnis der Konzentrationen zweier im Fließgleichgewicht miteinander stehenden Phasen an. Einem Molekül Toluol (log K_{OW} 2.69) im Wasser stehen 490 Moleküle im *n*-Oktanol gegenüber, für *n*-Hexan ist das Verhältnis entsprechend 1:12900. Physikalisch-chemische Angaben wichtiger Verbindungen sind der Tabelle 1 zu entnehmen.

Tabelle 1. Physikalische Eigenschaften von Kohlenwasserstoffen.

Substanz	**Löslichkeit [a] [mg/l]**	**log K_{OW}**	**Bindungsdissoziationsenergie [kJ·mol^{-1}] [e]**	
Benzol	1780	2.13 [b]	C_6H_5-H	461
Toluol	515	2.69 [b]	(C_6H_5)-CH_2-H	368
Ethylbenzol	152	3.15 [b]	$(C_6$-$H_5)(CH_3)$-CH-H	358
Cyclohexan	55	3.44 [c]	C_6-H_{11}-H	400
n-Hexan	9.5	4.11 [c]	CH_3-$(CH_2)_5$-H	410
n-Hexadekan	0.006	9.57 [d]		

[a] in Wasser (Mackay und Shiu 1981)
[b] (Leo et al. 1971)
[c] (Yaffe et al. 2002)
[d] (Khadikar et al. 2003)
[e] (McMillen und Golden 1982)

1.2 Toxizität

Kohlenwasserstoffe können auf physikalische und chemische Weise giftig wirken. Die physikalische Giftigkeit beruht darauf, daß sich lipophile Kohlenwasserstoffe bevorzugt in der Cytoplasmamembran lösen, akkumulieren und Funktion und Stabilität der Membran bis zur Lyse herabsetzen (Marquardt und Schäfer 1997). Beispielsweise kann nach Erbrechen von verschlucktem Lampenöl (C_8 – C_{16} *n*-Alkane) durch den Atemreflex Luft mit hohem Alkananteil (z.B. 800 mg insgesamt) in die Lunge gelangen, sich als Film auf die Alveolen legen und eine hämorrhagische Pneumonie mit möglicherweise tödlichem Ausgang verursachen (Hahn et al. 1998). Nicht-Erbrechen hätte wegen der schlechten Resorbierbarkeit im Magen-/Darmtrakt keine gravierenden Auswirkungen gehabt, es käme lediglich zu Durchfall (Bertram 1998). Für die physikalische Giftigkeit entscheidend ist also, wieviel Kohlenwasserstoff tatsächlich zum Wirkort kommt. Lampenöl *n*-Alkane (Löslichkeit 0.006

bis 9.5 mg/l Wasser, log K_{OW} 4-10) sind ungiftig, wenn eine wässrige Phase überquert werden muß, aber giftig, wenn sie gasförmig direkt an die Membran gelangen.

Die chemische Giftwirkung beruht hingegen auf der Ausbildung von Bindungen zu einem Molekül, daß dadurch denaturiert und damit biologisch unwirksam wird. Wegen der geringen Reaktivität der Kohlenwasserstoffe wird die Giftwirkung fast ausschließlich durch die beim Abbau gebildeten Metabolite hervorgerufen. Benzol wird z.B. zu Phenol und Catechol oxidiert, die nach weiteren Reaktionen kovalent an zelluläre Bestandteile wie die DNA binden und so kanzerogen und mutagen wirken (Marquardt und Schäfer 1997). Da ein einziges Molekül kanzerogen sein kann, ist die Benzol-Exposition zu vermeiden. Dem gegenüber steht die geringe Human-Toxizität von Toluol, das größtenteils an der Methylgruppe zum in der Lebensmittel-Konservierung eingesetzten Benzoat oxidiert wird (Marquardt und Schäfer 1997). Entsprechend wurde in vielen Anwendungen Benzol durch Toluol (oder andere Lösungsmittel) ersetzt.

Die Cytoplasmamembran von Bakterien dient der räumlichen Abtrennung gegen die Umwelt und dem Aufbau des chemi-osmotischen Gradienten für Energiegewinnung und Transport. Wegen dieser Prozesse enthält die Membran eine Vielzahl verschiedener Proteine, die zusammen 50-70 % der Trockenmasse der Membran ausmachen (Lengeler et al. 1999). Substanzen mit einem log K_{OW} zwischen 1.5 und 4 sind auf der einen Seite ausreichend wasser- bzw. mediumlöslich, um in relevanten Mengen zur Cytoplasmamembran zu gelangen und auf der anderen Seite ausreichend lipophil, um zu toxikologischen Mengen zu akkumulieren (Sikkema et al. 1995; Ramos et al. 2002). Diese Anreicherung erhöht die Fluidität und somit die Permeabilität der Membran und verändert die Raumumgebung und das Reaktionsmilieu von Membranproteinen. Die lethale Konzentration ist dabei geringer als diejenige, die zur vollständigen Lyse der Membran und damit einhergehend zum Verlust von Molekülen bis zur Größe von Proteinen und RNA führt (Jackson und de Moss 1965; Sikkema et al. 1995).

Mikroorganismen haben verschiedene Strategien zur Anpassung an Lösungsmittelstress entwickelt. Aktive Maßnahmen wie Effluxsysteme exportieren Kohlenwasserstoffe aus dem Cytoplasma oder der Membran ins Medium und werden durch den Protonengradienten angetrieben. Passive Maßnahmen verringern die Membranfluidität durch Erhöhung des Anteils *trans*-ungestättigter, gesättigter oder langkettiger Fettsäuren, oder durch Veränderung der Phospholipid-Kopfgruppen, die zur Entspannung der Membran-Doppelschicht führt (Weber und de Bont 1996; Ramos et al. 2002). Bei Organismen, die in einem 2-Phasen-

System aus Medium und z.B. Toluol wachsen, wie z.B. *Pseudomonas putida* Stamm DOT-T1E, wird die Expression von drei zur Familie der Resistance-Nodulation-Cell Division (RND) gehörenden Efflux-Systemen als maßgebliche Anpassung angesehen (Rojas et al. 2001; Segura et al. 2003). Die Herabsetzung der Membranfluidität beruht dagegen auf der Angleichung der Konformation der Fettsäuren. Gesättigte und *trans*-ungesättigte Fettsäuren ähneln sich sterisch, wohingegen sich *cis*-ungesättigte Fettsäuren durch einen 30°-Knick unterscheiden und den Schmelzpunkt entsprechend herabsetzen (Weber et al. 1994). Die Veränderung der Phospholipid-Kopfgruppen beeinflußt die Stabilität der Bilayer-Konfiguration gegenüber einer Monolayer mit ineinandergreifenden Phospholipiden (PL) oder einer micellären Struktur (Weber et al. 1994). Die stabilste Cytoplasmamembran bestünde demnach auf der Innenseite aus konus-förmigen PL wie Phosphatidylethanolamin, bei denen der Querschnitt der Kopfgruppe kleiner ist als der des Fettsäurerests und auf der Außenseite aus PL mit größerer Kopfgruppe wie Phosphatidylcholin. Bei der häufig beobachteten Veränderung der Zusammensetzung der PL-Kopfgruppen wird in der Regel nicht zwischen Innen/Außen-Lokalisierung unterschieden, da dies analytisch sehr aufwändig ist (Israelachvili et al. 1977; Weber et al. 1994).

Auf Ebene der Proteine sind Anpassungen an die Verringerung der chemischen Giftigkeit zu beobachten. Der Ursprung oder Mechanismus der Oxidation von Kohlenwasserstoffen zu reaktiven Peroxiden ist unklar, eine fehlgeleitete Elektronenübertragung von Cytochromen der Atmungskette aerober Organismen könnte die Ursache sein (Garbe und Yukawa 2001). Entsprechend erlangte eine *E. coli* Mutante vollständige Resistenz gegen Tetralin durch erhöhte Aktivität der Peroxid-Reduktase (AhpC) (Ferrante et al. 1995).

Insgesamt ist festzustellen, daß die BTEX-Kohlenwasserstoffe ein toxikologisch hohes Potential haben, da sie einerseits wegen guter Löslichkeit eine hohe Bioverfügbarkeit haben (Tab. 1) und andererseits im Falle des Benzols erwiesenermaßen kancerogen sind.

1.3 Geologische Entstehung

Erdöl entsteht in einem Millionen von Jahren dauernden Prozess in mehreren, zuerst mikrobiologisch dominierten Schritten und später durch steigende Temperaur (>50 °C) und erhöhten Druck aus organischem Material (Tissot und Welte 1984). Die Art der Biomasse beeinflußt dabei stark die Zusammensetztung des Vielstoffgemisches „Rohöl“. Leichte, viskose, schwefelarme Öle werden meist aus in Mündungsdeltas oder intrakontinentalen Becken sedimentiertem terrestrischem Material gebildet. In der Zusammensetzung dieser Öle

dominieren verzweigte und *n*-Alkane, die vermutlich aus Blattwachsen entstanden sind. Die im Ausgangsmaterial mengenmäßig weitaus bedeutsameren Kohlenhydrate und Lignine können wegen ihres oxidierteren Zustands nicht zur Ölbildung beitragen. Schwere, Aromaten-dominierte Öle sind meist aus in anoxischen Becken oder Auftriebsgebieten sedimentierten, marinen Organismen entstanden (Rullkötter 1993).

Mikrobieller Abbau führt zu der bereits früh beobachteten Korrelation zwischen verringerten Konzentrationen von *n*-Alkanen, Alkylbenzolen und Sulfat, und der Bildung von Sulfid (Rogers 1919; Bastin et al. 1926; Tissot und Welte 1984). Bei Erhitzung des Mutter- und Speichergestein auf über 80 bis 85°C kommt es dagegen möglicherweise im Zusammenhang mit dem Lösungsmitteldruck zu „Paläopasteurisierung“ (Connan 1984; Wilhelms et al. 2001; Townsend et al. 2003), der Abtötung anaerob Kohlenwasserstoff-abbauender Organismen, von der andere, hyperthermophile Organismen nicht betroffen sind (Stetter et al. 1993). Die Biodegradation von Rohöl wird dramatisch beschleunigt, wenn bei der sekundären Förderung sulfatreiches Seewasser zum Austreiben des Öls in das Speichergestein gepumpt wird (Myhr et al. 2002). Auch an undichten Lagerstätten oder an hydrothermalen Quellen wie z.B. im Guaymas Basin, wo durch Hitze und Wasserstrom die Ölentstehung und Migration stark beschleunigt sind, findet mikrobieller Abbau unter anaeroben Bedingungen im Sediment und unter aeroben Bedingungen in der Wassersäule nahe des Austritts statt (Rueter et al. 1994; Wenger und Isaksen 2002).

1.4 Biologische Bildung

Kohlenwasserstoffe können auf verschiedenen Wegen auch biologisch gebildet werden. Pflanzen synthetisierten Terpene, die aus Isopren-Einheiten bestehen und die, wie z.B. das für den Kieferngeruch charakteristische Pinen, zum Teil reine Kohlenwasserstoffe sind (Nuhn 1997). Außerdem kommen in der Cuticula von Pflanzen langkettige, ungeradzahlige *n*-Alkane und *n*-Alkene vor, die vermutlich durch Decarbonylierung geradzahliger Fettsäuren gebildet werden (Widdel und Rabus 2001). Große Mengen von Methan entstehen durch Disproportionierung von Acetat und noch bedeutsamer durch die respirative Reduktion von CO_2 durch Archeen (Schoell 1988). Die biologische Bildung von Alkylbenzolen ist weit weniger ausgeprägt. Aus Verbindungen wie z.B. Phenylalanin kann durch Defunktionalisierung unter anoxischen Bedingungen Toluol entstehen, dessen Konzentration im Hypolimnion von Seen bis zu 3 µM (Jüttner und Henatsch 1986) und in Klärwerken bis zu 0.45 mM (Mrowiec et al. 2005) betragen kann. Außerdem können unter methanogenen Bedingungen Terpen-

Kohlenwasserstoffe wie z.B. α-Phellandren oder Sabinen zu *p*-Cymol aromatisiert werden (Harder und Foss 1999).

1.5 Anthropogener Eintrag

Seit der Entstehung von Erdöl gelangt dieses an durch Teersände und Pechseen gekennzeichneten Stellen (z.B. in Kanada oder im Iran) an die Bodenoberfläche, ins Wasser oder die Luft (Wenger und Isaksen 2002), wo sie vermutlich von Anbeginn der Menschheit genutzt wurden (Boëda et al. 1998). Mit Beginn (etwa 1859) der industriellen Gewinnung von Kohlenwasserstoffen aus Erdöl z.B. zur chemischen Synthese, als Lösungs- oder Schmiermittel oder als Treib- und Brennstoff, gelangten diese verstärkt in die Umwelt. Spektakulär und allgemein bekannt sind Unfälle wie Tankerhavarien. Im Falle der Amoco Cadiz ergossen sich 1978 223000 t Rohöl an die Küste der Bretagne. Alltäglich sind Emissionen durch unvollständige Verbrennung oder durch Verdunstung aus Autotanks. Eine Studie aus den USA zeigt, daß 35 % aller untersuchten Bodentanks Leckagen aufwiesen und daß bei 40 % von ihnen Treibstoff in das Grundwasser gelangte (EPA 1986). Dies ist Besorgnis erregend, da die toxischen BTEX in der Benzin-Fraktion bis zu 25 % [v/v] betragen (Cline et al. 1991). BTEX, die in die Atmosphäre verdunsten, werden innerhalb weniger Tage durch Hydroxylradikale abgebaut (LAI 1996). Der biologische Abbau von BTEX verläuft unter oxischen Bedingungen relativ schnell. Gelangen die Kohlenwasserstoffe aber in wassergesättigte Schichten, ist die Sauerstoffversorgung so gering, daß sich schnell anoxische Bedingungen einstellen. Unter anoxischen Bedingungen verläuft der Abbau weit langsamer, sodaß Kohlenwasserstoffe ins Grundwasser gelangen können. Der biologische Abbau von Kohlenwasserstoffen wird in Kapitel 2 beschrieben.

2. Anaerober mikrobieller Abbau von Kohlenwasserstoffen bzw. Aromaten

Die Möglichkeit, Kohlenwasserstoffe unter oxischen Bedingungen als einzige Kohlenstoff- und Elektronenquelle zu benutzen, ist unter Mikroorganismen weit verbreitet (Schlegel und Zaborosch 1992; Madigan et al. 1997). Entscheidend für diese katabole Leistung ist die Nutzung von hoch reaktiven Sauerstoff-Spezies (aus O_2) als Ko-Substrate von Mono- und Dioxygenasen zur initialen Aktivierung dieser reaktionsträgen Substrate.

Monooxygenasen katalysiert z.B. in *Pseudomonas oleovorans* die Reaktion von *n*-Oktan, Sauerstoff und 2 Reduktionsequivalenten zu *n*-Oktanol und Wasser (Baptist et al. 1963). Das Substratspektrum der Monooxygenasen umfaßt *n*-Alkane von Methan bis C_{35}, verzweigte Alkane (Rapp und Gabriel-Jürgens 2003) und Cycloalkane (Schumacher und Fakoussa 1999). Aromaten können an der Seitenkette (Austin et al. 2003) sowie am Ring (Johnson und Olsen 1997) oxidiert werden. Dioxygenasen katalysiert z.B. in *Pseudomonas putida* die Oxidation vieler aromatischer Kohlenwasserstoffe zu Catecholen und die oxygenolytische Spaltung des aromatischen Rings (Harwood und Parales 1996).

2.1 Organismen

Neben der in A.1.3 beschriebenen selektiven Verwertung bestimmter Kohlenwasserstoffe in Ölreservoirs unter sulfidogenen Bedingungen gab es erste Hinweise auf anaerob Alkylbenzole verwertende Bakterien aus strikt anoxischen Mikrokosmen und Anreicherungskulturen (Kuhn et al. 1985; Zeyer et al. 1986; Grbic-Galic und Vogel 1987). Später wurden vor allem anaerob Toluol-abbauende Reinkulturen isoliert, die Nitrat, Eisen(III) oder Sulfat als terminalen Elektronenakzeptor nutzen (Tabelle 2). Neben Toluol werden zum Teil auch *m*-Xylol, *o*-Xylol, Ethylbenzol oder *p*-Cymol (*p*-Isopropyltoluol) verwertet. Wenige andere Stämme wachsen anaerob mit Benzol oder *n*-Alkanen. Bisher ist jedoch kein Organismus bekannt, der sowohl Alkane als auch Alkylbenzole verwerten kann. Alle Isolate gehören zu den *Proteobacteria* und stammen aus sehr diversen, Kohlenwasserstoff-belasteten aber auch unbelasteten Habitaten (kürzlich zusammengefaßt in Rabus 2005). Die im Vergleich zu Eisen(III) oder Nitrat mit ca. 28 mM hohe Sulfatkonzentration des Meerwassers ermöglicht die Oxidation großer Substratmengen. Dies ist vor allem im Hinblick auf Ölreservoirs von Bedeutung (siehe A.1.3). Weitergehende Untersuchungen der anaeroben Oxidation von Kohlenwasserstoffen wurden wegen ihres relativ schnellen Wachstums bevorzugt mit Nitrat-reduzierenden Bakterien durchgeführt.

Table 2. Anaerob Kohlenwasserstoff verwertende Reinkulturen.

Stamm und Protobacteria-Typ		Kohlenwasserstoff-Substrat	Aktivierungs-mechanismus[a]	Gene für Startreaktion[b]	Referenzen
Phototroph					
Blastochloris sulfoviridis ToP1	α	Toluol	BS		(Zengler et al. 1999)
Denitrifizierend					
Thauera aromatica K172	β	Toluol	BS	*bss*	(Tschech and Fuchs 1987; Seyfried et al. 1994; Anders et al. 1995; Leuthner et al. 1998)
Thauera aromatica T1	β	Toluol	BS	*bss*	(Evans et al. 1991; Evans et al. 1992; Coschigano et al. 1998)
Azoarcus sp. T	β	Toluol, *m*-Xylol	BS	*bss*	(Dolfing et al. 1990; Seyfried et al. 1994; Beller and Spormann 1998; Achong et al. 2001)
Azoarcus tolulyticus Tol-4	β	Toluol	BS		(Fries et al. 1994; Zhou et al. 1995; Migaud et al. 1996)
Thauera sp. DNT-1	β	Toluol	BS	*bss*	(Shinoda et al. 2004)
Magnetospirillum sp. TS-6	α	Toluol		*bss*	(Shinoda et al. 2005)
ToN1	β	Toluol			(Rabus and Widdel 1995)
mXyN1	β	Toluol, *m*-Xylol			(Rabus and Widdel 1995)
EbN1	β	Toluol, Ethylbenzol	BS, PE	*bss, ebd*	(Rabus and Widdel 1995; Rabus and Heider 1998; Rabus et al. 2001; Kube et al. 2004)
EB1	β	Ethylbenzol	PE	*ebd*	(Ball et al. 1996; Johnson et al. 2001)
PbN1	β	Ethylbenzol, Propylbenzol	PE		(Rabus and Widdel 1995; Kniemeyer and Heider 2001)
pCyN1	β	Toluol, p-Cymol, p-Ethyltoluol,			(Harms et al. 1999a)
pCyN2	β	p-Cymol			(Harms et al. 1999a)
Vibrio sp. NAP-4	γ	Naphthalin			(Rockne et al. 2000)
Dechloromonas aromatica RCB	β	Benzol			(Coates et al. 2001b)
HxN1	β	*n*-Hexan – *n*-Oktan			(Ehrenreich et al. 2000; Rabus et al. 2001; Wilkes et al. 2003)
OcN1	β	*n*-Oktan – *n*-Dodekan			(Ehrenreich et al. 2000)
HdN1	γ*	*n*-Tetradekan – *n*-Eikosan			(Ehrenreich et al. 2000)

Stamm und Protobacteria-Typ		Kohlenwasserstoff-Substrat	Aktivierungs-mechanismus[a]	Gene für Startreaktion[b]	Referenzen
Sulfat-reduzierend					
Desulfobacula toluolica Tol2	δ	Toluol	BS		(Rabus et al. 1993; Rabus and Heider 1998)
PRTOL1	δ	Toluol	BS		(Beller et al. 1996; Beller and Spormann 1997)
mXyS1	δ	Toluol, *m*-Xylol			(Harms et al. 1999b)
oXyS1	δ	Toluol, *o*-Xylol			(Harms et al. 1999b)
Desulfotomaculum sp. OX39	δ	Toluol, *o*-Xylol, *m*-Xylol	BS	*bss*	(Morasch et al. 2004)
EbS7	δ	Ethylbenzol	PES		(Kniemeyer et al. 2003a)
NaphS2	δ	Naphthalin			(Galushko et al. 1999)
Desulfatibacillum aliphaticivorans	δ	*n*-Tridekan – *n*-Oktadekan	MPS		(Cravo-Laureau et al. 2004; Cravo-Laureau et al. 2005)
Hxd3	δ	*n*-Dodekan – *n*-Eikosan	--		(Aeckersberg et al. 1991; So et al. 2003)
TD3	δ	*n*-Hexan – *n*-Hexadekan			(Rueter et al. 1994)
Pnd3	δ	*n*-Tetradekan – *n*-Heptadekan			(Aeckersberg et al. 1998)
AK-01	δ	*n*-Tridekan – *n*-Oktadekan	MPS		(So and Young 1999; Callaghan et al. 2003)
BuS5	δ	*n*-Propan, *n*-Butan	MPS		(Kniemeyer et al. 2003b)
Eisen(III)-reduzierend					
Geobacter metallireducens GS-15	δ	Toluol	BS	*bss*	(Lovley et al. 1989; Lovley and Lonergan 1990; Kane et al. 2002)
Geobacter grbiciae	δ	Toluol			(Coates et al. 2001a)

[a] Mechanismen der anaeroben Kohlenwasserstoffaktivierung, BS, Fumarat-Addition, ergibt Benzylsuccinat-Derivate; PE, Oxidation, ergibt 1-Phenylethanol-Derivate; PES, Fumarat-Addition, ergibt (Phenyl-)ethylsuccinat-Derivate; MPS, Fumarat-Addition, ergibt (Methyl-)pentylsuccinat-Derivate.

[b] Gene der die Startreaktion katalysierenden Proteine identifiziert, bss – Benzylsuccinat Synthase, ebd – Ethylbenzol-Dehydrogenase.

Der Modellorganismus der vorliegenden Arbeit ist das denitrifizierende Bakterium Stamm EbN1. Es wurde unter Nitrat-reduzierenden Bedingungen aus einer Mischung von Sedimenten Bremer Gewässer mit Ethylbenzol isoliert (Rabus und Widdel 1995a). Strikt anoxische Verhältnisse wurden durch Verwendung von Glasflaschen eingestellt, die mit Ascorbat-reduziertem Medium unter Begasung mit N_2/CO_2 (90:10, [v/v]) befüllt und mit Sauerstoff-undurchlässigen Butylstopfen verschlossen wurden. Um toxische Effekte der Substrate zu vermeiden, wurden Alkylbenzole mit Stickstoff-begastem, hochverzweigten, inerten HMN (2,2,4,4,6,8,8-Heptamethylnonan) verdünnt. Proben wurden mit Stickstoff-gespülten Spritzen aus den Wachstumskulturen entnommen (Rabus und Widdel 1995a).

Stamm EbN1 kann neben Ethylbenzol auch mit Toluol wachsen und beide Alkylbenzole direkt aus Rohöl verwerten (Rabus und Widdel 1996). Außerdem werden 1-Phenylethanol, Acetophenon, Benzoat und verschiedene andere Alkohole und Carbonsäuren zu CO_2 oxidiert, jedoch keine Zucker oder Aminosäuren (außer Phenylalanin) (Rabus und Widdel 1995a). Unter denitrifizierenden Bedingungen wird Nitrat unter vorübergehender Akkumulierung von Nitrit zu N_2 reduziert. Phylogenetisch bildet der Stamm EbN1 mit den meisten bislang isolierten anaerob Kohlenwasserstoff-abbauenden Denitrifizierern ein Cluster innerhalb der *Betaproteobacteria*, das in der Nähe von *Thauera selenatis* und *Azoarcus indigens* liegt. Ersterer zeichnet sich durch respiratorische Selenatreduktion aus, letzterer durch die Befähigung zur N_2-Fixierung und symbiotischen Assoziation mit Reispflanzen (Macy et al. 1993; Reinhold-Hurek et al. 1993; Fries et al. 1994; Rabus und Widdel 1995a). Wegen der besonderen metabolischen Eigenschaften werden Stamm EbN1 und seine Kohlenwasserstoff/ Aromaten-verwertenden Verwandten demnächst als eigene Gattung beschrieben (Rabus, in Vorbereitung).

2.2 Reaktionen und Abbauwege

2.2.1 Fumarat-abhängige Aktivierung. Aus Toluol wird durch radikalische Addition an Fumarat Benzylsuccinat gebildet, das wie in Abbildung 2 gezeigt in mehreren Schritten zu Benzoyl-CoA metabolisiert wird (Evans et al. 1992; Biegert et al. 1996; Migaud et al. 1996; Rabus und Heider 1998; Leuthner und Heider 2000; Leutwein und Heider 2001; Leutwein und Heider 2002). Die Startreaktion wird durch die cytoplasmatische Benzylsuccinatsynthase BssABC katalysiert (Biegert et al. 1996; Beller und Spormann 1997a), wobei die Aktivase BssD mit Hilfe von Adenosyl-Methionin ein Radikal generiert, das auf ein Glycin der α-Untereinheit der (BssA) (Radikalspeicher) übertragen wird. Von dort wird es auf das

katalytische Cystein im aktiven Zentrum und, den Reaktionszyklus startend, auf die Methylgruppe des Toluols übertragen. Letzteres addiert an die Doppelbindung des Fumarats. Der Kreis schließt sich durch Austausch des Radikals gegen den ursprünglich von der Methylgruppe abstrahierten Wasserstoff. Der Erhalt des Deuteriums von Methylgruppen-markiertem Toluol im Succinat-Rest des Produkts bestätigt diese Vorstellung vom Erhalt des Methyl-Wasserstoffs (Heider et al. 1998b; Krieger et al. 1999). Als Reaktionsprodukt entsteht stereospezifisch *R*-(+)-Benzylsuccinat. Bss ist sehr Sauerstoff-empfindlich, da die große Untereinheit (BssA) oxygenolytisch am Radikal-speichernden Glycin gespalten werden kann.

Ein ähnlicher Glycyl-radikalischer Reaktionsmechanismus wurde zuerst bei der Pyruvat-Format Lyase von *E. coli* aufgeklärt. Dieses Enzym spaltet Pyruvat homolytisch an einer C-C Bindung und wandelt es mit CoA in Acetyl-CoA und Formiat um (Knappe et al. 1984; Becker et al. 1999). Weitere Beispiele anaerober Radikalreaktionen sind die Ribonukleotid Reduktase NrdD (Fontecave et al. 1989) und *p*-Hydroxyphenylacetat Decarboxylase (Selmer und Andrei 2001). Die meisten anaeroben Bakterien sind folglich in der Lage, Glycyl-radikalische Reaktionen ausführen, was möglicherweise die Verbreitung der Fumarat-Addition zur anaeroben Aktivierung von Kohlenwasserstoffen begünstigte.

Für anaerob mit Toluol wachsende Stämme ist die Fumarat-Addition der einzige zur Zeit bekannte Mechanismus. Weitere Substrate für diesen Reaktionstyp (Tabelle 2) sind *o*- und *m*-Xylol, die zu den entsprechenden Methylbenzylsuccinaten aktiviert werden (Krieger et al. 1999; Morasch et al. 2004b), Ethylbenzol, aus dem (1-Phenylethyl)-succinat entsteht (Kniemeyer et al. 2003a) und *n*-Alkane wie z.B. *n*-Hexan (Rabus et al. 2001), *n*-Butan (Kniemeyer et al. 2003b) oder *n*-Hexadekan (Cravo-Laureau et al. 2005), die subterminal zu entsprechenden (1-Methylalkyl)succinaten aktiviert werden. Auch ko-metabolische Aktivierung von Alicyclen wie Cyclopentan und Methylcyclopentan durch Stamm HxN1 (Wilkes et al. 2003) oder Verwertung von Ethylcyclopentan (Rios-Hernandez et al. 2003) oder Cyclohexan (Musat 2005) in Sulfat- bzw. Nitrat-reduzierenden Anreicherungen wurde beobachtet.

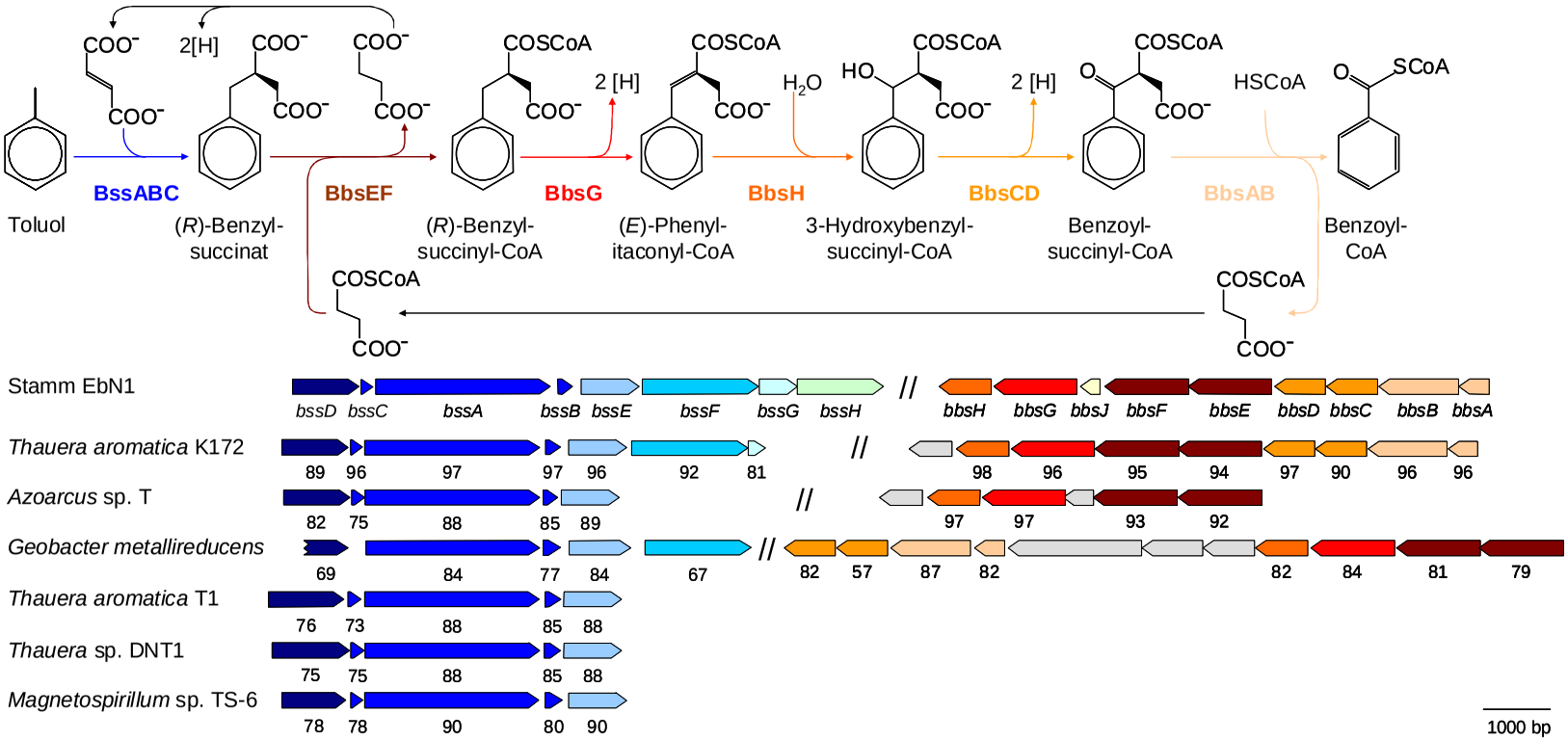

Abb. 2. Anaerobe Abbauwege für Toluol und die Organisation der kodierenden Gene in Stamm EbN1. Zum Vergleich sind Sequenzen anderer Stämme (Tab. 2) und deren Ähnlichkeit (%, BLASTP) aufgeführt. Enzym-Abkürzungen sind: BssABC, Benzylsuccinat Synthase; BbsEF, Succinyl-CoA:(*R*)-Benzylsuccinat-CoA Transferase; BbsG, (*R*)-Benzylsuccinyl-CoA Dehydrogenase; BbsH, Phenylithaconyl-CoA Hydratase; BbsCD, 2-[Hydroxy(phenyl)methyl]-succinyl-CoA Dehydrogenase; BbsAB, Benzoylsuccinyl-CoA Thiolase.

2.2.2 Dehydrogenierung. In den Nitrat-reduzierenden Stämme EB1 und EbN1 wurde ein weiterer auf Dehydrogenierung von Alkylbenzolen beruhender Mechanismus beschrieben. An der Methylen-Gruppe von Ethylbenzol wird durch die periplasmatische Ethylbenzol-Dehydrogenase (EbdABC) eine Hydroxyl-Gruppe eingeführt (Johnson et al. 2001; Kniemeyer und Heider 2001a). Das Produkt (*S*)-1-Phenylethanol wird wie in Abbildung 3 gezeigt via Acetophenon zu Benzoyl-CoA metabolisiert (Champion et al. 1999; Kniemeyer und Heider 2001b). Die Ethylbenzol-Dehydrogenase (EbdABC) gehört zu den Molybdän und Fe/S enthaltenden Enzymen, zeigt große Ähnlichkeit zur Selenatreduktase des nahe verwandten Stammes *Thauera selenatis* (Johnson et al. 2001; Kniemeyer und Heider 2001a) und aktiviert im Nitrat-reduzierenden Stamm PbN1 Propylbenzol (Kniemeyer und Heider 2001a). Wegen des hohen Redoxpotentials des Startenzyms von 0.3 bis 0.4 V kann dieser Mechanismus vermutlich nicht von Sulfatreduzierern, die üblicherweise Reaktionen in einem Redoxmilieu von <0 V ausführen, verwendet werden (Rabus et al. 2000).

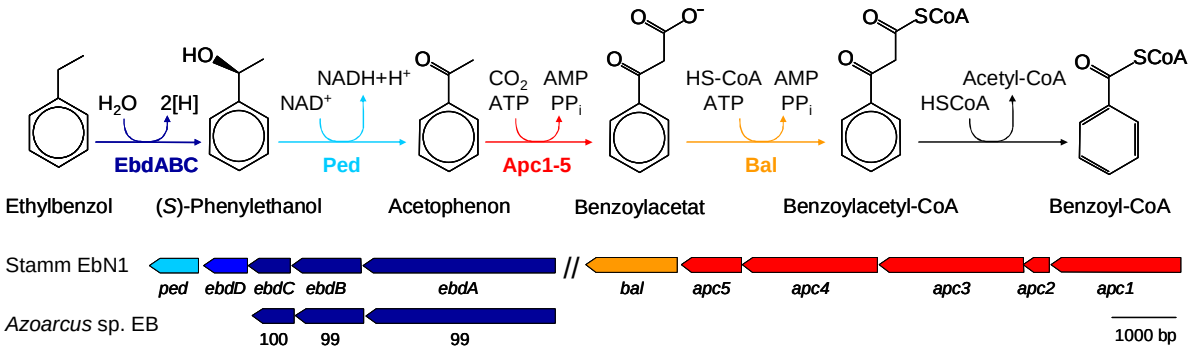

Abb. 3. Anaerobe Abbauwege für Ethylbenzol und die Organisation der kodierenden Gene in Stamm EbN1. Zum Vergleich sind Sequenzen eines anderen Stamms (Tab. 2)und deren Ähnlichkeit (%, BLASTP) aufgeführt. Enzym-Abkürzungen sind: EbdABC, Etylbenzol Dehydrogenase; Ped, (*S*)-1-Phenylethanol Dehydrogenase; Apc1-5, Acetophenon Carboxylase; Bal, Benzoylacetat-CoA Ligase.

2.2.3 Reduktive Dearomatisierung. Benzoat stellt für viele Aromaten-Verwerter ein hervorragendes Substrat dar. Nach Aufnahme in die Zelle wird es unter ATP-Verbrauch mit CoA aktiviert (Koch und Fuchs 1992; Harwood et al. 1999). Im Gegensatz zu den Dioxygenasen aerober Organismen verläuft die Ringspaltung in denitrifizierenden und phototrophen Organismen in mehreren O_2-unabhängigen Schritten (Abb. 4). Im ersten, als reduktive Dearomatisierung bezeichneten Schritt reduziert die Benzoyl-CoA Reduktase (BcrABCD) unter Hydrolyse von 2 ATP und Übertragung von 2 Elektronen Benzoyl-CoA zu

Cyclohexa-1,5-dien-carboxyl-CoA (Boll et al. 2002). Bioinformatische Untersuchungen legen nahe, daß bei Stamm EbN1 der weitere, *β*-Oxidations-artige Abbau zu Acetyl-CoA vermutlich über den von *T. aromatica* bekannten Weg verläuft (Harwood et al. 1999; Rabus et al. 2005).

Sulfat-reduzierende Bakterien wie *Desulfococcus multivorans* (Tabelle 2) oder der fermentierende *Syntrophus gentianae* erzielen aus der vollständigen Oxidation von Benzoat etwa 203 kJ/mol. Dies sind weniger als 10 % der Energie, die Nitrat-reduzierende Organismen aus der gleichen Oxidationsreaktion gewinnen (Thauer et al. 1977; Peters et al. 2004). Deshalb kann ein Mol Benzoat unter Sulfat-reduzierenden Bedingungen aus energetischen Gründen vermutlich nicht unter Verbrauch von 3 Mol ATP oxidiert werden. Neueste Untersuchungen deuten vielmehr auf Molybdat- und Selenocystein-haltige Enzyme, die nicht in phototrophen oder denitrifizierender Bakterien vorkommen, hin (Peters et al. 2004).

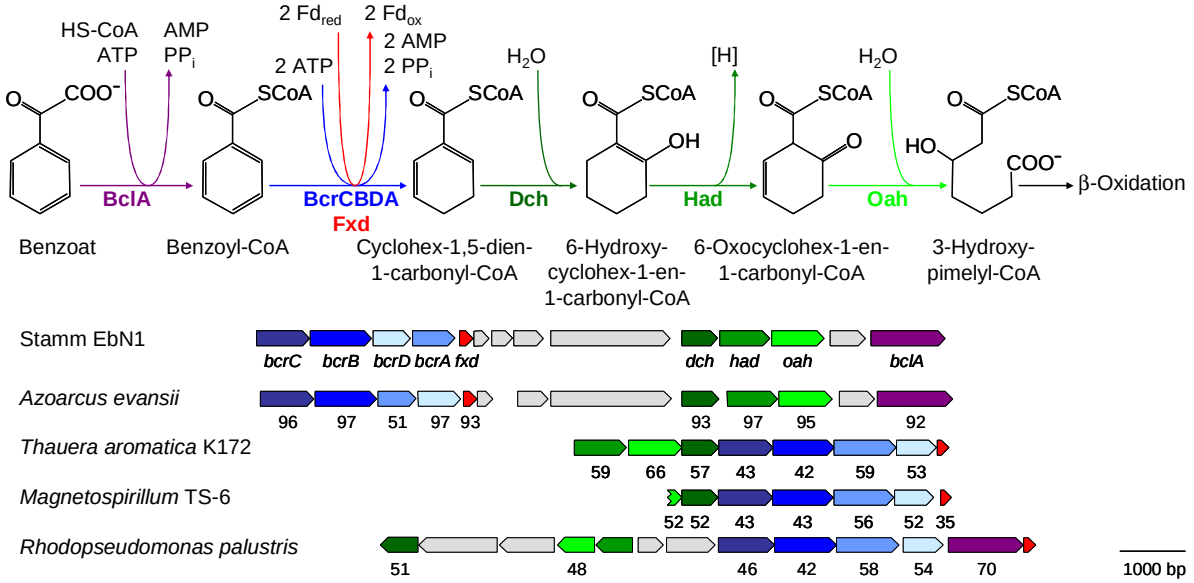

Abb. 4. Anaerober Benzoat-Abbauweg in Stamm EbN1 und die Organisation der kodierenden Gene. Zum Vergleich wird die Ähnlichkeit (%, BLASTP) zu Sequenzen anderer Stämme angegeben. Enzym-Abkürzungen sind: BclA, Benzoat-CoA Ligase; BcrCBDA, Benzoyl-CoA Reduktase; Fxd, Ferredoxin; Dch, Cyclohexa-1,5-dien-1-carbonyl-CoA Hydratase; Had, 6-Hydroxycyclohex-1-en-1-carbonyl-CoA Dehydrogenase; Oah, 6-Oxocyclohex-1-en-1-carbonyl-CoA Hydrolase.

2.3 Gene

Von einigen Organismen des anaeroben Toluol- und Ethylbenzolabbaus sind Teile der genetischen Sequenz der Abbauwege bekannt. Stamm EbN1 ist der erste anaerob Kohlenwasserstoff-abbauende Organismus, dessen Genom vollständig sequenziert wurde (Rabus et al. 2005). Auf einem zirkulären Chromosom (4.3 Mb) und zwei Plasmiden (0.21 und 0.22 Mb) befinden sich 4603 vorhergesagte Proteine. Darunter konnten ca. 150 Proteine zehn anaeroben und vier aeroben Abbauwegen aromatischer Verbindungen zugeordnet werden. Die Gene der meisten Abbauwege sind Operon-artig organisiert und haben eine zum Teil sehr hohe Ähnlichkeit zu Genen anderer Organismen (Tabelle 2, Abb. 2,3,4). Die große Anzahl mobiler genetischer Elemente legt nahe, daß das Genom des metabolisch vielseitigen Stamms EbN1 durch lateralen Gentransfer geprägt ist. Gene für über 30 2-Komponenten Regulator-Systeme sowie eine Reihe FNR-artiger Regulatoren sollten eine fein abgestufte Substrat- bzw. Redox-abhängige Regulation der Genexpression ermöglichen.

2.3.1 Toluol-Abbau. Mehr oder weniger vollständige Informationen zu Genen (und deren Organisation) des anaeroben Toluol-Abbaus liegen von acht Stämmen vor (Tab. 2, Abb. 3AB). Die Gene sind in zwei Operons organisiert: Das *bss*-Operon enthält die Benzylsuccinat Synthase kodierenden Gene, und das *bbs*-Operon die des weiteren Abbaus zu Benzoyl-CoA kodiert. Bei Stamm EbN1 und *G. metallireducens* sind die beiden Operons lediglich durch einen Abstand von 6,5 bzw. 4,6 kb voneinander getrennt.

Das *bss*-Operon enthält in Stamm EbN1 die Gene *bssDCABEFGH*. Sequenzvergleiche zwischen allen bekannten *bssABC* Genen (Benzylsuccinat-Synthase kodierend) zeigte eine große Ähnlichkeit der katalytischen BssA-Untereinheit, diese Ähnlichkeit korrelierte nicht vollständig mit der auf 16S-rDNA basierenden Phylogenie, z.B. Stamm EbN1 versus *Azoarcus* sp. T. Der Vergleich der *bssD*-Sequenz mit dem Aktivase-Gen der Pyruvat-Formiat Lyase (PFL) in *E. coli* unterstützt die Funktion von Bss als Adenosylmethionin-abhängig Radikal-generierendes Protein (Kube et al. 2004). Das stromabwärts liegende *bssE* gehört vermutlich zu einer Klasse von Chaperonen, die für die korrekte Zusammensetzung und Funktion von Enzymen benötigt wird. Orthologe der nächsten Gene, *bssFGH* sind z.T. aus *bss*-Operons anderer Stämme bekannt. Außer dem Genprodukt von *bssH* mit Ähnlichkeit zu Antibiotika-Resistenz assoziirten Transportern der ‚Major Facilitator Superfamily', konnte bisher keine Funktion zugeordnet werden. Da Toluol membrangängig ist, könnte es sich um ein Efflux-System für das in höheren Konzentrationen toxische Substrat handeln (Kube et al. 2004).

Das *bbs*-Operon enthält die Gene *bbsABCDEFJGH* für die Enzyme, die die Reaktionen von Benzylsuccinat zu Benzoyl-CoA katalysieren (Abb. 2). Die Ähnlichkeit zu den nahe verwandten Stämmen *T. aromatica* und *Azoarcus* sp. T ist größer als die zu *G. metallireducens*. Bis auf das in *T. aromatica* stromabwärts von *bbsH* liegende *bbsI* treten in Stamm EbN1 alle Gene in gleicher Reihenfolge auf. Die Funktion der Genprodukte von *bbsI* und des ausschließlich bei Stamm EbN1 identifizierten *bbsJ* ist bislang unbekannt. Das Produkt des in Stamm T an Stelle von *bbsJ* vorhandenen Gens weist keinerlei Ähnlichkeit zu BbsJ auf.

2.3.2 Ethylbenzol-Abbau. Informationen zur genetischen Sequenz und Organisation des Ethylbenzol-Abbauwegs liegen von den Stämmen EbN1 und EB1 vor (Tab. 2, Abb. 3) (Johnson et al. 2001; Rabus et al. 2002b). Von Stamm EbN1 ist bekannt, daß die Gene in zwei Operons organisiert sind. Das Operon für den oberen Ethylbenzol-Abbauweg (von Ethylbenzol bis Acetophenon) enthält die Gene *ebdABCDped*. Die beiden bekannten Sequenzen für die Ethylbenzol-Dehydrogenase *ebdABC* unterscheiden sich lediglich in vier Nukleotiden bzw. sechs Aminosäuren (Rabus et al. 2002b). Darüber hinaus besteht eine Ähnlichkeit von *ebdABCD* zu den Genen anderer Enzyme mit Molybdän-Cofaktor, wie der Selenat-Reduktase (*serABCD*), Nitrat-Reduktase (*narGHIJ*), Dimethylsulfoxid-Dehydrogenase (*dmsABCD*) und Trimethylamin-*N*-Oxid-Reduktase (*torABCD*) (Johnson et al. 2001; Rabus et al. 2002b). Wie in den genannten Genen ist auch in *ebdA* ein Leaderpeptid kodiert, um daß vollständig gefaltete Enzym mit Hilfe des Twin-Arginin-Translocation System in das Periplasma zu exportieren (Johnson et al. 2001; Rabus et al. 2002b). Der Einbau des Molybdän-Cofaktors bzw. die Reifung der Ethylbenzol-Dehydrogenase (EbdABC) wird vermutlich durch das „private“ Chaperon EbdD unterstützt.

Im Operon für den unteren Abbauweg von Ethylbenzol (von Acetophenon zu Benzoyl-CoA) befinden sich die Gene *apc12345bal* (Abb. 3). Das Gen für die Benzoylacetyl-CoA Thiolase ist bislang nicht bekannt. Die Produkte von *apc1234* ähneln einer Aceton-Carboxylase, das Genprodukt von *bal* gehört höchstwahrscheinlich zur Familie der AMP-bildenden CoA-Ligasen und gleicht der aeroben Phenylacetat-CoA-Ligase von *Azoarcus evansii* (Rabus et al. 2002b). Stromabwärts von *bal* befinden sich in dichter Folge fünf weitere vorhergesagte offene Leserahmen (Orfs). Die höchste Ähnlichkeit des zweiten Orfs besteht zu den bereits oben erwähnten Transportproteinen der ‚Mayor Facilitator’ oder ‚Resistance Nodulation’ Familie. Als Substrat kommen Ethylbenzol, Acetophenon oder das periplasmatisch gebildete (*S*)-1-Phenylethanol in Frage. Alle drei Verbindungen sind jedoch

nicht-ionisch. Im Falle von Ethylbenzol und Acetophenon könnte auch eine Export-Funktion zur Entgiftung in Frage kommen.

2.3.3 Benzoat Abbau. Die anaerobe Verwertung von Benzoat ist bei *Azoarcus evansii*, *Rhodopseudomonas palustris* und *Thauera aromatica* K172 gut verstanden (Harwood et al. 1999; Boll et al. 2002; Shinoda et al. 2005). Ein entsprechender Sequenzvergleich zwischen diesen Organismen und Stamm EbN1 bzw. *Magnetospirillum* sp. TS-6 ergibt eine fast gleiche Gen-Anordnung und eine hohe Ähnlichkeit von mehr als 90 % bezogen auf die Orthologe von *A. evansii* (Abb 2C, 3E). Die Gene der anderen Stämme sind denen von Stamm EbN1 nur noch zu durchschnittlich 50 % ähnlich und haben eine andere Reihenfolge.

2.4 Regulation

Bereits früh wurde an *Desulfobacula toluolica* Tol2 beobachtet, daß ein Substratwechsel von Benzoat zu Toluol zu einer anfänglichen Verzögerung des Wachstums führt. Als Ursache wurde die Induktionszeit für die Bildung der Proteine des Toluolabbaus angenommen (Rabus et al. 1993). Die Substrat-spezifische Bildung von Proteinen wurde bei den Stämmen *T. aromatica* K172 (Toluol und andere aromatische Verbindungen) und EbN1 (Toluol, Ethylbenzol und Acetophenon) festgestellt (Heider et al. 1998a; Champion et al. 1999). Gleiches gilt für die Expression der *bss*-Gene in *T. aromatica* T1 und K172 (Coschigano 2000; Hermuth et al. 2002), die vermutlich durch das 2-Komponenten Regulatorsystem TdiSR aktiviert wird. Die *tdiSR*-Gene wurden in unmittelbarer Nachbarschaft von den *bss*-Genen der Stämme *T. aromatica* T1 und K172 sowie der *Azoarcus*-ähnlichen Stämme T und EbN1 gefunden (Coschigano und Young 1997; Leuthner und Heider 1998; Achong et al. 2001; Kube et al. 2004). Sequenzanalyse des TdiS Sensors ergab, daß das Protein über zwei PAS-Domänen verfügt, für die Stimuli wie *p*-Aminosalicylat, Redoxpotential, Licht und Kohlenwasserstoffe beschrieben wurden (Leuthner und Heider 1998). Diese ähneln denen des TodS-Sensors. Das 2-Komponenten System TodSR reguliert die Expression des chromosomalen *tod*-Operons in den Stämmen *P. putida* F1 und DOT-T1E; Das *tod*-Operon enthält alle Gene für den aeroben Abbau von Toluol zu Pyruvat und Acetyl-CoA (Lau et al. 1997). Aufgrund der großen Ähnlichkeit der Promotor-Regionen der *bss* und *bbs*-Operons und dem Unvermögen, Benzylsuccinat als Substrat zu verwerten, wurde eine koordinierte Regulation der Operons vorgeschlagen (Kube et al. 2004).

Wegen der unterschiedlichen Proteinmuster bei Wachstum mit Ethylbenzol bzw. Acetophenon wurde eine sequentielle Regulation des Ethylbenzol-Abbauwegs postuliert (Champion et al. 1999). Bioinformatisch wurden in Stamm EbN1 in der Nähe der beiden katabolen Operons zwei 2-Komponenten-Systeme identifiziert und *tcs1/tcr1* bzw. *tcs2/tcr2* genannt (Rabus et al. 2002b). Tcs2 ähnelt im Sequenzvergleich TdiS, wobei die erste PAS-Domäne und die das Regulatorprotein aktivierende Histidin-Kinase-Domäne eine deutlich größere Ähnlichkeit zueinander haben als die zweite PAS-Domäne (Kube et al. 2004). Tcs1 zeigt hohe Ähnlichkeit zu entsprechenden Sensor-Komponenten in pflanzenpathogenen Stämmen, von denen in einem Fall die Erkennung von *p*-Hydroxyacetophenon gezeigt wurde. Daher reguliert Tcs2/Tcr2 in Antwort auf Ethylbenzol die Expression des oberen Ethylbenzol-Operons (*ebd/ped*) und Tcs1/Tcr1 in Antwort auf Acetophenon das untere Operon (*apc/bal*). Auf ähnliche Weise ist der plasmidkodierte (pWW0) aerobe Abbauweg für Toluol bzw. Xylol in *P. putida* mt-2 reguliert. Der Regulator XylR steuert den oberen Teil des Abbauwegs von Toluol zu Benzoat und XylS den *meta*-Weg für den weiteren Abbau zu Acetaldehyd und Pyruvat (Ramos et al. 1997b).

Der anaerobe Benzoat-Abbau in Stamm EbN1 wird wahrscheinlich durch den Repressor BzdR reguliert, der sich wie in *Azoarcus* sp. CIB (87 % Ähnlichkeit) unmittelbar vor dem Benzoat-Operon befindet (Barragán et al. 2004). BzdR gehört zur Familie der HTH-XTR-Regulatoren und ermöglicht in Anwesenheit von Benzoyl-CoA die Expression des Benzoat-Operons (Barragán et al. 2005). Im Gegensatz dazu wird in *Rhodopseudomonas palustris* der anaerobe Benzoat-Abbau durch BadR (Benzoat-abhängig) und AadR (Fnr-artiger Regulator für Anaerobiosis) aktiviert (Egland und Harwood 1999). Da Stamm EbN1 unter oxischen Bedingungen Benzoat mit Hilfe des O_2-abhängigen Benzoyl-CoA Oxidationskomplexes (Box) metabolisiert und BoxR ein Regulator mit großer Sequenzähnlichkeit (69 %) zu BzdR ist, wird es vermutlich auch in Stamm EbN1 noch einen weiteren, Redox-abhängigen Regulator des Benzoatabbaus geben (Gescher et al. 2002; Rabus et al. 2005).

2.5 Bedeutung in der Umwelt

Die generelle Bedeutung der zuvor beschriebenen mikrobiellen Abbauprozesse wird bereits deutlich durch die bislang bekannte Vielfalt der isolierten Stämme sowie ihrer Habitate, geographischen Verbreitung und dem großen Spektrum anaerob verwerteter Kohlenwasserstoffe. Darüber hinaus gibt es eine stetig wachsende Anzahl an Studien, in denen Fumarat-Addukte von Toluol, Xylol-Isomeren, *n*-Alkanen (z.B. Hexan), Alicyclen (z.B. Ethylcyclopentan) aber

auch höher substituierte Aromaten oder polycyclische Aromaten (z.B. Methylnaphthalin) in kontaminierten Boden- oder Wasserproben nachgewiesen wurden (Gieg et al. 1999; Beller 2000; Elshahed et al. 2001; Gieg und Suflita 2002; Martus und Püttmann 2003; Rios-Hernandez et al. 2003; Griebler et al. 2004). Diese Indizien werden dadurch bekräftigt, dass

a) bisher keine weiteren natürlichen oder anthropogenen Quellen für die Aryl- oder Alkylsuccinate bekannt sind (Beller 2000),
b) die Konzentration entsprechender Kohlenwasserstoffe mit zunehmender Entfernung vom Schadensherd ebenso abnimmt, wie die Konzentration mikrobieller Elektronen-akzeptoren (Gieg et al. 1999; Beller 2000; Martus und Püttmann 2003),
c) im noch verbliebenen Anteil eines Kohlenwasserstoffs im Vergleich zum Ausgangs-materials das Verhältnis von ^{13}C zu ^{12}C zunimmt (Wilkes et al. 2000; Meckenstock et al. 2004; Morasch et al. 2004a).

Das Vorkommen Kohlenwasserstoff-abbauender Stämme im natürlichen Habitat kann direkt mittels Hybridisierung der 16S rRNA mit Oligonucleotid-Sonden untersucht werden. Mit diesem Verfahren wurde festgestellt, daß in einem mit Toluol und Nitrat versetzten Aquifermikrokosmos *Azoarcus tolulyticus* einen Anteil von ca. 1 bis 2 % (Hess et al. 1997) bzw. *Azoarcus* spp. 6 % (Pelz et al. 2001) der Gesamtpopulation einnimmt. Von größerer Aussagekraft bezüglich der Aktivität sind Sonden für katabole Gene wie z.B. *bssA*, das die katalytische Untereinheit der Benzylsuccinat Synthase kodiert. So wurde in Mikrokosmen mit Proben Treibstoff-verunreinigter Aquifere festgestellt, daß nach Toluol-Zugabe die Anzahl der Bakterien, in deren Genom der Benzylsuccinat-Abbauweg kodiert war, 100- bis 1000-fach anstieg (Beller et al. 2002).

3. Ziele der vorliegenden Arbeit

Am Beginn dieser Arbeit stand die Frage, wie sich Mikroorganismen der freien Umwelt an variable Bedingungen anpassen. Diese weitläufige Problematik wurde beispielhaft an der Regulation des anaeroben Alkylbenzolabbaus bzw. der physiologischen Anpassung an diese toxischen Verbindungen im denitrifizierenden Stamm EbN1 untersucht. Beide Fragestellungen wurden mit einer Kombination aus physiologischen und proteomischen Ansätzen verfolgt.

Zur Substrat-abhängigen Regulation der Abbauwege. Zunächst sollen auf der Basis von Wachstumsversuchen die prinzipielle Induktion von Abbauleistungen nachgewiesen werden. Grundlage dafür bilden Kulturen von Stamm EbN1, die über mehrere Passagen an das anaerobe Wachstum mit Benzoat angepasst sind. Nach Wechsel auf die Substrate Toluol, Ethylbenzol, einem Gemisch der beiden Alkylbenzole, sowie Benzoat soll dann hochauflösend das Wachstum und der Verbrauch von organischem Substrat und Nitrat verfolgt werden. In einem zweiten Schritt sollen die beobachteten Regulationsprozesse auf proteomischer Ebene weitergehend untersucht werden. Dabei wird die 2-dimensionale Differenz-Gelelektrophorese (2D DIGE) eingesetzt, um die Substrat-abhängige Bildung Abbauweg-spezifischer Proteine quantitativ zu erfassen und Proteine für die nachfolgende massenspektrometrische Identifizierung zu gewinnen.

Zur Toxizität der Alkylbenzole. Die Anpassung von Stamm EbN1 an die Toxizität von Toluol bzw. Ethylbenzol soll unter Bedingungen induzierter bzw. nicht induzierter Abbauleistung untersucht werden. Im ersten Fall werden Wachstumsverhalten, Nitratverbrauch, Veränderung des Proteinmusters und der Phospholipide bei Wachstum mit sublethalen, d.h. das Wachstum nicht vollständig inhibierenden Konzentrationen der Alkylbenzolsubstrate untersucht. Im zweiten Fall werden Kulturen, die an das anaerobe Wachstum mit Succinat angepaßt sind, während des linearen Wachstums plötzlich sublethalen Konzentrationen von Toluol bzw. Ethylbenzol ausgesetzt. Auch hier soll der Einfluß auf Wachstumsparameter und Proteome untersucht werden.

B Ergebnisse und Diskussion

1. Substrat-abhängige Regulation der anaeroben Abbauwege für Toluol und Ethylbenzol

Zunächst wurden Kulturen von Stamm EbN1 über 5 Passagen an das anaerobe Wachstum mit jeweils einem der folgenden Substrate angepaßt: Toluol, Ethylbenzol, Toluol + Ethylbenzol und Benzoat. Mit diesen vier verschieden-adaptierten Kulturen wurden dann jeweils neue Folgekulturen inokuliert, die als Substrat entweder Toluol, Ethylbenzol, Toluol + Ethylbenzol oder Benzoat enthielten. Dabei wurden die Alkylbenzole verdünnt in einer inerten Trägerphase zugegeben. Auf diese Weise lagen (i) nicht toxische Konzentrationen und (ii) ausreichende Gesamtmengen für eine vollständige Reduktion des Nitrats vor.

1.1 Physiologische Befunde zur Regulation

Die Verwertung der Alkylbenzole durch die unterschiedlich adaptierten Zellen ist in Abb. 5A-D dargestellt. **Benzoat-adaptierte Zellen** (Abb. 5A) konnten die Alkylbenzole nicht sofort verwerten, sondern erst nach ca. 15 h. Unabhängig davon, ob die Alkylbenzole als Einzelsubstrate oder im Gemisch zugegeben wurden, begann die Verwertung von Toluol und Ethylbenzol gleichzeitig und verlief mit ähnlicher Rate. Keines der beiden Alkylbenzole unterdrückte also die Induktion der Abbauleistung für das jeweils andere.

Bei **Toluol-adaptierten Zellen** (Abb. 5B) begann die Verwertung von Toluol als Einzelsubstrat oder im Gemisch sofort nach Versuchsbeginn. Die Induktionszeit der Abbauleistung für Ethylbenzol war im Fall der Einzelsubstrat-Zugabe etwas länger und im Fall der Zugabe des Alkylbenzol-Gemisches ähnlich der von Benzoat-adaptierten Zellen. Die Induktion der Abbauleistung für Ethylbenzol erfolgte parallel zur fortschreitenden Toluolverwertung. Ähnliches gilt für **Ethylbenzol-adaptierte Zellen** (Abb. 5C), wobei die Induktion der Abbauleistung für Toluol deutlich länger dauerte und Toluol erst nach ca. 55 h mit hoher Rate verbraucht wird. In Ansätzen mit dem Alkylbenzol-Gemisch war die Induktionszeit ähnlich lange wie in Benzoat-adaptierten Zellen und beide Alkylbenzole wurden gleichzeitig verwertet. Bei **Alkylbenzol-Gemisch adaptierten Zellen** wurden Toluol und Ethylbenzol schnell und gleichzeitig verbraucht (Abb. 5D).

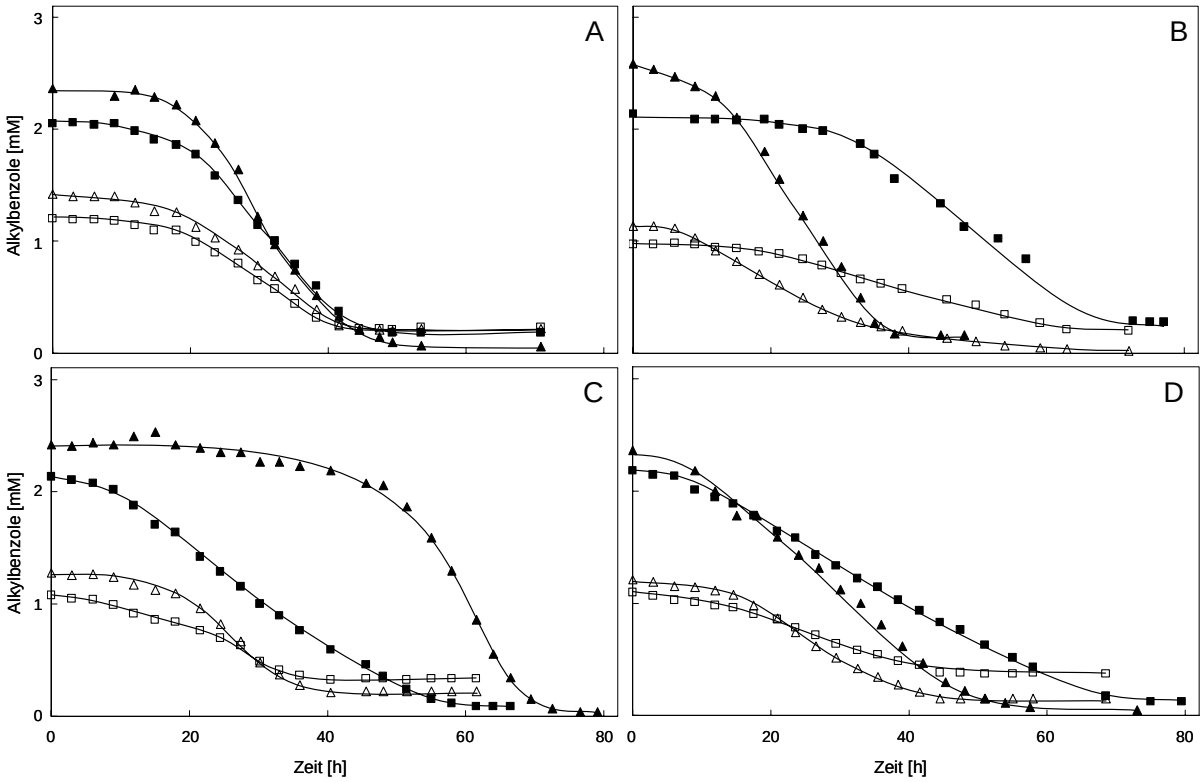

Abb. 5. Wachstum von unterschiedlich adaptierten Zellen des Stamms EbN1 auf Toluol und Ethylbenzol unter Nitrat-reduzierenden Bedingungen. Anaerob adaptiert an (A) Benzoat, (B) Toluol, (C) Ethylbenzol, (D) Toluol und Ethylbenzol in äquimolarer Mischung. Zeichen: ▲, Toluol als Einzelsubstrat; ■, Ethylbenzol als Einzelsubstrat; △, Toluol und □, Ethylbenzol als Substratgemisch.

Die gleichzeitige Verwertung von Toluol und Ethylbenzol durch die vier unterschiedlich adaptierten Kulturen bedeutet, daß Stamm EbN1 die entsprechenden Abbauleistungen gleichzeitig ausbilden kann. Außerdem zeigt dieses Ergebnis, daß keines der beiden Alkylbenzole ein präferentielles Substrat darstellt und es keine Katabolit-repressionsartige Differenzierung zwischen den beiden Alkylbenzolen gibt. In zusätzlichen Experimenten mit 5-fach höheren Konzentrationen von Toluol und Ethylbenzol wurden prinzipiell gleiche Ergebnisse erzielt. Über Repression durch andere Substrate, wie z.B. für Succinat oder Glukose beim aeroben Kohlenwasserstoffabbau beobachtet (Duetz et al. 1996; Cases et al. 1999), kann auf Basis dieser Experimente keine Aussage gemacht werden. Wie schon zuvor beschrieben (Rabus und Widdel 1995a) verlief das Wachstum bei allen eingesetzten Alkylbenzol-Konzentrationen nur anfangs exponentiell, später aber eher linear. Mit Benzoat oder Acetat wurde hingegen exponentielles Wachstum beobachtet. Die Diffusion der Alkylbenzole ist ein Versuchs-immanentes Kriterium. Sie bewirkt für die Alkylbenzole ein Verteilungsgleich-gewicht zwischen mehreren unterschiedliche Kompartimenten: Trägerphase, Medium, Periplasmamembran, Periplasma, Cytoplasmamembran und Cytoplasma. Die Alkylbenzole stehen in einem Fließgleichgewicht zwischen diesen Kompartimenten, sodaß als Ursache für

das lineare Wachstum eine Diffusions-Limitierung der Substratversorgung zu erwarten ist. Das Fließgleichgewicht und die Konzentration in den Kompartimenten wird in Teil II B.2.1 erörtert.

1.2 Proteomische Untersuchungen

An Toluol, Ethylbenzol, dem Alkylbenzol-Gemisch, Benzoat und Acetophenon-adaptierte Zellen von Stamm EbN1 wurden in der Mitte der linearen Wachstumsphase geerntet. Diese Proben dienten dazu, die Regulation auf RNA-Ebene mittels real-time-RT-PCR bzw. DNA-Microarray und auf Protein-Ebene mittels 2D-DIGE zu untersuchen. Benzoat-adaptierte Zellen dienten als Referenzzustand, während alle anderen Substratbedingungen die Testzustände darstellten. In diesem Abschnitt werden im wesentlichen Ergebnisse der 2-DE erörtert. Die Ergebnisse der Transkriptom-Analyse sind in den entsprechenden Veröffentlichung (Teil II B.1, B.2) dargestellt.

Die Extrakte löslicher Proteine wurden für die 2D-DIGE-Analyse mit Cy-Dyes markiert. Dabei wird bei durchschnittlich 2 % jeder Proteinspezies ein Fluorophor kovalent an der ε-Aminogruppe eines Lysins pro Polypeptidkette gebunden. Es handelt sich um ein sogenanntes „Minimal-Labeling“, da pro Proteinspezies nur 2 % des Proteins markiert werden. Die eingesetzten Fluoreszenzfarbstoffe besitzen diskrete, nicht überlappende Absorptions- und Emissionsmaxima. Daher können verschiedene Proteinextrakte (in der Regel Test- und Referenzzustand) in ein und demselben Gel separiert werden. Dadurch wird die Gel-zu-Gel Variation deutlich reduziert. Ein zusätzlicher interner Standard, der alle zu untersuchenden Proteinextrakte enthält, dient dazu, einen genauen Abgleich aller Spot-Karten über alle Gele zu erreichen. Insgesamt erlaubt die 2D DIGE Methode relative Unterschiede in der Proteinhäufigkeit mit hoher statistischer Sicherheit zu bestimmen. Um die Untereinheiten der Ethylbenzol-Dehydrogenase (isoelektrischer Punkt (IP) zwischen pH 7.4 und pH 9.2) elektrophoretisch zu erfassen, wurden zusätzlich zu den üblicherweise verwendeten Gelen mit einem pH-Gradienten von 4 – 7 solche mit 3 – 10 eingesetzt. Von den über 150 Proteinspots mit mehr als 2.5-fach erhöhter Häufigkeit wurden die nach Coomassie-Färbung klar erkennbaren Spots manuell ausgestochen und mit MALDI-TOF-MS analysiert. Alle identifizierten Proteine, die mit dem anaeroben Alkylbenzolabbau in Verbindung gebracht werden konnten, sind in Tabelle 3 aufgelistet. Diese Proteine wurden jeweils mindestens drei mal aus unabhängigen Proben mit einem durchschnittlichen MASCOT-Score von mindestens 100 identifiziert.

Die Veränderung der relativen Häufigkeit von zehn, im *bss*- oder *bbs*-Operon kodierten Proteinen wird in Tabelle 3 gezeigt, wobei durch Identifizierung des stromabwärts von *bssE* kodierten BssG das *bss*-Operon um drei Gene erweitert werden konnte (Kube et al. 2004) und somit die Beteiligung von 17 Genprodukten für den anaeroben Toluol-Abbau nahe liegt. BssG konnte nicht in DIGE-Gelen, sondern ausschließlich auf Coomassie- und Silber-gefärbten Gelen als gegenüber mit Benzoat gewachsenen Zellen differentiell reguliert erfaßt werden. Eine mögliche Erklärung könnte sein, daß das Protein nur ein markierbares Lysin enthält (Tab. 3). In den **Toluol-adaptierten Zellen** wurde eine 4- (BbsG) bis 155-fache (BbsH) Erhöhung der relativen Häufigkeit der im *bss*- oder *bbs*-Operon kodierten Proteine festgestellt. Darüber hinaus wurde auch eine bis zu 40-fache (EbdC) Erhöhung der relativen Häufigkeit von Proteinen des Ethylbenzol- und Acetophenon-Abbauwegs in den Toluol-adaptierten Zellen festgestellt.

Von den 11 für die anaerobe Ethylbenzolverwertung vorhergesagten Genprodukten (Rabus et al. 2002b) wurden 7 mit einer 4- bis 242- fach erhöhten Häufigkeit in **Ethylbenzol-adaptierten Zellen** identifiziert. Eine Ko-Induktion der *bss*- bzw. *bbs*-Genprodukte wurde nicht beobachtet (Häufigkeitsveränderung <|2|).

Bei **Alkylbenzol-Gemisch adaptierten Zellen** waren alle bisher identifizierten Proteine der beiden Abbauwege 3- bis 138-fach häufiger als im Referenzzustand (Benzoat-adaptierte Zellen). In **Acetophenon-adaptierten Zellen** waren ausschließlich die für den Acetophenon-Abbau spezifischen Proteine (Apc/Bal) 6- bis 104- fach hochreguliert, inklusive Orf 84, welches am stromabwärtigen Ende des *apc/bal* Genclusters kodiert ist. Mit Ausnahme von Orf 68 (3-fach reguliert) war die Häufigkeit aller anderen Proteine der beiden Alkylbenzol-Abbauwege nicht signifikant verändert. Interessanterweise zeigen die identifizierten Untereinheiten der Acetophenon-Carboxylase aus Acetophenon-adaptierten Zellen ein etwas verändertes elektrophoretisches Trennverhalten (anderer isoelektrischer Punkt und anderes Molekulargewicht) gegenüber den anderen Substratbedingungen. Die massenspektrometrische Analyse ergab keine Erklärung (z.B. posttranslationale Modifikation) für diesen Befund. In einer früheren Arbeit wurden mit einem anderen Prinzip der isoelektrischen Fokussierung ein ähnliches Trennverhalten beobachtet (Champion et al. 1999).

Tabelle 3. Mittels 2D-DIGE bestimmte Veränderung der relativen Häufigkeit von massenspektrometrisch identifizierten Proteinen, die bei Stamm EbN1 am anaeroben Alkylbenzolabbau beteiligt sind.

Protein[a]	Anzahl der Lysinreste	Güte der MS-Identifizierung[b]	-fache Veränderung in Zellen angepaßt an[c]			
			Toluol	Ethylbenzol	Toluol + Ethylbenzol	Acetophenon
Toluol-Abbauweg						
BssD	9	226	9	1	9	1
BssE	13	383	14	1	13	1
BssG	1	163	-[d]	-[d]	-[d]	-[d]
BbsF	10	180	9	1	7	1
BbsG	18	176	4	1	3	1
BbsH	13	278	155[e]	2[e]	138[e]	1
BbsC	9	149	77	-2	65	1
BbsD	9	280	34	2	27	1
BbsA	13	231	27	1	24	1
BbsB	15	117	25	-2	19	1
EbA1936	15	271	46	1	45	1
EbA1932	26	246	54	1	44	1
Oberer Ethylbenzol-Abbauweg						
C1A68	37	222	163[e]	281[e]	63[e]	3[e]
EbdC	16	114	40	242	92	1
EbdD	7	391	3	14	8	1
Unterer Ethylbenzol-Abbauweg						
Apc1	20	423	3	8	5	72[e]
Apc3	30	188	4	13	9	36
Apc4	30	466	13	88	32	104[e]
Apc5	5	249	5	34	17	6
Bal	8	425	2	4	3	11
C1A84	15	216	6	28	15	9

[a] Reihenfolge wie im Abbauweg (Abb. 2,3).
[b] Mittelwerte von mind. 3 unabhängigen Identifizierungen, je größer die Zahl des MASCOT-Scores, desto höher die Güte.
[c] Häufigkeit als Mittelwerte von 5 Gelen im Vergleich zu Benzoat-angepaßten Zellen
[d] Häufigkeit nicht bestimmbar, da Protein in Coomassie- und Silber-gefärbten Gelen auftrat, aber nicht in DIGE Gelen.
[e] Mittelwert, Protein trat in 2 Punkten mit unterschiedlichem isoelektrischen Punkt auf.

Die Ergebnisse der real-time RT-PCR und des DNA-Microarrays stimmen mit den vorgestellten proteomischen Ergebnissen überein. Insgesamt erlaubte der integrative Ansatz von physiologischen Anpassungsexperimenten und funktionaler Genomik (RNA- und Protein-Ebene) das bioinformatisch vorhergesagte Regulationsmodell für die beiden Alkylbenzol-abbauwege zu untermauern: Koordinierte versus sequentielle Regulation der Operons des Toluol- bzw. Ethylbenzolabbaus. Die Ethylbenzol- bzw. Acetophenon-abhängige Regulation des oberen bzw. unteren Teils des Ethylbenzol-Abbauwegs ist durchaus sinnvoll, wenn man bedenkt, daß das Pflanzenmetabolit Acetophenon im natürlichen Boden- oder Sediment-Habitat verfügbar ist.

1.3 Selektivität der Regulatorsysteme

Die Induktion der Operons des Ethylbenzol-Abbauwegs durch Toluol könnte auf eine reduzierte Substratspezifität des Tcs2-Sensors für Ethylbenzol hindeuten. Die Unterschiede in der zweiten PAS-Domäne von TdiS bzw. Tcs2 wurden bereits als mögliche Ursache für die Toluol- bzw. Ethylbenzol-Spezifität des jeweiligen Sensors diskutiert (Kube et al. 2004). Es wäre allerdings denkbar, daß das kleinere Toluol-Molekül in die Bindungstasche von Tcs2 passt, während dies umgekehrt für das größere Ethylbenzolmolekül beim TdiS-Sensor nicht möglich ist. Ein ähnlicher Effekt wäre auch für Toluol und den Acetophenon-spezifischen Tcs1-Sensor vorstellbar. Eine mögliche Kreuzreaktivität von TdiS mit Tcr2 und Tcr1 muß jedoch auch in Betracht gezogen werden. In *Azoarcus* sp. T1 wurde kürzlich die Induktion des *bss*-Operons durch das als Wachstumssubstrat nicht verwertbare Benzylsuccinat beobachtet (Coschigano und Bishop 2004). Im Gegensatz dazu konnte unter identischen Versuchsbedingungen in Stamm EbN1 weder eine erhöhte Expression von *bssA* noch Veränderung der relativen Häufigkeit der identifizierten Bss- und Bbs-Proteine mittels real-time RT-PCR bzw. 2DE festgestellt werden. Das etwas promiske Verhalten des Sensors für den Ethylbenzol-Abbauweg passt zu der physiologischen Beobachtung, daß es nach Wechsel des Substrats von Toluol zu Ethylbenzol ca. 20 h bis zum Abbau mit hoher Rate dauert. Bei umgekehrtem Substratwechsel dauert es jedoch deutlich mehr als 30 h.

1.4 Weitere Alkylbenzol-regulierte Proteine

Neben dem erstmaligen Nachweis der Genprodukte von *bssE* und *bssG* konnten mittels DNA-Microarray und 2DE weitere Substrat-spezifisch exprimierte Gene mit den Abbauwegen von Toluol, Ethylbenzol und Acetophenon in Verbindung gebracht werden. Die Expression aller bei Stamm EbN1 zwischen *bss*- und *bbs*-Operon liegenden Gene (z.B. *orf200*) war ähnlich stark hochreguliert, wie die *bss* und *bbs* Gene. Gleichermassen sind die Gene stromabwärts des *apc/bal*-Clusters (z.B. *orf90*) sowie die das *ebd/ped*-Operon flankierenden Gene orf57 und *orf68* bei Wachstum mit Ethylbenzol bzw. Acetophenon hochreguliert

Durch zweidimensionale Gelelektrophorese konnten mit EbA1936 (46-fach) und EbA1932 (54-fach) zwei weitere Toluol-abhängig regulierte Proteine unbekannter Funktion identifiziert werden. Die kodierenden Gene gehören möglicherweise mit zu einem weiteren Toluol-regulierten Operon, welches ca. 8 kbp stromaufwärts vom *bbs*-Operon liegt. Die Ko-Regulation dieses neuen Operons mit den bisher bekannten *bss*- und *bbs*- Operons wird auch

durch eine gemeinsame Konsensussequenz in den stromaufwärts der Promotoren gelegenen Bereichen aller drei Operons unterstützt. Orthologe von EbA1936 und EbA1932 sind in *T. aromatica* K172 direkt stromaufwärts von *tdiSR* kodiert. Die erstaunlichen Mengen von EbA1932 und EbA1936 legen eine Funktion im anaeroben Toluolabbau nahe. Eine irrtümliche Expression (z.B. durch zufällige Insertion des Promotor-Konsensus) kann zum gegenwärtigen Zeitpunkt nicht ausgeschlossen werden.

Zusätzlich zu den biochemisch bereits charakterisierten bzw. bioinformatisch vorhergesagten Proteinen des Ethylbenzol-Abbaus wurden die mit 281- bzw. 28-fach erhöhter Häufigkeit auftretenden Proteine Orf68 und Orf84 gefunden. Das Gen *orf68* befindet sich 272 bp stromaufwärts von *ebdA* und gehört trotz Ko-Expression auf Grund des relativ großen Genabstands vermutlich nicht zum *ebd/ped* Operon.

2. Molekular-physiologische Antwort auf Lösungsmittelstress

Unbelastete oder kontaminierte Böden und Süßwassersedimente sind die natürlichen Habitate der anaerob Kohlenwasserstoff-abbauenden Stämme der *Azoarcus*/*Thauera* Verwandschaftsgruppe. Durch Schwankungen des Wasserstands, veränderte Strömungsverhältnisse oder variablen Eintrag kann es zu Veränderungen der Kohlenwasserstoffkonzentration am Standort kommen. Die Anpassung von Stamm EbN1 an veränderte Toluol- und Ethylbenzolkonzentration wurde unter Bedingungen untersucht, bei denen die entsprechenden Abbauleistungen ausgeprägt (B.2.1) bzw. nicht vorhanden waren (B.2.2).

2.1 Reaktion auf sublethale Konzentrationen der Alkylbenzol-Wachstumssubstrate

Stamm EbN1 wird routinemäßig mit 2 % Toluol bzw. 5 % Ethylbenzol (jeweils [v/v] in Heptamethylnonan, HMN) kultiviert. An diese Bedingungen angepaßte Kulturen dienten als Inokulum für Ansätze mit verringerter, normaler und erhöhter Alkylbenzolkonzentration. Bei Letzterer ist Wachstum grade noch möglich, weshalb sie hier als sublethal definiert wird. So wurden Mischungen aus 0.5, 2 und 6 % Toluol bzw. 2, 5 und 8 % Ethylbenzol in HMN eingesetzt, die zu Gleichgewichtskonzentrationen von 70, 240 und 740 µM Toluol und 80, 210 und 315 µM Ethylbenzol im Medium führten.

2.1.1 Physiologische Effekte

Bei Toluolkonzentrationen von 70 bzw. 240 µM Toluol zeigte sich ein ähnliches Wachstumsverhalten. In Gegenwart von 740 µM Toluol war das Wachstum hingegen deutlich verlangsamt, auch wenn schließlich eine höhere finale optische Dichte erreicht wurde.

Der Verlauf der **Toluol**-Konzentration während des Inkubationszeitraums war bei den verschiedenen Ansätzen unterschiedlich. Im Ansatz mit 70 µM Toluol sank die Konzentration während des linearen Wachstums unter die Nachweisgrenze von 2.2 µM und erreichte mit Eintritt in die stationäre Phase 30 µM. In Kulturen mit 240 µM fiel die Konzentration temporär auf 160 µM, bei Wachstum mit 740 µM Toluol war keine signifikante Veränderung festzustellen.

Unterschiede waren ebenfalls beim Verbrauch von Nitrat bzw. der intermediären Bildung von Nitrit zu beobachten. In den Ansätzen mit 70 bzw. 240 µM Toluol wurde Nitrat relativ schnell verbraucht und 3.8 bzw. 3 mM Nitrit intermediär ausgeschieden und schließlich vollständig verbraucht. Beim Ansatz mit 740 µM wurde Nitrat hingegen wesentlich langsamer verbraucht, lediglich max. 1.5 mM Nitrit ausgeschieden und letzteres nicht vollständig verbraucht.

Mit ansteigender **Ethylbenzol**-Konzentration verringerten sich sowohl die Wachstumsrate als auch die maximal erreichte optische Dichte deutlich. Die Ethylbenzolkonzentration fiel in den Ansätzen mit 80 bzw. 210 µM zwischenzeitlich (während des linearen Wachstums) auf 10 bzw. 170 µM, wohingegen sie im sublethalen Ansatz unverändert bei 315 µM blieb.

In ähnlicher Weise verringerten sich die Raten des Nitratverbrauchs und die Konzentration des intermediär gebildeten Nitrits (von 5.9 über 4.5 auf 2.9 mM). Im Unterschied zum sublethalen Ansatz mit Toluol war bei 315 µM Ethylbenzol Nitrit von Beginn an nachweisbar, akkumulierte mit dem Beginn einer zweiten Phase schnelleren Wachstums zu nennenswerten Mengen, führte jedoch nach vollständigem Nitrat-Verbrauch wie bei 740 µM Toluol nicht zu Wachstum. Die Ursache der bevorzugten Nitrat-Reduktion liegt vermutlich in der effizienteren Energiekonservierung der cytoplasmatisch/membrangebundenen Lokalisation der Nitrat-Reduktase (NarGHI) gegenüber der periplasmatischen Nitrit-Reduktase NirS (Nicholls 1992). Entsprechend zeigte *Paracoccus denitrificans* in Gegenwart von Pestiziden ein verändertes Ausmaß der Nitritbildung (keine- bzw. erhöhte Bildung; Saetz et al. 2003).

Die Berechnung der Alkylbenzolkonzentration in der Membran wurde durch die von Sikkema et. al (1994) beobachtete Korrelation des logarithmierten Verteilungsgleichgewichts von Membran und Puffer zum log K_{OW} ermöglicht. Nach Einsetzen der log K_{OW} von 2.69 und 3.15 für Toluol bzw. Ethylbenzol ergeben sich log K_{MW} von 1.97 bzw. 2.42. Die in den sublethalen Ansätzen gemessenen Mediumkonzentrationen von 740 µM Toluol und 315 µM Ethylbenzol ergeben folglich eine Membrankonzentration von 69 mM Toluol und 83 mM Ethylbenzol. Bei der Übertragung dieser Berechnung auf die *in vivo* Situation in Organismen muß jedoch beachtet werden, daß Efflux-Systeme die Membrankonzentrationen reduzieren können. Im Genom von Stamm EbN1 sind mehrere dieser Systeme vorhergesagt. Außerdem muß im aktuellen Experiment mit Stamm EbN1 der quantitative, katabole Alkylbenzol-verbrauch und die biochemischen Besonderheiten der involvierten Abbauwege berücksichtigt werden: Die Ethylbenzol-Dehydrogenase ist periplasmatisch lokalisiert, verbraucht also einen

Teil des Ethylbenzols, bevor es die Cytoplasmamembran erreicht hat. Im Gegensatz dazu befindet sich die Benzylsuccinat-Synthase im Cytoplasma, kann Toluol also erst verwerten, nachdem es in die Membran aufgenommen wurde. Auf der anderen Seite haben die Intermediate des Toluol-Abbaus ein weit geringeres toxisches Potential als 1-Phenylethanol oder Acetophenon, die ersten beiden Intermediate des Ethylbenzol-Abbauwegs.

2.1.2 Proteomische Veränderungen

Die proteomischen Untersuchung der Anpassung von Stamm EbN1 an veränderte Alkylbenzolkonzentrationen ergab, daß sich die relative Häufigkeit von 158 Proteinen mindestens 2.5-fach veränderte. Von diesen regulierten Proteinen konnten 56 identifiziert werden. Die meisten Veränderungen wurden in Kulturen beobachtet, die mit sublethalen Konzentrationen von Toluol (740 µM) bzw. Ethylbenzol (315 µM) wuchsen. Als Referenzzustand dienten Zellen, die mit Standardkonzentrationen der Alkylbenzole (240 µM bzw. 210 µM) kultiviert wurden. EbA7011 und EbA1861 zeigten mit 66- bzw. -37-facher Veränderung der relativen Häufigkeit den größten Effekt. Die Funktion von EbA7011 ist bislang unbekannt. EbA1861 ist ein periplasmatisches Bindeprotein, welches vermutlich an der Fe^{2+}-Aufnahme beteiligt ist. Es wäre also denkbar, daß sublethale Alkylbenzolkonzentrationen die Konzentration zellulärer Eisenionen bzw. deren Metabolismus beeinflussen. Diese Vermutung wird durch folgende weitere Befunde unterstützt. Die relative Häufigkeit des Bindeproteins eines Fe^{3+}-Aufnahmesystems war reduziert und die eines bei oxidativem Stress gebildeten Aconitase-Isoenzyms (AcnA) mit stabilerem [4Fe/4S]-Cluster (Varghese et al. 2003) erhöht. Auch Superoxid Dismutase (SodB) trat in erhöhter relativer Häufigkeit auf. Üblicherweise katalysiert dieses Protein die Zersetzung von O_2-Anionen, um die Bildung von hochreaktiven Hydroxyl-Radikalen zu verhindern. Stamm EbN1 wurde jedoch in O_2-freiem, Nitrat-haltigen, Ascorbat-reduzierten Medium kultiviert. Unter diesen Bedingungen ist das einzige zu erwartende, in der Literatur beschriebene Substrat von Sod das hochreaktive Nitroxyl-Anion, welches aus NO entstehen kann (Hughes 1999; Reif et al. 2001).

Die Hypothese der vermehrten Bildung von NO stimmt überein mit der Beobachtung der verminderten Ausscheidung von Nitrit und der erhöhten Häufigkeit der Nitrit-Reduktase (NirS, 3-fach), deren Anteil an den separierten Proteinen in den sublethalen Ansätzen 6 % betrug. Diese drastische Mengenzunahme an NirS könnte zur Kompensierung eines zu erwartenden Energiedefizits dienen. Die Akkumulation von Alkylbenzolen in der Cytoplasma-

membran könnte zu einer erhöhten Protonen-Durchlässigkeit und damit zu einem Abbau des Protonengradienten führen. Außerdem könnte der Protonengradient zunehmend zur Energetisierung von Effluxsystemen in Anspruch genommen werden.

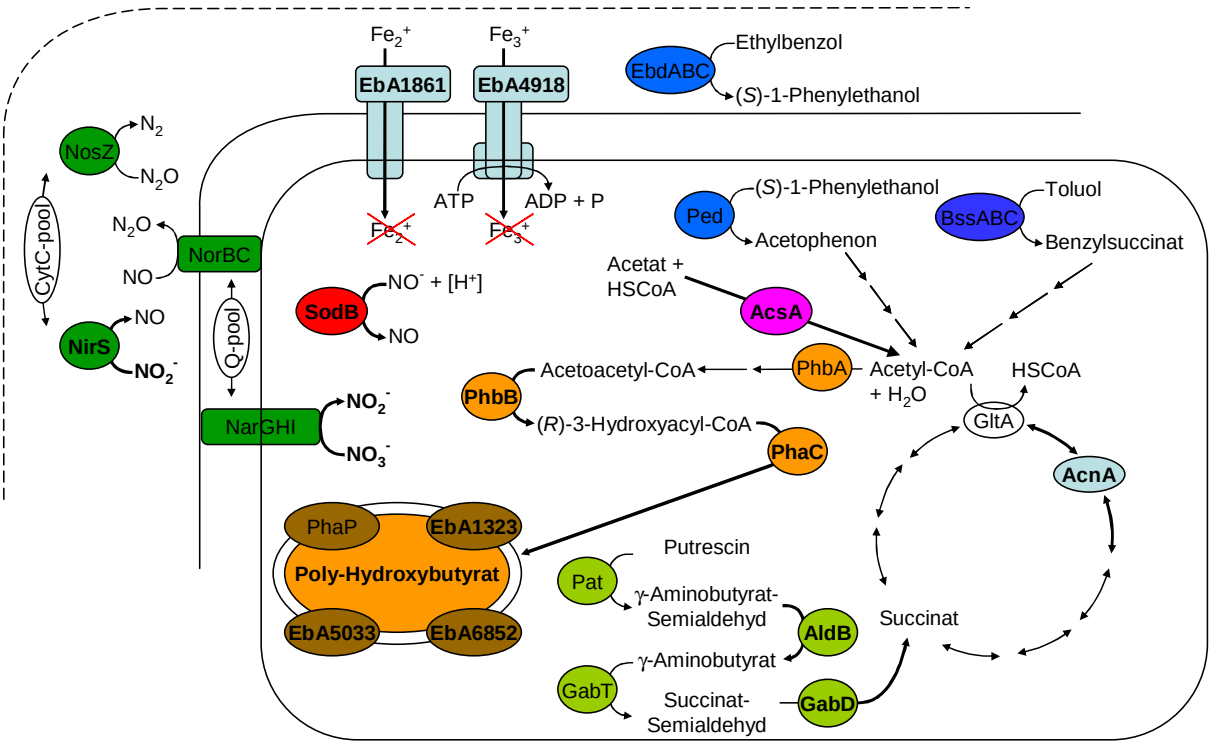

Abb. 6. Änderung der relativen Protein-Häufigkeiten bei Wachstum mit sublethalen Alkylbenzolkonzentrationen und dazugehörige Prozesse (fett gedruckt). Abkürzungen bedeuten: NarGHI, Nitratreduktase; NirS, Nitritreduktase; NorBC, NO-Reduktase; NosZ, N_2O-Reduktase; EbA1861, EbA4918, mögliche periplasmatische Eisen-Bindeproteine; EbdABC, Ethylbenzoldehydrogenase; Ped, (S)-1-Phenylethanol-Dehydrogenase; BssABC, Benzylsuccinat-Synthase; SodB, Superoxid-Dismutase; PhbA, Acetyl-CoA-Acetyltransferase; PhbB, Acetoacetyl-CoA-Reduktase; PhaC, PHB-Polymerase; PhaP, EbA1323, EbA5033, EbA6852, Phasine; Pat, Putrescin-Transaminase; AldB, mögliche Aminobutyraldehyd-Dehydrogenase; GabT, 4-Aminobutyrat-Transaminase; GabD, Succinat-Semialdehyd-Dehydrogenase; AcsA, Acetyl-CoA-Synthase; AcnA, Aconitase Isoenzym; GltA, Citrat-Synthase.

Die Häufigkeit der Arginin-Deiminase ArgA und der am Ende des Abbauwegs stehenden Succinatsemialdehyd-Dehydrogenase (GabD) ist in aerob Glukose verwertenden, Toluol-exponierten Zellen von *P. putida* DOT-T1E erhöht (Segura et al. 2005). Die Autoren leiten aus diesem Befund die Möglichkeit einer Energiegewinnung durch ArgA ab. In Stamm EbN1 wurde ebenfalls eine erhöhte Häufigkeit von GabD und einer möglichen, auch in dem Stoffwechselweg vorkommenden γ-Aminobutyrat-Semialdehyd-Dehydrogenase (AldB) festgestellt. Diese Alternative der Energiegewinnung scheint im Fall der aktiv wachsenden Zellen von Stamm EbN1 jedoch physiologisch unwahrscheinlich.

Die relativen Häufigkeiten der bekannten Proteine beider Alkylbenzol-Abbauwege waren bei den sublethalen Konzentrationen nur geringfügig verändert. Daher scheint Stamm EbN1 einen verstärkten Abbau von Toluol bzw. Ethylbenzol nicht zur Entgiftung einzusetzten. Eine interessante Beobachtung ist die Zunahme an Proteinen des Ethylbenzolabbaus in Ansätzen mit 740 µM Toluol. Dieser Nebeneffekt beruht vermutlich auf der bereits diskutierten (B.1) relaxierten Spezifität der Tcs1 und Tcs2 Sensoren.

In Zellen, die unter sublethalen Bedingungen kultiviert wurden, traten außerdem vermehrt Proteine der Polyhydroxyalkanoat (PHA)-Speicherstoff-Synthese auf. Die Acetoacetyl-CoA-Reduktase (PhbB) und die Poly-β-Hydroxyalkanoat-Synthase (PhaC) katalysieren die letzten zwei Reaktionsschritte der PHA-Synthese aus Acetyl-CoA. Das PHA-Polymer bildet Tröpfchen, die von einer Phasin-haltigen Membran umschlossen werden. In *Ralstonia eutropha* korreliert die Menge von Phasinen und PHA (Wieczorek et al. 1995). In Stamm EbN1 wurden in dieser Studie drei Phasin-ähnliche Proteine (EbA1323, EbA5033 und EbA6852) entdeckt, die bei Wachstum auf 740 µM Toluol bis zu 27-fach hoch reguliert waren; eine erhöhte Abundanz war auch in Ansätzen mit 315 µM Ethylbenzol zu beobachten. Dieser proteomische Befund wurde nachfolgend durch den chemisch-analytischen Nachweis von PHA bestätigt.

2.1.3 Bildung von Polyhydroxyalkanoaten

Die massenspektrometrische Analyse ergab in Ansätzen mit 740 µM Toluol einen Polyhydroxybutyrat-Anteil von 5.2 %, und in solchen mit 210 bzw. 315 µM Ethylbenzol einen Anteil von 2.0 bzw. 10.3 %, bezogen auf die Zelltrockenmasse. *R. eutropha* bildet bei Nährstoff-Limitation bzw. in der stationären Wachstumsphase sogar bis zu 90 % der Zelltrockenmasse. Bei Stamm EbN1 findet die PHA-Bildung jedoch unter vollkommen gegensätzlichen Bedingungen statt, nämlich während des linearen Wachstums in Gegenwart von sublethalen Alkylbenzolkonzentrationen. Diese PHA-Bildung kann man im Zusammenhang mit der beeinträchtigten Denitrifikation folgendermaßen interpretieren: Acetyl-CoA aus der Alkylbenzol-Oxidation wird in die PHA-Bildung umgeleitet, da NAD bzw. FAD (reduziert durch vollständige Oxidation von Acetyl-CoA im TCA-Zyklus) auf Grund der beeinträchtigten Denitrifikation nicht mehr ausreichend regeneriert werden können. Möglicherweise könnten die lipohilen PHA-Tröpfchen auch als cytoplasmatischer Speicher für Alkylbenzole dienen. Dieser Alternative wurde im nachfolgend beschriebenen „Alkylbenzol-Schock“-Experiment (B.2.2) nachgegangen.

2.1.4 Anpassungen der Membran

Der Einfluß von sublethalen Alkylbenzolkonzentrationen auf die Cytoplasmamembran wurde durch Analyse intakter Phospholipide untersucht. Die Phospholipide von Stamm EbN1 enthielten unter allen Bedingungen zu über 95 % C16-**Fettsäuren**. Mit steigender Toluol-Konzentration stieg der Anteil gesättigter Fettsäuren (16:0) von 35 auf 45 %, während nur geringe Unterschiede durch Ethylbenzol hervorgerufen wurden. Gesättigte Fettsäuren haben einen höheren Schmelzpunkt als ungesättigte und stabilisieren daher die Membran.

Bei den PL-**Kopfgruppen** wurden deutliche Veränderungen der Zusammensetzung beobachtet. Mit ansteigender Alkylbenzol-Konzentration wurde auf Kosten von Phosphatidyl-ethanolamin (PE) bis zu 3.5 % Phosphatidylcholin (PC) gebildet. PC war bei Wachstum mit 70 µM Toluol, 4 mM Benzoat bzw. 5 mM Succinat nicht nachweisbar. PC und Phospatidyl-glycerin (PG) besitzen im Vergleich zu PE größere Kopfgruppen und stabilisieren die Cytoplasmamembran bei Einlagerung in die äußere Schicht (Weber und de Bont 1996; Murzyn et al. 2005).

2.2 Reaktion auf Alkylbenzol-Shock

Die reine Schockantwort von Stamm EbN1 auf sublethale Konzentrationen von Toluol bzw. Ethylbenzol wurde in Succinat-adaptierten, wachsenden Kulturen untersucht. Bei einer optischen Dichte von 0.2 wurden jeweils Konzentrationen zwischen 0.7 und 5.8 mM Toluol und 0.3 und 1.67 mM Ethylbenzol eingestellt. Die Schockantwort wurde anhand von Wachstum, Denitrifizierung und Alkylbenzolkonzentration verfolgt.

2.2.1 Physiologische Effekte

Die mit 0.7 mM **Toluol** versetzte Kultur zeigte gegenüber der Kontrolle ein kaum beeinträchtigtes Wachstum. Nach Zugabe von 1, 1.2 und 1.5 mM Toluol kam es zu deutlich beeinträchtigtem Wachstum, verzögertem Nitrat-Verbrauch und verringertem bis stagnierenden (bei 1.5 mM Toluol) Verbrauch von ausgeschiedenem Nitrit. Nach Zugabe von 3 bzw. 5.8 mM Toluol nahm die OD ab und Nitrat wurde kaum noch verbraucht. Zehn Stunden nach Toluol-Zugabe wurde Toluol-freies Medium mit 5 % (v/v) der jeweiligen Kulturen beimpft. Die zuvor mit bis zu 1.5 mM Toluol behandelten Kulturen wuchsen über Nacht, während die Passage des Ansatzes mit 3 mM Toluol erst nach vier Tagen Trübung zeigte. Das zuvor mit 5.6 mM Toluol behandelte Inokulum zeigte auch nach 14 Tagen kein Wachstum mehr.

Bei 0.3 mM **Ethylbenzol** glichen Verbrauch von Nitrat und ausgeschiedenem Nitrit dem des Kontrollansatzes. Nach Zugabe von 0.4 bzw. 0.5 mM Ethylbenzol kam es bereits zu einem deutlich verzögerten Nitrit-Verbrauch. Nach Zugabe von 0.6 mM wurden die Effekte deutlicher: Das Wachstum stagnierte, Nitrat wurde nur noch langsam verbraucht, Nitrit gar nicht mehr. Nach Zugabe von 0.8 mM Ethylbenzol nahm die Trübung ab, wenngleich Nitrat noch verbraucht wurde. Nach Zugabe von 1.1 bzw. 1.6 mM Ethylbenzol kam auch der Nitrat-Verbrauch vollständig zum Erliegen. Passagierung der Kulturen in frisches, Ethylbenzol-freies Medium führte in allen Fällen zu robustem Wachstum.

In Kulturen, zu denen 1.2 mM Toluol oder 0.5 mM Ethylbenzol gegeben wurde, stellte sich –gemittelt über den Versuchszeitraum von 10 h– nach Alkylbenzol-Zugabe Konzentrationen von 0.98 mM Toluol bzw. 0.37 mM Ethylbenzol ein. Unter diesen Bedingungen war eine ähnlich deutliche Beeinträchtigung von Wachstum und Verbrauch von Nitrat und Nitrit wie beim Wachstum von Stamm EbN1 mit 0.315 mM Ethylbenzol zu beobachten. Daher werden diese beiden Alkylbenzolkonzentrationen als sublethal angesehen. Entsprechend Abschnitt

B.2.1 ergeben sich für das hier beschriebene Schock-Experiment Membrankonzentrationen von 91 mM (0.98 mM im Medium) Toluol und 97 mM (0.37 mM im Medium) Ethylbenzol, die damit durchschnittlich 24 % höher liegen als beim tatsächlichen Wachstum auf diesen beiden Alkylbenzolen.

2.2.2 Proteomische Veränderung

Exponentiell mit Succinat-wachsende Kulturen von Stamm EbN1 wurden mit 0.97 mM Toluol bzw. 0.37 mM Ethylbenzol versetzt und 45, 250 und 600 Minuten nach dieser Alkylbenzol-Zugabe wurden Zellen für die Proteomanalyse geerntet. Als Referenzzustand wurden unbehandelte Zellen zum gleichen Zeitpunkt geerntet, um Wachstumsphasen-abhängige Effekte berücksichtigen zu können. Insgesamt wurden -5.37- bis 11.95-fache Veränderungen der relativen Häufigkeit von insgesamt 81 Proteinen in Antwort auf Alkylbenzol-Schock festgestellt. Dieses Ausmaß an Veränderungen liegt im Bereich bisheriger Beobachtungen mit anderen Organismen: Nach Zugabe von Benzol zu einer mit Glukose wachsenden Kultur von *E. coli* wurden 23 Proteine neu gebildet, von denen zwei auch bei Kohlenstoff-Limitation beobachtet wurden (Blom et al. 1992). Bei aeroben Wachstum von *P. putida* mt-2 mit Toluol wurden 22, mit der Anpassung an Kohlenstoff-Limitation in Zusammenhang stehende Proteine beobachtet (Vercellone-Smith und Herson 1997). Von den 81 in diesem Experiment mit Stamm EbN1 veränderten Proteinen wurden 51 ausschließlich in sublethalen Alkylbenzolkonzentrationen ausgesetzten Zellen beobachtet, wobei 7 bzw. 19 spezifisch für Toluol bzw. Ethylbenzol waren. Zwei Proteine wurden nur in unbehandelten Zellen regulierten, die Häufigkeit der anderen 28 Proteine wurde abhängig von der Wachstumsphase verändert. Die meisten Wachstumsphasen-abhängig veränderten Proteine wurden beim Vergleich der 45 und 250 min Proben beobachtet, d.h. deutlich vor Eintritt in die stationäre Phase. Das steht zwar im Gegensatz zu dem bei *E. coli* beobachteten Zusammenhang von Wachstumsrate und RpoS (σ^S)-vermittelter Anpassung an die stationäre Phase (Ihssen und Egli 2004), stimmt aber mit Beobachtungen bei *Listeria monocytogenes* überein, wo die Hälfte der Proteine zur Anpassung an die stationäre Phase schon in der Mitte der exponentiellen Phase gebildet wurden (Weeks et al. 2004).

2.2.3 Streß-abhängig regulierte Proteine

Von den insgesamt 81 regulierten Proteinen konnten 34 identifiziert werden. Drei direkt im Zusammenhang mit Stress stehende Proteine sind die Hitzeschock-induzierten Proteine HtpG, IbpA und ClpB. Dieser Befund war unerwartet, da weder Benzol-exponierte Zellen von *E. coli* (Blom et al. 1992) noch aerob Toluol-verwertende Zellen von *P. putida* mt-2 (Vercellone-Smith und Herson 1997) Hitzeschock-induzierte Proteine bildeten. Jedoch wurden in einem Lösungsmittel-sensiblen *E. coli*-Stamm 30 min nach *n*-Hexan-Zugabe die Transkripte für die Hitzeschock-Proteine HslJ, HtpX und IbpAB beobachtet, in einer resistenten Mutante jedoch nicht (Hayashi et al. 2003). Es könnte sich dabei also um eine vorübergehende Reaktion moderat toleranter Organismen handeln, da Stamm EbN1 bei Wachstum mit Toluol und Ethylbenzol unter sublethalen Bedingungen keine Bildung von Hitzeschock-Proteinen zeigte und deren Erhöhung durch Toluol- bzw. Ethylbenzol-Schock nach 250 bzw. 600 min zurück ging.

Für das an der *N*-Acetyl-D-Fucosamin-Synthese und somit an der O-Antigen-Bildung der Lipopolysaccharide (LPS) beteiligte WbjB wurde nach Ethylbenzol-Schock eine etwa 12-fach erhöhte Häufigkeit ermittelt. Das ist interessant, weil nach Toluol-Exposition bei *P. putida* Idaho der raue, O-Antigen-lose LPS-Typ gefunden wurde, bei der sensiblen Mutante MW1200 jedoch der glatte Typ (Pinkart et al. 1996). Womöglich liegt hier eine Beeinflussung der Permeabilität der äußeren Membran vor. Andererseits bewirkt die Deletion eines Gens für ein anderes an der O-Antigen-Bildung beteiligtes Protein keine veränderte Lösungsmittel-sensibilität (Junker et al. 2001). Darüber hinaus wurden abhängig von der Wachstumsphase die im Zusammenhang mit Stress stehenden Proteine KatA, Dps und OmpC, eine bifunktionale Katalase, ein vor DNA–Oxidation schützendes Protein und ein Porin der Periplasmamembran reguliert.

Proteine der Denitrifizierung. Das Vorläuferprotein der periplasmatischen cd_1-Nitrit-Reduktase (NirS) hatte zu allen Zeitpunkten unter sublethalen Bedingungen eine etwa 2-fach erhöhte Häufigkeit. Demgegenüber stieg die Häufigkeit der ebenfalls periplasmatischen Lachgas (N_2O)-Reduktase (NosZ) nach Alkylbenzol-Schock kontinuierlich an. In den unbehandelten Kontrollen tritt NirS erst bei den 250 bzw. 600 Minuten Zeitpunkten nach vollständigem Nitratverbrauch mit stark erhöhter Häufigkeit auf, während NosZ relativ unverändert bleibt. Die Reduktion von Nitrat durch NarGHI ermöglicht eine deutlich höhere

Energiekonservierung als die nachfolgenden Reduktionsschritten durch die Nitrit– (NirS), Stickstoffmonoxid– (NorBC) und Lachgas (NosZ) –Reduktasen (Nicholls 1992). Expressionsstudien mit *Paracoccus denitrificans* (Baumann et al. 1996), *Pseudomonas stutzeri* (Härtig et al. 1999) und *Pseudomonas fluorescens* (Philippot et al. 2001) zeigten, daß die Gene dieser verschiedenen Reduktasen nicht koordiniert reguliert werden, wobei aus energetischen Gründen *nar* vor *nir* induziert wird. Die Gene der weiteren Schritte wurden jedoch ebenfalls früher als *nir* exprimiert, um der Akkumulation des hochreaktiven NO vorzubeugen. Erhöhte NirS- und NosZ-Häufigkeiten bei aktivem Nitrat-Verbrauch 45 min nach Toluol- bzw. Ethylbenzolschock deuten also auf erhöhten Energiebedarf oder beeinträchtigte Enzymfunktion hin.

Metabolismus. Erhöhte Häufigkeiten von AceE, Icd2 und PpcK zeigen an, daß das zur Succinat-Verwertung benötigte Acetyl-CoA via Malat und Pyruvat gewonnen wird, wobei letzteres auch für die Glukoneogenese verwendet wird. Dieser Weg wird auch von *E.coli* bei Wachstum mit Succinat benutzt, wobei unter O_2-Limitation ein Teil des Acetyl-CoA zur ATP-Gewinnung in Acetat überführt wird (Edwards et al. 2001). Die Acetyl-CoA-Synthase (AcsA) war sowohl bei Alkylbenzol-Schock als auch bei Wachstum mit sublethalen Alkylbenzolkonzentrationen hochreguliert. Dieses Enzym aktiviert Acetat unter ATP-Verbrauch zu Acetyl-CoA. In über Nacht Toluol exponierten, mit Glukose wachsenden Zellen von *P. putida* DOT-T1E war die Abundanz von Proteinen des *tod* Toluol-Abbauwegs deutlich erhöht (Segura et al. 2005). Auch nach zehnstündiger Einwirkung sublethaler Toluol- bzw. Ethylbenzol-Konzentrationen auf Succinat-verwertende Kulturen von Stamm EbN1 konnten keine erhöhten Häufigkeiten entsprechender kataboler Proteine festgestellt werden. Das bedeutet, daß die in Teil I B.1 beschriebene ca. 15 stündige Induktionszeit nach Substratwechsel von Benzoat zu Toluol bzw. Ethylbenzol auch in aktiv wachsenden Zellen nicht wesentlich verkürzt wurde, oder daß Katabolit-Repression vorlag.

2.3 Relevanz der eingesetzten Alkylbenzolkonzentrationen

Die höchste bisher *in-situ* beobachtete BTEX–Konzentration betrug 30 mg/l (Aronson und Howard 1997; Teutsch et al. 1997). In Laborversuchen stellte sich in einer 1:20 (v/v) Mischung verschiedener Benzinsorten (durchschnittlich 21 % (v/v) BTEX) mit Grundwasser eine durchschnittliche Konzentration von 130 mg/l ein (Cline et al. 1991). Zur besseren Vergleichbarkeit sei erwähnt, daß 100 mg/l Toluol bzw. Ethylbenzol einer molaren

Konzentration von 1.09 mM bzw. 0.94 mM entsprechen. Stamm EbN1 kann mit bis zu 0.315 mM Ethylbenzol und 0.74 mM Toluol als Substrat wachsen und toleriert bis zu 0.37 mM bzw. 0.98 mM dieser Substanzen im Schockexperiment. Das Wachstum von EbN1 und verwandten Stämmen auf einer 1:40 Mischung aus Rohöl und Mineral-Medium unter Nitrat-reduzierenden Bedingungen wurde gezeigt (Rabus und Widdel 1996). Es könnte also sein, daß an Orten, die mit Erdöl, Diesel, Kerosin und vielleicht sogar Benzin-kontaminiert sind, Stamm EbN1 metabolisch aktiv ist und Alkylbenzole auch unter natürlichen Bedingungen abbauen kann.

3. Ausblick

Die Regulation der anaeroben Abbauwege für Toluol und Ethylbenzol wurde in der vorliegenden Arbeit in Abhängigkeit vom Vorhandensein bzw. der Konzentration des jeweiligen Alkylbenzols untersucht. Es konnte unter anderem gezeigt werden, daß keines der beiden Alkylbenzole die Verwertung des jeweils anderen unterdrückt. Eine interessante Frage für weiterführende Untersuchungen ist in diesem Zusammenhang der Effekt von anderen, gut verwertbaren Substraten(z.B. Benzoat oder Succinat) auf die Regulation der beiden Alkylbenzol-Abbauwege. Unterliegen sie auf dieser Ebene einer Art Katabolit-Repression? Da die Eingangsenzyme (Benzylsuccinat-Synthase und Ethylbenzol-Dehydrogenase) der beiden Abbauwege Sauerstoff-empfindlich sind, sollte eine O_2-abhängige Regulationsebene integriert sein. Vorversuche (Wöhlbrand, Affeld, Rabus, unveröffentlicht) deuten bereits in diese Richtung. Der Einfluß von O_2 und die Möglichkeit einer Katabolit-Repression lassen sich am Besten unter Wachstumsraten-kontrollierten Bedingungen im Chemostaten untersuchen.

Im Hinblick auf die durchgeführten Experimente zur Lösungsmittel-Toleranz stellen sich drei weiterführende Fragen: (1) Gibt es eine vergleichbare molekular-physiologische Antwort auf Stress durch nicht verwertbare Kohlenwasserstoffe (z.B. Xylol, *n*-Hexan) oder (2) phenolische Wachstumssubstrate (Phenol, *p*-Cresol)? (3) Welchen Einfluß können Exportsysteme, z.B. aus der RND-Familie, auf die Toleranz gegenüber Alkylbenzolen bzw. phenolischen Verbindungen nehmen?

Die globalen Analysen auf RNA– und Protein-Ebene haben die Ko-Regulation einiger neuer, bislang nicht mit dem anaeroben Alkylbenzolabbau in Verbindung gebrachter Proteine gezeigt. Eine mögliche Funktion der entsprechenden Genprodukte in den jeweiligen Abbauwegen könnte durch einen genetischen Ansatz (knock-out Mutagenese) nachgewiesen werden.

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Teil II Publikationen

A Publikationsliste mit Erläuterungen

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

1. **Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1**
   Michael Kube, Johann Heider, Judith Amann, Peter Hufnagel, Simon Kühner, Alfred Beck, Richard Reinhardt, Ralf Rabus
   Archives of Microbiology (2004) **181**:182-194
   Durchführung der zweidimensionalen Gelelektrophorese.

2. **Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1**
   Simon Kühner, Lars Wöhlbrand, Ingo Fritz, Wasco Wruck, Claus Hultschig, Peter Hufnagel, Michael Kube, Richard Reinhardt, Ralf Rabus
   Journal of Bacteriology (2005) **187**:1493-1503
   Mitwirkung an der Versuchsplanung, Durchführung der Wachstumsexperimente, der HPLC- sowie GC-Analytik und der zweidimensionalen Gelelektrophorese. Redaktionelle Mitarbeit an der Erstellung des Manuskripts.

3. **Response of a denitrifying bacterium, strain EbN1 to sublethal concentrations of alkylbenzene growth substrates**
   Simon Kühner, Kai Affeld, Roland Meyer, Klaus-Gerhard Zink, Thomas Halder, Kenny Kuchta, Alexander Steinbüchel, Ralf Rabus
   Manuskript in Vorbereitung
   Entwicklung des Konzepts zusammen mit Ralf Rabus. Durchführung der physiologischen Versuche und chromatographischen Analysen zum Teil durch Anleitung von Kai Affeld und Roland Meyer. Durchführung der zweidimensionalen Gel-

elektrophorese und der Computer-gestützten Datenauswertung. Erstellung des Manuskripts zusammen mit Ralf Rabus.

4. **Solvent (alkylbenzene) tolerance of an aromatic-degrading, denitrifying bacterium, strain EbN1**

   Simon Kühner, Matthias Wietz, Diana Radovan, Thomas Halder, Ralf Rabus

   Manuskript in Vorbereitung

   Entwicklung des Konzepts zusammen mit Ralf Rabus. Durchführung der physiologischen Versuche und chromatographischen Analysen zum Teil durch Anleitung von Matthias Wietz und Diana Radovan. Durchführung der zweidimensionalen Gelelektrophorese und der Computer-gestützten Datenauswertung. Erstellung des Manuskripts zusammen mit Ralf Rabus.

B Publikationen

1

Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1

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Abstract

The organisation of all genes required for the anaerobic conversion of toluene to benzoyl-CoA was investigated in denitrifying *Azoarcus*-like strain EbN1. All these genes are clustered within 25.3 kb of contiguous DNA sequence, which includes only a few intervening sequences. The toluene-catabolic genes are organised in two apparent operons. One contains the genes (*bssCAB*) for the three subunits of benzylsuccinate synthase, which initiates anaerobic toluene degradation by converting toluene to (*R*)-benzylsuccinate. The BssCAB proteins of strain EbN1 are most similar to those of *Thauera aromatica* strain K172. The *bssCAB* genes are part of a larger putative operon (*bssDCABEFGH*), which contains the *bssD* gene encoding the activase for benzylsuccinate synthase and four genes (*bssEFGH*) for proteins of unknown function. RT-PCR experiments showing continuation of transcription over the three largest intergenic regions of the *bss* operon support the assumed structure. Moreover, BssG was identified as toluene-induced protein. Downstream of the *bss* genes, another large putative operon (*bbsA-H*) was identified which contains all genes required for β-oxidation of benzylsuccinate to benzoyl-CoA, e.g. *bbsEF* encoding succinyl-CoA:-(*R*)-benzylsuccinate CoA-transferase. Immediately upstream of the *bss*-operon, genes for a two-component regulatory system were identified; their products may sense toluene and induce the expression of both catabolic operons. The order and sequences of the *bss* and *bbs* genes are highly similar among toluene-degrading denitrifiers. The *bss* and *bbs* genes of the Fe^{III}-reducing *Geobacter metallireducens* display less sequence similarity and are organised differently. The genes between the *bss* and *bbs* operons and in the flanking regions differ between strain EbN1 and the other strains.

Introduction

Oxygen-independent degradation pathways of hydrocarbons have been demonstrated as novel metabolic capacities in bacteria during the last decade (for overview see Heider et al. 1999; Spormann and Widdel 2000; Widdel and Rabus 2001; Widdel et al. 2003). While aerobic bacteria initialize the degradation of hydrocarbons exclusively by O_2-dependent mono- or di-oxygenase reactions, anaerobic bacteria must employ fundamentally different activation mechanisms. The best understood and apparently most widespread of these anaerobic mechanisms is the radical-catalysed addition of the hydrocarbons to fumarate, yielding substituted succinate derivatives. This reaction has been recognised for the activation of several alkyl-substituted benzenes as well as for *n*-alkanes (for overview see Heider et al. 1999; Spormann and Widdel 2000; Widdel and Rabus 2001; Widdel et al. 2003).

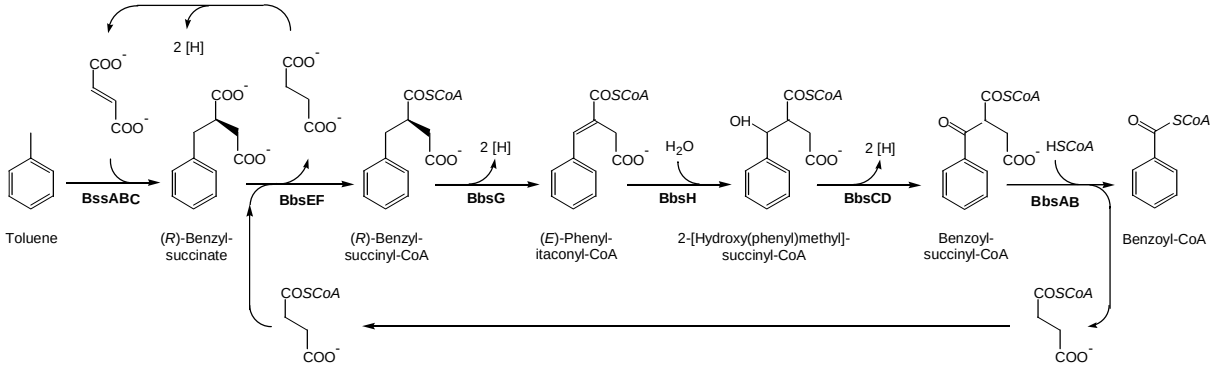

Fig. 1. Proposed reaction sequence for the anaerobic degradation of toluene in denitrifying strain EbN1 to the level of benzoyl-CoA (modified from: Boll et al. 2002; Leuthner and Heider 2000). The fumarate cosubstrate of benzylsuccinate synthase is recycled during activation of benzylsuccinate and subsequent β-oxidation to benzoyl-CoA. The latter is further oxidized via ring cleavage to carbon dioxide (not shown). Reducing equivalents ([H]) are used for the reduction of nitrate to dinitrogen. Enzyme names of shown (bold) gene products are as follows: BssABC, benzylsuccinate synthase; BbsEF, succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase; BbsG, (*R*)-benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase; BbsAB, benzoylsuccinyl-CoA thiolase. For coding genes of these enzymes see Fig. 2.

Our understanding of the fumarate-dependent hydrocarbon activation and the consecutive degradation pathways is most advanced in the case of toluene (Fig. 1). Formation of (*R*)-benzylsuccinate from toluene and fumarate is catalysed by the glycyl-radical enzyme benzylsuccinate synthase, a heterohexameric enzyme of $\alpha_2\beta_2\gamma_2$ composition, whose subunits are encoded by the *bssCAB* genes (Beller and Spormann 1998; Coschigano et al. 1998; Leuthner et al. 1998; Achong et al. 2001; Kane et al. 2002). The glycyl radical present in activated benzylsuccinate synthase (Krieger et al. 2001; Duboc-Toia et al. 2003) is obviously

generated by an *S*-adenosylmethionine (SAM) dependent activating enzyme, as known for pyruvate formate-lyase and anaerobic ribonucleotide reductase (for overview see: Sawers and Watson 1998). The gene for the putative activating enzyme, *bssD*, is encoded immediately upstream of the *bssCAB* genes. Expression of the *bss* genes is probably controlled by a two-component regulatory system and the respective genes (*tdiSR*) are mostly located in close proximity to the *bss* operon (Coschigano and Young 1997; Leuthner and Heider 1998; Achong et al. 2001). It should be noted that although the genes involved in anaerobic toluene degradation in *Thauera aromatica* strain T1 have originally been designated *tutEFDGH* (Coschigano 2000), we will use the *bss* nomenclature to avoid confusion. Further degradation of (*R*)-benzylsuccinate to benzoyl-CoA in *Thauera aromatica* strain K172 follows a modified β-oxidation pathway (Leuthner and Heider 2000; Leutwein and Heider 2001, 2002), which is initiated by activation of (*R*)-benzylsuccinate to the CoA-thioester. Benzylsuccinyl-CoA is oxidised to benzoylsuccinyl-CoA and cleaved to benzoyl-CoA and succinyl-CoA. The latter is used for the activation of benzylsuccinate in a CoA-transfer reaction, thus releasing succinate for the regeneration of fumarate via succinate dehydrogenase. All enzymes required for β-oxidation of benzylsuccinate are encoded in the *bbs* operon. The sizes and genetic compositions of the regions between the *bss* and *bbs* operons are currently unknown. Further degradation of benzoyl-CoA proceeds via reductive dearomatisation, hydrolytic ring cleavage, β-oxidation to acetyl-CoA units and terminal oxidation to CO_2 (Harwood et al. 1999; Boll et al. 2002).

Among the known bacteria that degrade toluene anaerobically, *Azoarcus*-like strain EbN1 is unique in utilising ethylbenzene as an alternative hydrocarbon substrate (Rabus and Widdel 1995). Despite of chemical and structural similarities between both hydrocarbons, their anaerobic degradation pathways differ completely. Whereas toluene catabolism follows the common route (Fig. 1), ethylbenzene is anaerobically hydroxylated and dehydrogenated to acetophenone, which is then carboxylated and converted to benzoyl-CoA as the first common intermediate of both pathways (Rabus and Heider 1998; Kniemeyer and Heider 2001). Since both pathways are regulated independently (Rabus and Heider 1998; Champion et al. 1999), strain EbN1 is a useful study organism to gain insights into the largely unexplored principles of specific substrate sensing and gene activation in the anaerobic hydrocarbon metabolism. The genes for the ethylbenzene degradation pathway including those for putative regulators have recently been identified on a 56-kb contig from strain EbN1

(Rabus et al. 2002a). Here, we describe the organisation of the genes needed for the anaerobic toluene catabolism, which were identified on a different contig from this bacterium.

Materials and methods

Bacterial strain, growth conditions and isolation of genomic DNA. The denitrifying bacterium *Azoarcus*-like strain EbN1 (β-Proteobacteria) has been isolated from anoxic freshwater mud sampled in Bremen, Germany (Rabus and Widdel 1995). Strain EbN1 was cultivated and cells were harvested as previously described (Rabus and Widdel 1995). The procedure for isolation of genomic DNA was modified (Rabus et al. 2002a) from methods reported by others (Ausubel et al. 1992; Zhou et al. 1996).

Construction of shotgun libraries, DNA sequencing and sequence assembly. Two shotgun libraries with average insert sizes of 1.5 kb and 3.5 kb were generated for DNA sequencing. Obtained sequences were assembled. Regions of weak quality within the analysed contig were improved by resequencing and primer walking. Final sequence quality was based on three independent reads and sequencing of both strands. This procedure was recently described in more detail (Rabus et al. 2002a). The nucleotide sequence has been deposited at EMBL under the accession number BX682953.

Gene prediction, functional assignment and data management. The program ORPHEUS (Frishman et al. 1998) was used for gene prediction. The program was adjusted, falsely predicted orfs were removed and orfs refined as previously described (Rabus et al. 2002a).

Similarity searches were carried out by the BLAST programs (Altschul et al. 1997) and screening of the amino acid sequences of the predicted orfs against the non-redundant protein database and the translated nucleotide database sequences. The predicted orfs were functionally assigned with the INTERPRO system (Apweiler et al. 2001) and screening against the Clusters of Orthologous Groups of proteins (COGs; Tatusov et al. 2001). To evaluate the reliability of gene annotation (i.e. functional assignment), an additive scoring tool was used. More details about this procedure are given in Rabus et al. (2002a). Results of the automated orf prediction and functional assignment were manually controlled for the entire contig (36.3 kb). Multiple alignments were generated with ClustalW (Thompson et al. 1994), and used to determine identities of amino acids with GAP (Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, Wisc, USA).

The methods used for functional assignment (BLAST, INTERPRO and the additive scoring tool) are all implemented in the annotation platform HTGA (High Throughput

Genome Annotation; Rabus et al. 2002a). More details about the design of the HTGA system are provided on the Web page http://www.micro-genomes.mpg.de/ebn1/.

The genomic sequence of *Geobacter metallireducens* is currently determined by the DOE Joint Genome Institute (www.jgi.doe.gov). DNA sequence data from the draft version (07. November 2002) are accessible at NCBI under accession number NZ_AAAS01000001 (www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table.cgi). A sequence region of about 60 kb of the *G. metallireducens* genome was used to compare *bss* and *bbs* genes among different toluene-degrading bacteria.

RT-PCR experiments. Total RNA was prepared from toluene-grown cells of strain EbN1 by the hot-phenol method as described by Aiba et al. (1981). The dried RNA was dissolved in water, and potential contaminating DNA was removed by treating the samples with RNase-free DNase (Promega, Mannheim, Germany) according to the manufacturers instructions. Quality of RNA preparations was controlled using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), and cDNA was synthesised with gene specific reverse primers by H Minus M-MuLV reverse transcriptase (MBI Fermentas, St. Leon Roth, Germany) according to the manufacturers protocol. PCR was carried out in 50 µl scale under standard conditions with REDTaq Polymerase (Sigma-Aldrich, Munich, Germany) and the synthesised cDNAs as templates (2 µl of individual reverse transcription products). To prove the absence of DNA in the RNA preparations, controls (cDNA synthesis) were carried out in the absence of reverse transcriptase. To cover the intergenic region between *bssA* and *bssB*, reverse primer bssBrev (TTACACGTGGTCGCGGAA) and forward primer bssAfor (GACCTGATCGTGCGGGTATC) were used (expected product of 470 bp). Likewise, primers bssErev (GGCTCAGGGTCTCGGTATTCA) and bssBfor (GGATACCCATCATGA GCGCA) were used for the intergenic region between *bssB* and *bssE* (expected product of 435 bp), and primers bssFrev (GATGATGTCGCCGGTGTTG) and bssEfor (ACGTCGGTC TCGGCAAGAT) for that between *bssE* and *bssF* (expected product of 285 bp). The sizes of the obtained RT-PCR products were analyzed by agarose gel electrophoresis.

Two-dimensional gel electrophoresis and analysis by mass spectrometry. Differential analysis of protein patterns by two-dimensional gel electrophoresis was based on cells of strain EbN1 anaerobically grown with toluene or benzoate. Samples were prepared and separated by two-dimensional gel electrophoresis as recently described (Rabus et al. 2002b;

Gade et al. 2003). Gels were loaded with 50 and 500 µg protein, respectively, and staining was performed with silver and colloidal Coomassie Brilliant Blue, respectively.

Toluene-specific protein spots were selected and excised manually using a cutting tool with a 1.5 mm needle. Proteolytic digests were performed on a PROTEINEER dp digest and sample preparation robot (Bruker Daltonik GmbH, Bremen) using a commercial digestion kit (DP 96 Kit; Bruker), containing all necessary buffers, porcine trypsin as proteolytic enzyme and α-cyano-4-hydroxycinnamic acid as MALDI matrix. The PROTEINEER dp run consisted of several washing steps, incubation for 4h with trypsin, extraction, and thinlayer sample preparation on an AnchorChip600 MALDI target (Bruker).

MS fingerprint and MS/MS fragment spectra were acquired with an ultraflex TOF/TOF instrument (Bruker), equipped with a gridless reflectron, Lift cell and Scout MTP ion source in positive ionization mode. In a single automated run, MS spectra were acquired and based on the resulting peak lists up to 10 precursor ions were selected and submitted to Lift MS/MS without manual interference. The combined information of the fingerprint and fragment spectra was submitted to a protein database search (Mascot search engine; Matrix Science Ltd, London) against a database containing the genetic information described in this study. The MS-tolerance was set to 30 ppm for external calibration.

Results and discussion

The present study describes a shotgun DNA sequencing approach to identify genes of strain EbN1 (related to *Azoarcus*, β-Proteobacteria) that are involved in the anaerobic conversion of toluene to benzoyl-CoA. The general pathway of anaerobic toluene catabolism is depicted in Fig. 1. DNA fragments from shotgun sequencing were screened for genes related to toluene metabolism by similarity of the gene products to known protein sequences of *T. aromatica* strains K172 (Leuthner et al. 1998; Leuthner and Heider 2000) and T1 (Coschigano et al. 1998) and *Azoarcus* sp. strain T (Achong et al. 2001). By this approach, a 36,333 bp contig was assembled from a total of 831 sequence reads. Based on the criteria described in the Materials and methods section and a recent study (Rabus et al. 2002a), 35 orfs were finally predicted on the contig (Fig. 2). INTERPRO/COG references, BLASTP hits and assigned functions for each orf are listed in Table 1. Bioinformatical annotation of gene function was complemented by a combined physiological/RT-PCR/proteomic approach. Identified genes correlated to anaerobic toluene degradation are indicated at each reaction step of the pathway (Fig. 1).

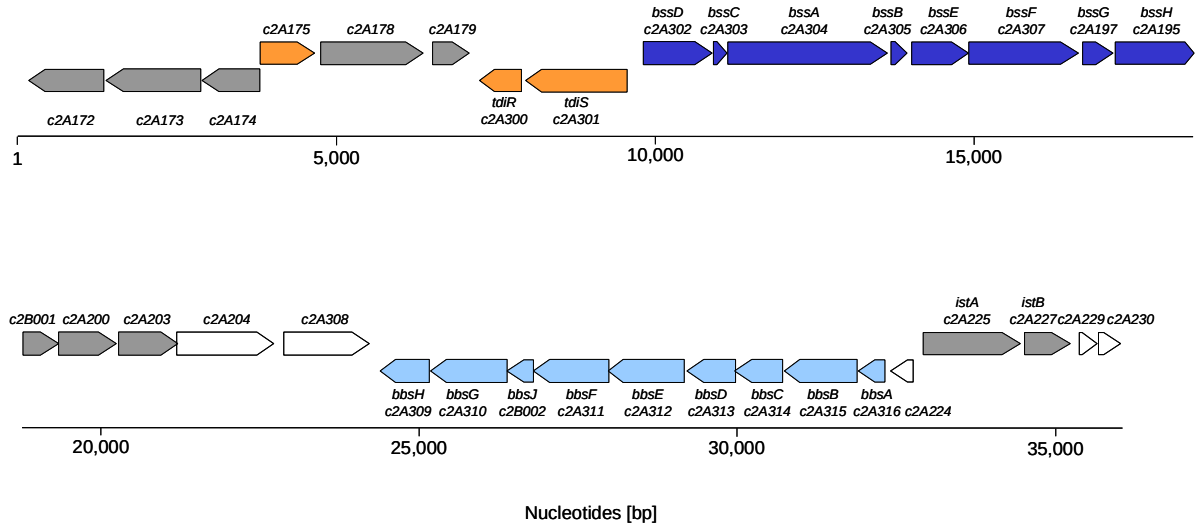

Fig. 2. Scale model of gene organisation in the investigated contig from denitrifying strain EbN1. Putative functions of the depicted orfs are listed in Table 1. Color coding: blue, genes related to anaerobic oxidation of toluene to benzoyl-CoA (hues indicate initial reaction and β-oxidation-like reaction sequence as explained in the text); orange, regulatory proteins; grey, orfs with assigned putative functions; white, hypothetical proteins. The scale indicates nucleotide position on the DNA fragment.

Table 1 Annotated ORFs of studied contig from denitrifying strain EbN1.

Open reading frame	Length (amino acids)	INTERPRO / COG references[a]	BLASTP hit used for annotation[b]				Putative function
			Gene	Organism[c]	E-value	Acc. no[d]	
c2A172	402[e]	IPR002155 / COG0183	*paaJ*	Ecoli	1e-102	P77525	β-Ketoadipyl CoA thiolase
c2A173	506	IPR006108, IPR006176 / COG1250	*paaH2*	Ralso	1e-128	CAD15715	3-Hydroxyacyl-CoA dehydrogenase
c2A174	256[e]	IPR001753	*orf2*	Thaar	1e-84	CAC28159	Enoyl-CoA hydratase
c2A175	285	IPR005471 / COG1414	*pa1630*	Pseae	1e-67	AAG05019	Transcriptional regulator
c2A178	550	COG0644	*etf*	Ralso	0	CAD15269	Electron transfer flavoprotein-ubiquinone oxidoreductase
c2A179	204[e]	IPR001451 / COG0663	*paaY*	Azoev	3e-65	AAG28963	Bacterial transferase
c2A300	218	IPR000792, IPR001789	*tdiR*	Azosp	3e-99	AAK50368	Two-component response regulator; *tdiR* (*tcr3*)
c2A301	544[e]	IPR000014, IPR004358	*tdiS*	Azosp	0	AAK50369	Two-component sensor; *tdiS* (*tcs3*)
c2A302	331[e]	IPR001450, IPR001989 / COG1180	*bssD*	Thaar	1e-143	CAA05050	Benzylsuccinate synthase activating enzyme; *bssD*
c2A303	57	-	*bssC*	Thaar	3e-18	CAA05051	γ-Subunit of benzylsuccinate synthase; *bssC*
c2A304	861	IPR004184 / COG1882	*bssA*	Thaar	0	CAA05052	α-Subunit of benzylsuccinate synthase; *bssA*
c2A305	74	-	*bssB*	Thaar	2e-30	CAA05053	β-Subunit of benzylsuccinate synthase; *bssB*
c2A306	287	IPR001687 / COG0714	*bssE*	Thaar	1e-151	CAD12889	Chaperone; *bssE*
c2A307	568	-	-	-	-	-	Hypothetical protein; „*bssF*“
c2A197	190	-	*fn0848*	Fusnu	2e-9	AAL95044	Hypothetical protein; „*bssG*“
c2A195	426[e]	IPR005828, IPR007114 / COG0477	*bcr_2*	Pasmu	2e-35	AAK03617	Possible transporter; „*bssH*“
c2B001	194	-	-	-	-	-	Hypothetical protein
c2A200	307	-	*fn0847*	Fusnu	2e-58	AAL95043	TPR-repeat-containing protein
c2A203	315	COG0451	*fn1299*	Fusnu	1e-19	AAL95495	Sugar dehydratase

c2A204	520[e]	IPR000515, IPR005847	*ra0050*	Sinme	1e-38	AAK64708	Hypothetical protein
c2A308	454	IPR003812 / COG3177	*tou1*	Neime	4e-19	AAF06681	Hypothetical protein
c2A309	256	IPR001753 / COG1024	*bbsH*	Thaar	1e-131	AAF89843	Putative E-phenylitaconyl-CoA hydratase; *bbsH*
c2A310	406	IPR006090, IPR006091 / COG1960	*bbsG*	Thaar	0	AAF89842	Benzylsuccinyl-CoA dehydrogenase; *bbsG*
c2B002	94	-	-	-	-	-	Hypothetical protein; „*bbsJ*“
c2A311	409	IPR003673 / COG1804	*bbsF*	Thaar	0	AAF89841	Subunit of Benzylsuccinate CoA-transferase; *bbsF*
c2A312	410	IPR003673 / COG1804	*bbsE*	Thaar	0	AAF89840	Subunit of Benzylsuccinate CoA-transferase; *bbsE*
c2A313	248	IPR002198 / COG1208	*bbsD*	Thaar	1e-126	AAF89839	Subunit of 2-[hydroxy(phenyl)methyl]-succinyl-CoA DH; *bbsD*
c2A314	250	IPR002198 / COG1208	*bbsC*	Thaar	4e-82	AAF89838	Subunit of 2-[hydroxy(phenyl)methyl]-succinyl-CoA DH; *bbsC*
c2A315	389	IPR002155 / COG0183	*bbsB*	Thaar	0	AAF89837	Subunit of benzylsuccinyl-CoA thiolase; *bbsB*
c2A316	148	IPR002878 / COG1545	*bbsA*	Thaar	9e-65	AAF89836	Subunit of benzylsuccinyl-CoA thiolase; *bbsA*
c2A224	86[e]	-	-	-	-	-	Hypothetical protein
c2A225	519[e]	IPR001584, IPR007102 / COG4584	*istA*	Psefl	e-148	CAA55959	Putative transposase for insertion sequence; *istA*
c2A227	244	IPR001687	*istB*	Pseal	6e-70	P95446	Putative transposase subunit; *istB*
c2A229	70[e]	-	-	-	-	-	Hypothetical protein
c2A230	125	-	*yajo*	Rhisp	5e-6	P55515	Hypothetical protein

[a]References relate to INTERPRO (http://www.ebi.ac.uk/interpro/; Apweiler et al. 2001) and COG databases (http://www.ncbi.nlm.nih.gov/COG/; Tatusov et al. 2001)
[b]Hits were obtained from BLASTP-comparison of predicted proteins from strain EbN1 with SWISS-PROT and TrEMBL-databases (Bairoch and Apweiler 2000)
[c]Abbreviations of names of organisms is according to the list of organism identification codes (SWISS-PROT). Azoev, *Azoarcus evansii*; Azosp, *Azoarcus* sp. strain T; Ecoli, *Escherichia coli*; Fusnu, *Fusobacterium nucleatum*; Neime, *Neisseria meningitidis*; Pasmu, *Pasteurella multocida*; Pseae, *Pseudomonas aeruginosa*; Pseal, *Pseudomonas alcaligenes*; Psefl, *Pseudomonas fluorescens*; Ralso, *Ralstonia solanacearum*; Rhisp, *Rhizobium* sp. strain NGR234; Sinme, *Sinorhizobium meliloti*; Thaar, *Thauera aromatica*
[d]Accession numbers allow retrieval of sequences from NCBI database (http://www.ncbi.nlm.nih.gov/Entrez/index.html)
[e]ORFs were manually shortened to improve alignments and to avoid overlaps with upstream genes

Genes of the benzylsuccinate synthase operon: *bssDCABEFGH*. Benzylsuccinate synthase was previously purified from *T. aromatica* strain K172 and demonstrated to catalyse the formation of (*R*)-benzylsuccinate from toluene and fumarate (Leuthner et al. 1998). Three genes, *bssA*, *bssB* and *bssC*, were identified, which apparently code for the α- (BssA), β- (BssB) and γ-subunits (BssC) of the heterohexameric enzyme. Identification was based on similarity to translated gene sequences from *T. aromatica* strains K172 (Leuthner et al. 1998) and T1 (Coschigano et al. 1998) and *Azoarcus* sp. strain T (Achong et al. 2001). Active benzylsuccinate synthase of strain EbN1 is assumed to carry a radical on Gly825 of the α-subunit (BssA). The Gly825 probably functions in storing the radical and in generating a transient catalytically active thiyl residue on Cys489. As first step of catalysis, the thiyl radical may then abstract a hydrogen atom from the methyl group of toluene (Heider et al. 1999; Himo 2002). A similar radical-based mechanism has previously been demonstrated for pyruvate formate-lyase (Becker et al. 1999) and anaerobic ribonucleotide reductase (Eklund and Fontecave 1999). Amino acid sequences of the subunits of all currently known benzylsuccinate synthases display a high degree of similarity (see Table 2 for BssA). The *bss* gene products of *Azoarcus*-like strain EbN1 are most similar to those of *T. aromatica* strain K172 (> 94% identity), and slightly less similar to those of *Azoarcus* strain T, *T. aromatica* strain T1 and *Thauera* sp. strain DNT-1 (around 80% identity). The sequences from the latter three denitrifying strains are again > 95% identical to each other, whereas the *bss* gene products of the phylogenetically more remote *G. metallireducens* are only 71-73% identical to those of any of the denitrifying strains (Fig. 3).

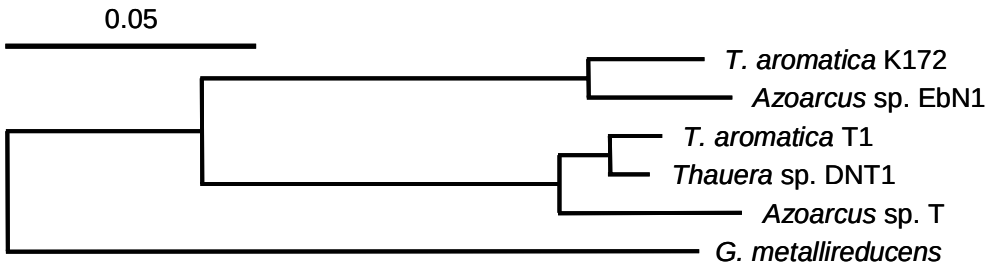

Fig. 3. Phylogenetic tree of BssA subunits of benzylsuccinate synthases from different strains of bacteria. Sequences were retrieved from the databases and trimmed to the first common start codon. Accession numbers were: AY032676, *Azoarcus*-like strain T; AJ001848, *T. aromatica* strain K172; AF113168, *T. aromatica* strain T1; AB066263, *Thauera* sp. strain DNT-1; NZ_AAAS01000001, *G. metallireducens.*

Directly upstream of the *bssC* gene, the *bssD* gene coding for an activating enzyme required for glycyl radical generation was detected as part of the *bss* operon. As shown for

bssD from *T. aromatica* strain K172 and *Azoarcus* sp. strain T, the start codon of *bssD* appears to be a GTG codon, rather than an in-frame ATG codon located 34 codons further upstream. This is evident from the lack of similarity of the translated sequence between the ATG and GTG codons to other activating enzymes and from the location of the predicted promoter (see below). The BssD protein sequence from *Azoarcus*-like strain EbN1 is again most similar to the ortholog from *T. aromatica* strain K172 (79% identity) and less similar to those of *Azoarcus* sp. strain T, *T. aromatica* strain T1, *Thauera* sp. DNT-1 and *G. metallireducens* (50 to 63% identity). All BssD proteins contain three conserved Cys-motives in the N-terminal part of the proteins. The first motif (position 29-36 in strain EbN1) has a C-x_3-C-x_2-C structure characteristic for the emerging class of SAM-dependent radical generators (Sofia et al. 2001), whereas the other two (positions 55-65 and 89-99 in strain EbN1) corresponded to the consensus sequences (C-x_2-C-x_2-C-x_3-C) of typical [Fe_8S_8] ferredoxins. Sequence similarity with the PFL-activating enzyme from *E. coli* suggests that BssD is involved in introducing the glycyl radical into BssA. The C-x_3-C-x_2-C motif of PFL-activase was demonstrated to coordinate an atypical [Fe_4S_4] cluster that is required for the activity of the enzyme (Külzer et al. 1998) and for direct binding and reduction of SAM to methionine and an adenosyl radical (Walsby et al. 2002).

Downstream of the *bssDCAB* genes, at least four further genes of a putative continued operon are located, which we designate *bssEFGH*. The derived *bssE* product contains a Walker-type ATP/GTP binding site motif and is similar to an emerging class of chaperone-like ATPases required for assembly, operation and disassembly of protein complexes (Neuwald et al. 1999). Moreover, orthologs of *bssE* were independently shown to be part of the *bss* operons of three other bacterial strains (Coschigano 2000; Achong et al. 2001; Hermuth et al. 2002). The *bssF* gene was predicted to be part of the operon, because genes coding for highly similar proteins are found directly downstream of *bssE* in all known and sufficiently far sequenced *bss* operons (intergenic distances ranging from 0 to 47 bases). The derived gene products do not show similarity to other known proteins, precluding any prediction of possible function. An extended transcript of the *bss* operon containing *bssE* and *bssF* was recently shown for *Azoarcus* sp. strain T, and the same study revealed an additional RNA 5'-end in front of the *bssF* gene. To date, it is unknown whether this 5'-end reflects transcription initiation within the coding sequence of *bssE* or RNase processing of the extended *bss* transcript (Achong et al. 2001). The next gene encoded in the DNA sequence, *bssG*, is very closely spaced to *bssF* with an intergenic region of only 9 bases. A gene coding for an orthologous protein is also present immediately downstream of *bssF* in *T. aromatica*

strain K172, but not in the operon of the more distantly related Fe^{III}-reducing *G. metallireducens*. The *bss* operons of other denitrifying toluene degraders are not sequenced sufficiently far to detect possible orthologs. Finally, a further gene, *bssH*, is located immediately downstream of *bssG*. Although no *bssH* orthologs are known from other strains due to insufficient sequence information, the observed one-base-overlap of the GTG start codon of *bssH* with the TGA stop codon of *bssG* strongly suggests the presence of a common transcript. The *bssH* gene product displays sequence similarity to members of the major facilitator superfamily (MFS) and the Bcr/CflA subfamily of drug resistance transporters. Some members of these transporter families are involved in uptake/efflux of aromatic compounds (Saier 2000). Because a hydrophobic compound like toluene is expected to diffuse freely across the cytoplasmic membrane, one may speculate that BssH functions in export of toxic levels of toluene from the cytosol rather than in toluene uptake. An alternative function in specific transport of benzylsuccinate appears unlikely, since strain EbN1 does not grow with benzylsuccinate.

In strain EbN1, the largest intergenic regions of the putative *bssDCABEFGH* operon are between *bssA* and *bssB* (97 bp), between *bssB* and *bssE* (119 bp) and between *bssE* and *bssF* (44 bp). These intergenic regions may be regarded long enough to cause rho-dependent transcriptional termination, and the first two even contain putative RNA secondary structures resembling rho-independent termination signals (Fig. 4B). Therefore, we tested for continuation of transcription over these intergenic gaps by RT-PCR. Using primer pairs covering the intergenic regions, we indeed obtained RT-PCR products of the expected sizes from total RNA of toluene-grown cells of strain EbN1, which were absent when the reverse transcriptase reaction was omitted (Fig. 4A). The last two genes of the predicted operon (*bssGH*) are so closely spaced to their preceding genes (intergenic distances 9 and 0 bases) that termination of transcription downstream of *bssF* or *bssG* is not plausible. In support of the suggested operon organisation, we specifically detected the BssG protein in toluene-grown cells by two-dimensional gel electrophoresis (data not shown) and identified it unambiguously by mass spectrometry of tryptic peptides (Fig. 5). The assumed ATG codon of *bssG* is the only reasonable start codon preceding the coding sequence of the first identified peptide (starting at amino acid 22). In contrast to this ATG start codon, two alternative GTG codons (codon positions 9 and 20) are not associated with possible ribosome binding sites.

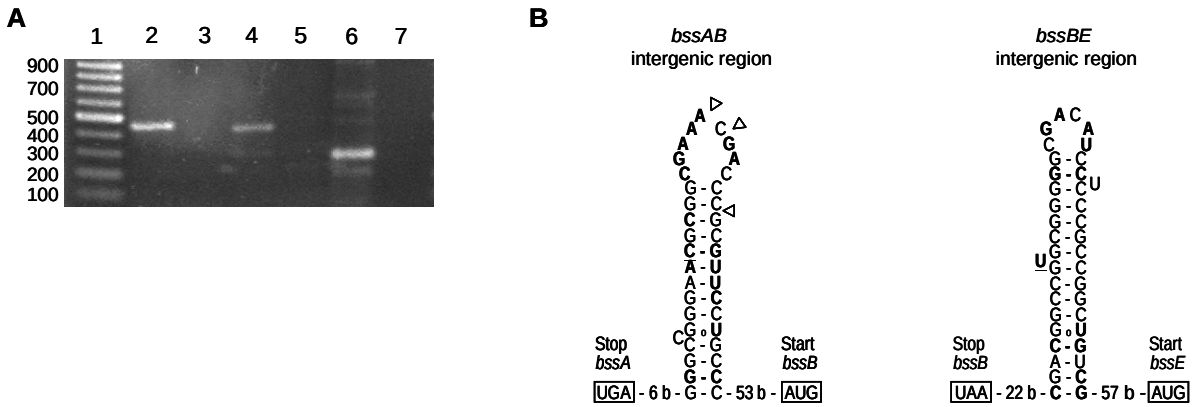

Fig. 4. Transcription of intergenic regions in the *bss* operon. (**A**) RT-PCR analysis. *Lane 1*: size-marker. *Lane 2*: RT-PCR reaction product of the intergenic region between *bssA* and *bssB* (expected product of 470 bp). *Lane 4*: RT-PCR reaction product of the intergenic region between *bssB* and *bssE* (expected product of 435 bp). *Lane 6*: RT-PCR reaction product of the intergenic region between *bssE* and *bssF* (expected product of 285 bp). *Lanes 3, 5, 7*: corresponding controls where reverse transcriptase was absent from the reaction mixtures. (**B**) Predicted RNA secondary structures in the intergenic regions. Analogous, but not identical RNA structures are also present in the corresponding intergenic regions from *T. aromatica* strain K172 (Hermuth et al. 2002). **N**, changed base; ◁, deleted base; **N**, inserted base.

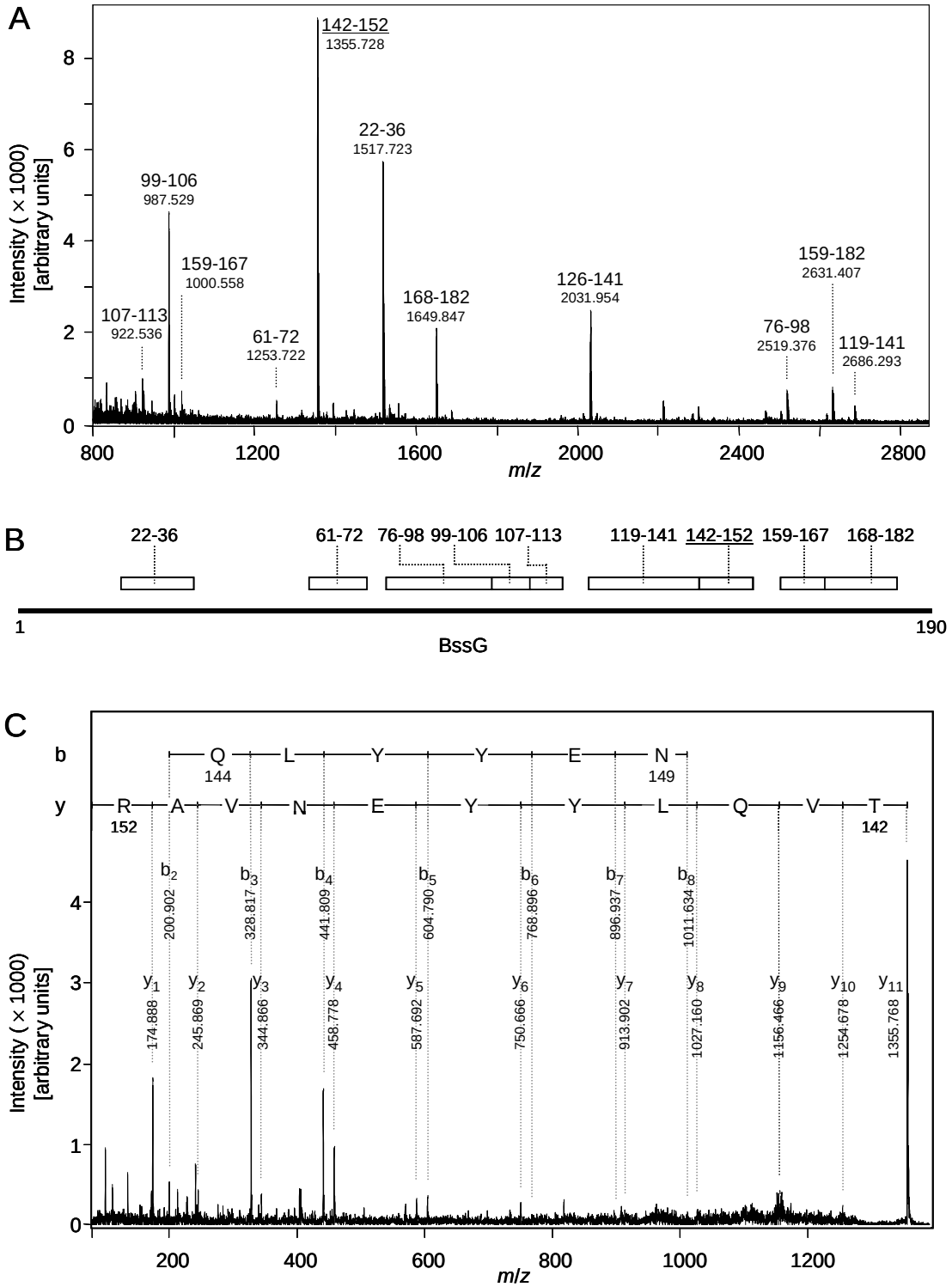

Fig. 5. Identification of the *bssG* gene product by mass spectrometry. Tryptic peptides of the 2DE-separated protein were analyzed by MALDI MS and MS/MS (ultraflex TOF/TOF). The *bssG* gene product was identified unambiguously. (**A**) MALDI MS-spectrum. MS-peaks are assigned with the BssG sequence position (top) and the peptide mass (bottom). (**B**) BssG sequence map. Peptides confirmed by mass spectrometry are symbolised by bars, and positions in the BssG sequence are indicated. The sequence coverage is 65%. (**C**) MALDI MS/MS spectrum of peptide TVQLYYENVAR (position 142-152). MS/MS-peaks are assigned with the ion-type (top) and the peptide mass (bottom). Cleavage occurs most frequently at the peptide bond resulting in b-ions (fragments starting from N-terminus) and y-ions (fragments starting from C-terminus).

At present it cannot be decided whether the *bss* operon may even continue beyond the *bssH* gene. One of two possible translational start codons of the gene following *bssH* (*c2B001*; see Fig. 2 and Table 1) overlaps with the *bssH* stop codon, indicating transcriptional read-through (if used). The other one is located 336 bases downstream, allowing for enough space for transcription termination of the *bss* operon and expression of the downstream genes as independent operon. In any case, the three genes (*c2A200*, *c2A203* and *c2A204*; see Fig. 2 and Table 1) following *bssH* are expected to be part of the same transcription unit, based on the short intergenic regions between the genes (0 to 22 bases). Thus the *bss* operon of strain EbN1 is predicted to consist of eight genes and might even contain up to 12 genes (corresponding to 8.7 or 12.8 kb).

Operon encoding enzymes for β-oxidation of benzylsuccinate: *bbsA-H*. Further degradation of (*R*)-benzylsuccinate proceeds via β-oxidation to benzoyl-CoA and succinyl-CoA (see Fig. 1). To date, this reaction sequence has only been studied with *T. aromatica* strain K172. Based on N-terminal sequences of toluene-induced, electrophoretically separated proteins, nine genes (*bbsA-I*) were identified which form the *bbs* operon in this strain (Leuthner and Heider 2000). The *bbsEF* genes code for the two subunits of succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase, *bbsG* for the subunit of 2-(*R*)-benzyl-succinyl-CoA dehydrogenase, as shown with the purified and characterised enzymes (Leutwein and Heider 2001, 2002). The next three enzymes of the pathway are encoded by *bbsH* (phenylitaconyl-CoA hydratase), *bbsCD* (two subunits of an alcohol dehydrogenase), and *bbsAB* (two subunits of a thiolase), respectively (C. Feil, K. Hermuth and J. Heider, unpublished data).

Using the *bbs* gene sequences from *T. aromatica* strain K172, orthologs (*2cA309-316*) of all *bbs* genes (except for *bbsI*, the only unaccounted gene in the operon of *T. aromatica* strain K172) were also identified in *Azoarcus*-like strain EbN1. Therefore, the functions of all eight conserved *bbs* genes of strain EbN1 can be annotated. The *bbs* gene products of both strains displayed high sequence similarity (share of identical residues ranging from 82.7 to 95.0%), and strong similarity is also retained on the DNA level (89.1% identity for the overall operon). The *bbs* operons of *Azoarcus*-like strain EbN1 and *T. aromatica* strain K172 mainly differ in the length of the intergenic region between *bbsF* and *bbsG*. Closer inspection of this gap revealed the presence of another putative gene in the *bbs* operon of strain EbN1 (termed *bbsJ*) which is lacking in strain K172. The *bbsJ* gene codes for a protein of 9.8 kDa that contains a CxxC and a CRC motif, but does not show apparent similarity to entries in the

current databases. Still, it must be regarded as an expressed gene of the operon, based on its good ribosome binding sequence and its short intergenic distances to the preceding and the following gene. Most interestingly, DNA alignment of the *bbs* operons from strains EbN1 and K172 shows that there is recognisable nucleotide similarity between the rather large *bbsFG*-intergenic gap of strain K172 and the last quarter of the *bbsJ* gene from strain EbN1. One possible interpretation of this finding would be that the *bbs* operon of *T. aromatica* strain K172 has evolved from a *bbsJ*-containing operon by a deletion of most of that gene and some subsequent deterioration of the remaining "scar" sequence. Genes coding for *bbsA-H* orthologs were also detected in the current draft version of the *G. metallireducens* genome. However, the degree of similarity between corresponding *bbs* genes of *G. metallireducens* and *T. aromatica* strain K172 was lower (derived amino acid sequence identities ranging from 39.1 to 75.5%). Moreover, three additional genes are inserted into the *bbs* operon of *G. metallireducens*. Two of these clearly code for the subunits of an electron transferring flavoprotein (ETF), which is expected to serve as physiological electron acceptor for benzylsuccinyl-CoA dehydrogenase (Leutwein and Heider 2002), the third codes for a protein similar to a domain of heterodisulfide reductases, thiol:fumarate oxidoreductases or succinate dehydrogenases and may therefore also be involved in electron transfer from benzylsuccinyl-CoA to the respiratory chain. The organisation of the *bbs* operon in *Azoarcus*-like strain EbN1, *T. aromatica* strain K172 and *G. metallireducens* is shown in Fig. 6.

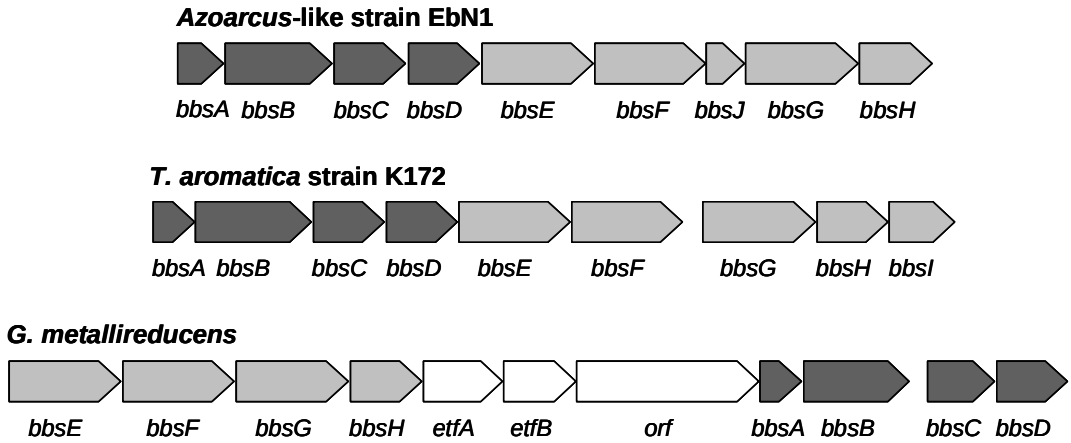

Fig. 6. Organisation of *bbs* genes in different toluene-degrading bacteria.

Conserved promoter structures of the *bss* and *bbs* operons. The 5'-flanking DNA sequences of the *bss* and *bbs* operons of strain EbN1 were analysed for similarity to the

characterised promoter regions of the orthologous operons from *T. aromatica* strain K172 (Leuthner and Heider 2000; Hermuth et al. 2002) and *Azoarcus* sp. strain T (Achong et al. 2001), as well as to the upstream sequences of other sequenced *bss* operons. As shown in Fig. 7, several conserved sequence motives can be identified upstream of the *bss* and *bbs* operons of strain EbN1 and all other known *bss* and *bbs* operons from denitrifying bacteria. Two of these motives are similar to typical –10 and –35 boxes of *E. coli* and are underlined in Fig. 7. The spacing of these two sequence motives varies by one to two bases in the different operons, but would be consistent with RNA polymerase binding in all cases. Further conserved motives of all sequences are located in the regions between bases –40 to –50 and around base –65, relative to the mapped (or presumed) transcriptional starts. The strong conservation of these sites and their location just upstream of the RNA polymerase binding site identifies these motives as prime candidates for binding of regulatory protein(s) that may be involved in induction of gene expression in response to anaerobiosis and/or toluene availability. It should be noted that the putative promoter region suggested for *T. aromatica* strain T1 in Fig. 7 differs from a previously mapped promoter 305 bases upstream of the assumed GTG start codon of the corresponding *bssD* ortholog ("*tutE*"; Coschigano 2000). The transcriptional start point of *bssD* proposed in Fig. 7 would not have been detected under the experimental conditions employed. The occurrence of internal 5´-ends of RNA species in the *bss* operon, as detected upstream of *bssC* in *T. aromatica* strains K172 and T1 (Hermuth et al. 2002; Coschigano 2000) and upstream of *bssF* in *Azoarcus* sp. strain T (Achong et al. 2001), can not be predicted for strain EbN1 because the respective DNA sites are not sufficiently conserved.

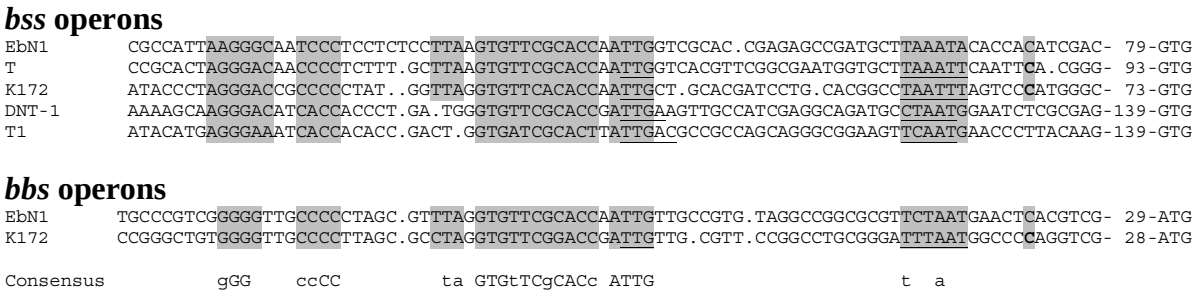

Fig. 7. Promoter consensus of *bss* and *bbs* operons from denitrifying bacteria. Bacterial strains depicted are *Azoarcus*-like strains EbN1 and T, as well as *Thauera* strains K172, T1 and DNT-1. Mapped transcription starts are labeled in bold, the distances to the respective translation start codons is indicated by numbers. Sequences similar to known *E. coli* –10 and –35 boxes are underlined. Conserved sequences that may either be involved in regulator- or RNA polymerase-binding are indicated by shading.

Regulatory proteins for the *bss* and *bbs* operons. Directly upstream of the *bss* operon of *Azoarcus*-like strain EbN1, two adjacent genes, *tdiR* and *tdiS*, code for a two-component regulatory system. The gene organisation and the corresponding gene products most closely resemble the TdiSR system of *Azoarcus* sp. strain T (Achong et al. 2001; 79 and 81% identity). The next similar proteins in the database are orthologous two-component systems from *T. aromatica* strain K172 and T1 (Leuthner and Heider 1998; Coschigano et al. 1997; 68 and 69% identity). The sensor component consists of two sensory PAS domains and a histidine kinase domain, each occupying about a third of the protein (Leuthner and Heider 1998). PAS domains are implicated in monitoring light, redox or hydrocarbon stimuli in diverse sensory proteins (Taylor and Zhulin 1999), and are obviously well suited to serve the regulatory requirements in the present case. The regulator consists of domains for a response regulator and a helix-turn-helix motif (Leuthner and Heider 1998). All known TdiSR-like systems are encoded in direct neighbourhood of the respective *bss*/*tut* genes and are suggested to be involved in transcriptional control of the toluene catabolic genes. Thus, it appears likely that the TdiSR system also regulates transcription of the *bss* and *bbs* operons in *Azoarcus*-like strain EbN1. The involvement of two-component regulatory systems in transcriptional control of (aerobic) toluene metabolism was first demonstrated for the TodST-system of *Pseudomonas putida* F1 (Lau et al. 1997). The sensor and regulator components of the predicted anaerobic regulatory systems even show significant similarity with their aerobic counterparts (Leuthner and Heider 1998). Recently, we reported the presence of another TdiSR-like two-component regulatory system in strain EbN1, whose genes are in close proximity to those coding for ethylbenzene dehydrogenase (Rabus et al. 2002a). The components of this putative ethylbenzene-responsive regulatory system are 35-40% identical to those of the known TdiSR systems and share the same domain organisation. Remarkably, the first (PAS) and the last (His kinase) domains of the putative ethylbenzene sensor are much more similar (41-43% identity) to those of the known toluene sensors than the second (PAS) domain (16% identity). Thus, strain EbN1 possesses two TdiSR-like two-component regulatory systems, which may be able to discriminate (possibly via the second PAS domain) between toluene and ethylbenzene, thereby allowing a finely tuned, substrate-dependent regulation of the respective degradation pathways.

Other genes. The genes *c2A174*, *c2A173* and *c2A172* probably encode enzymes involved in β-oxidation. The orf *c2A174* codes for an enoyl-CoA hydratase-type enzyme of unknown function, and *c2A173* for an alcohol dehydrogenase that is apparently a fusion protein of two subdomains, each similar to "short chain" alcohol dehydrogenases (Jörnvall et al. 1995). Similar fusions are known in other operons containing genes for β-oxidation enzymes, e. g. in the operons for aerobic phenylacetate catabolism (Luengo et al. 2001; Mohamed et al. 2002). Finally, *c2A172* codes for a standard thiolase of unknown function. The divergently transcribed gene *c2A175* apparently codes for a transcriptional regulator, which may be involved in regulation of the β-oxidation related genes. It belongs to the IclR family of bacterial regulatory proteins (INTERPRO entry IPR005471). Members of this family have been implicated in regulation of organic acids catabolism: acetate utilization via the glyoxylate bypass in *E. coli* (Sunnarborg et al. 1990) or protocatechuate degradation in *Acinetobacter* sp. strain ADP1 (Popp et al. 2002).

The distance to the next gene (*c2A178*) is long enough (93 bases) to expect an independent promoter for it. The gene product of this gene is clearly an electron transferring flavoprotein (ETF): ubiquinone oxidoreductase, which is required for channeling the redox equivalents derived from acyl-CoA dehydrogenase reactions (as reduced ETF) into the respiratory chain. The gene is located within 20 kb of the next gene coding for an ETF-reducing enzyme, namely benzylsuccinyl-CoA dehydrogenase ("BbsG"). The gene product of *c2A179* is similar to the *caiE* and *paaY* gene products whose genes are correlated to the operons involved in carnitine metabolism and aerobic phenylacetate catabolism, respectively.

Insertion sequence: *isE1*. Upstream of the *bbs* operon an insertion element (IS*E1*) was detected, which contains the *istA* and *istB* genes, whose products are highly similar to known transposases/cointegrases and correlated helper proteins, respectively. Sequence similarities of *istA* with IS1162 of *Pseudomonas fluorescens* (Solinas et al. 1995) and *istB* with IS1474 of *P. alcaligenes* (Yeo and Poh 1997) indicate affiliation to the IS21 family. The coding region (*istAB*) is flanked by 13 bases imperfectly (underlined) matching inverted repeats (32914-TGCGGATTCCGAC / GTCGGAATGCGCA -35670). They are highly similar to conserved terminal parts of inverted repeats in other members of the IS21 family (http://www-is.biotoul.fr/) like IS408 (TGCG T/G ATT C/T C) and IS1162 (TGCG T/G ATTTTC). The occurrence of a conserved mismatch in the fifth position from the start/end of the terminal region of the inverted repeat is striking. The short inverted repeats in *isE1* are possibly the

result of a deletion. Introduction of a single gap in the right inverted repeat allows to enlarge the inverted repeats (TGCGGATTCCGACCCAACGTGACCGC/GCGGTCACGTTCGTCG GAATGCGCA) to 26 bases. These longer inverted repeats possibly represent the original sequence also containing a conserved region (AACGTGA) of the inverted repeats of IS408 (http://www-is.biotoul.fr/). The inverted repeats are directly flanked by 7 bases of direct repeats (GGCTGTG), which probably originated from duplication of the target site. Such an insertion element appears to be absent downstream of the *bbs* operons in *G. metallireducens* or *T. aromatica* strain K172.

Genetic organisation. The order of the first six genes of the *bss* operon *(bssDCABEF*) is identical in all currently known examples (except for *T. aromatica* strain T1, where only partial sequence information on *bssF* is available). The five operons from denitrifying strains can be arranged into two subgroups differing in the length of the intergenic distance between *bssB* and *bssE*. In the operons of *Azoarcus*-like strain EbN1 and *T. aromatica* strain K172, these genes are separated by a transcribed intergenic region of 122 bases which may contain a stable RNA structure (Hermuth et al. 2002). This distance is much shorter in the operons from *Azoarcus* sp. strain T, *T. aromatica* strain T1 and *Thauera* sp. strain DNT-1 (47 bases). The different operon organisations are correlated with the respective benzylsuccinate synthases belonging to different similarity subgroups (see preceding section on the *bss* operon), and therefore allow for a facile PCR-based discrimination of the type of *bss* operon in denitrifying toluene-degrading strains (S. Zorn, K. Verfürth, J. Heider, unpublished). In the *bss* operon of the phylogenetically remote *G. metallireducens* the *bssG* and *bssH* genes are lacking, as well as the following genes found in strain EbN1.

The gene organisation of the *bbs* operons in strains EbN1 and K172 differ in the presence of the *bbsJ* gene between *bbsF* and *bbsG* in strain EbN1 and the presence of the *bbsI* gene as the last gene of the operon of strain K172. The function of the *bbsI* and *bbsJ* gene products in β-oxidation of benzylsuccinate is not known; the two proteins share no similarity. Interestingly, the preliminary sequence of the *bbs* operon of *G. metallireducens* only codes for orthologs of BbsA-H, but does not contain genes for a BbsI- or BbsJ-like protein. Moreover, this operon starts with the *bbsEFGH* genes, followed by three inserted genes (see above section on *bbs* operon) not present in the other *bbs* operons, and ends with the *bbsABCD* genes. The different *bbs* operon organisations of *Azoarcus*-like strain EbN1, *T. aromatica* strain K172 and *G. metallireducens* are shown in Fig. 6.

In strain EbN1, five genes for proteins of unknown function are predicted to be located between the predicted *bssDCABEFGH* and *bbsABCDJEFGH* operons. Four of these genes may be cotranscribed with the *bss* operon, but their relevance for anaerobic toluene catabolism is unknown. In *G. metallireducens*, the intercalating sequence between the *bss* and *bbs* operons is about 4.4 kb and codes for six predicted genes. The orf following *bssF* in *G. metallireducens* codes for a TodX-like protein, which is part of the toluene catabolic operon and implicated in toluene transport in *Pseudomonas* sp. (Wang et al. 1995). Even though this TodX-like protein does not display pronounced similarity with BssH from *Azoarcus*-like strain EbN1, the presence of genes coding for potential toluene transporters in (or next to) the *bss* operons in both organisms is remarkable. The other five genes of *G. metallireducens* are apparently not involved in toluene degradation and are disparate from the corresponding genes in strain EbN1. Moreover, the known flanking genes of the *bbs*-operons (both flanks) and the *bss*/*tdiSR*-operons (only 5´-flank) of *T. aromatica* strain K172 also differ from those of strain EbN1 and *G. metallireducens*. Therefore, the operons involved in anaerobic toluene metabolism seem to be embedded in quite different genomic contexts in varying bacterial species.

Similar to the genes involved in anaerobic toluene metabolism, those of anaerobic ethylbenzene metabolism of strain EbN1 are organised in two apparent operons, (*ebdABCDped* and *apc1-5bal*; Rabus et al. 2002a). The intercalating sequence (about 16 kb) between these operons contains genes coding for two different two-component regulatory systems, which are probably involved in sequential regulation of the upper (*ebdABCDped*) and lower part (*apc1-5bal*) of the degradation pathway. Strain EbN1 grows with either ethylbenzene or acetophenone, which is in accordance with a sequential regulation of these two operons (Rabus et al. 2002a). In contrast, strain EbN1 grows with toluene, but not with benzylsuccinate, which may at least partially be explained by a strictly coordinated regulation of the *bss* and *bbs* operons (mediated by the *tdiSR* gene products and toluene as inducer).

Conclusions

A unique property of strain EbN1 is its capacity to degrade toluene and ethylbenzene anaerobically via two completely different pathways. The identification of the corresponding genes will enable more detailed investigations into the substrate-dependent regulation of these two pathways. Based on the observed expression of "*bssG*" in toluene metabolising cells, the *bss* operon can be predicted to include this gene. This demonstrates the benefit of proteomic approaches for the annotation of hypothetical proteins. Considering the high degree of conservation among benzylsuccinate synthases, it will be interesting to compare them to the so far unknown subunit sequences of (1-methylpentyl)succinate synthase, the proposed *n*-hexane activating enzyme, of *Azoarcus*-like strain HxN1 (Rabus et al. 2001). These two types of enzymes might differ on the sequence level, since they activate chemically different hydrocarbons and form products with different stereochemical properties. The high sequence similarity of the *bss* and *bbs* operons of different species may be utilised for environmental studies to monitor expression of genes involved in anaerobic degradation of aromatic hydrocarbons. In fact, expression of *bssA* has only recently been determined in fuel-contaminated groundwater by real-time RT-PCR (Beller et al. 2002). Such *in situ* expression studies together with determination of key metabolites, e.g. benzyl-succinates (Beller 2000; Elshahed et al. 2001), could substantially advance our analytical capabilities to assess biodegradation of hydrocarbons in oil reservoirs or bioremediation efforts at contaminated sites.

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Substrate-dependent regulation of anaerobic degradation pathways for toluene and ethylbenzene in a denitrifying bacterium, strain EbN1

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Abstract

Anaerobic biodegradation of toluene and ethylbenzene is of environmental concern and biochemical interest due to toxicity and novel reactions, respectively. The denitrifying strain EbN1 is unique in anaerobically degrading both alkylbenzenes via different pathways converging at benzoyl-CoA. The organization of genes involved in both pathways was only recently determined in strain EbN1. In the present study, global expression analysis (DNA-microarray and proteomics) indicated involvement of several thus far unknown proteins in the degradation of both alkylbenzenes. E.g. *orf68* and *orf57*, framing the *ebd* operon, are implicated in ethylbenzene degradation, and *ebA1932* and *ebA1936*, located 7.2 kb upstream of the *bbs* operon, in toluene degradation. In addition, expression studies were now possible on the level of the complete pathways. Growth experiments demonstrated that degradative capacities for toluene and ethylbenzene could be simultaneously induced, regardless of the substrate used for adaptation. Regulation was studied on the RNA (real-time RT-PCR and DNA-microarray) and protein (2-dimensional difference gel electrophoresis) level, using cells adapted to anaerobic growth with benzoate, toluene, ethylbenzene, or a mixture of toluene and ethylbenzene. Expression of the two toluene-related operons (*bss* and *bbs*) was specifically induced in toluene-adapted cells. In contrast, genes involved in anaerobic ethylbenzene degradation were induced in ethylbenzene- and toluene-adapted cells, suggesting that toluene may act as gratuitous inducer. Agreeing with the predicted sequential regulation of the ethylbenzene pathway, Ebd proteins (encoding subunits of ethylbenzene dehydrogenase) were formed in ethylbenzene-, but not in acetophenone-adapted cells, while Apc proteins (subunits of predicted acetophenone carboxylase) were formed under both conditions.

Introduction

Alkylbenzenes are abundant constituents of crude oil and fuels, and are widely used solvents and starting compounds in chemical synthesis. Among them BTEX (benzene, toluene, ethylbenzene and xylenes) have attracted particular attention, due to their toxicity. Environmental pollution by crude oil or fuels often affects (leads to) anoxic systems such as groundwater aquifers, demanding knowledge about bacterial degradative capacities under anoxic conditions. Over the recent years, numerous pure cultures of novel bacteria were isolated which degrade alkylbenzenes completely to CO_2 in the absence of molecular oxygen (for overview see: Heider et al. 1999; Spormann and Widdel 2000; Widdel and Rabus 2001; Widdel et al. 2003). Most of them are denitrifying isolates belonging to a recently recognized phylogenetic group clustering with the known genera *Azoarcus* and *Thauera* within *Betaproteobacteria*. Due to the remarkable degradative capacities of its members, this group is currently described as a new genus, and the *Azoarcus*-like strain EbN1 as a new species therein (Rabus et al., unpublished).

Biodegradation of alkylbenzenes has to overcome their exceptional chemical stability. Aerobic bacteria achieve this by employing highly reactive oxygen species in mono- or dioxygenase catalyzed reactions. Understanding of the fundamentally different reactions used by anaerobic bacteria was only obtained during the recent years (for overview see: Heider et al. 1999; Spormann and Widdel 2000; Widdel and Rabus 2001; Widdel et al. 2003).

Benzylsuccinate synthase catalyzes the initial formation of benzylsuccinate from toluene and fumarate (Fig. 1A). The glycyl-radical of the active enzyme is generated by a *bssD*-encoded activase (Achong 2001; Beller and Spormann 1998; Coshigano et al. 1998; Duboc-Toja et al. 2003; Kane et al. 2002; Krieger et al. 2001; Leuthner et al. 1998). Benzylsuccinate is further degraded to the common aromatic intermediate benzoyl-CoA via a modified β-oxidation pathway (Leuthner and Heider 2000, 2001, 2002). In denitrifying bacteria, anaerobic degradation of ethylbenzene (Fig. 1B) is initiated by the molybdenum-containing ethylbenzene dehydrogenase forming (*S*)-1-phenylethanol (Ball et al. 1996; Johnson and Spormann 1999; Johnson et al. 2001; Kniemeyer and Heider 2001; Rabus and Heider 1998; Rabus and Widdel, 1995; Rabus et al. 2002). The maturation of this periplasmic enzyme is presumably mediated by the *ebdD* product. Subsequent conversion to benzoyl-CoA is proposed to involve a carboxylation reaction and thiolytic removal of an acetyl-CoA moiety. Further degradation of the common intermediate benzoyl-CoA begins with a reductive

dearomatization catalyzed by benzoyl-CoA reductase (Boll et al. 2002) and proceeds via hydrolytic ring cleavage and β-oxidation to acetyl-CoA units. The latter are then terminally oxidized to CO_2 (Boll et al. 2002; Harwood et al. 1999).

Strain EbN1 is unique among known alkylbenzene degraders for its capacity to anaerobically degrade toluene as well as ethylbenzene. These alkylbenzenes are not only utilized when supplied as pure substances (Rabus and Widdel 1995), but also directly from crude oil (Rabus and Widdel 1996). Strain EbN1 employs the above described pathways, which were previously suggested to be regulated by their respective substrates (Champion et al. 1999). Only recently, the complete genetic blueprints for both pathways including three related two-component regulatory systems were identified in strain EbN1 (Kube et al. 2004; Rabus et al. 2002).

Regulation of anaerobic hydrocarbon degradation is to date only poorly understood. Initial studies were concerned with the *bss*-operon of anaerobic toluene degradation (Coshigano 2000; Hermuth et al. 2002). The genomic reconstruction of anaerobic toluene and ethylbenzene metabolism in strain EbN1 (Kube et al. 2004; Rabus et al. 2002) allowed for the first time to investigate regulation of anaerobic hydrocarbon degradation on the level of the complete pathways. Physiological adaptation experiments were combined with global expression profiling (DNA-microarrays and proteomics) to pursue the following lines of research: (i) influence of adaptation substrates on induction of pathways; (ii) simultaneous induction and activity of both pathways; (iii) differing modes of regulation for the two pathways; (iv) specificities of the predicted sensors for their respective substrates; (v) search for additional gene products previously not correlated with the two pathways.

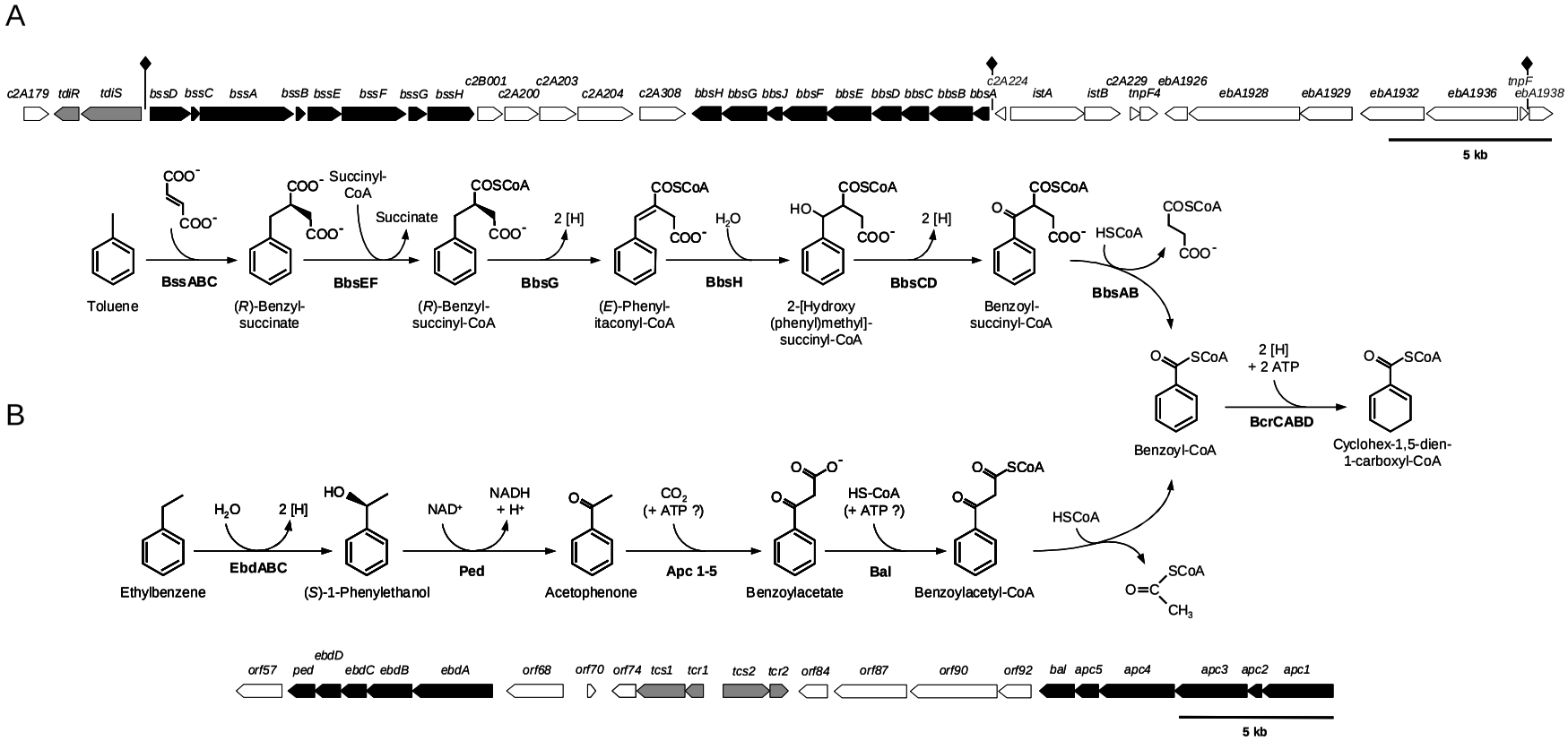

Fig. 1. Anaerobic toluene and ethylbenzene degradation in strain EbN1 proceed via different reaction sequences to the first common intermediate benzoyl-CoA. (A) Pathway of anaerobic toluene degradation (modified from: Boll et al., 2002; Leuthner and Heider, 2000). Enzyme names of indicated gene products (shown in bold; (Kube et al., 2004)) are as follows: BssABC, benzylsuccinate synthase; BbsEF, succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase; BbsG, (*R*)-benzylsuccinyl-CoA dehydrogenase; BbsH, phenylitaconyl-CoA hydratase; BbsCD, 2-[hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase; BbsAB, benzoylsuccinyl-CoA thiolase. (B) Pathway of anaerobic ethylbenzene degradation (modified from: Ball et al., 1996; Kniemeyer and Heider, 2001; Rabus and Heider, 1998; Rabus and Widdel, 1995). Enzyme names of indicated gene products (shown in bold; (Rabus et al., 2002)) are as follows: EbdABC, ethylbenzene dehydrogenase; Ped, (*S*)-1-phenylethanol dehydrogenase; Apc1-5, acetophenone carboxylase; Bal, benzoylacetate CoA-ligase. Anaerobic degradation of benzoyl-CoA is initiated by benzoyl-CoA reductase (BcrCABD) and then further oxidized via reductive ring cleavage to carbon dioxide (not shown; (Boll et al., 2002)). Reducing equivalents ([H]) are used for the reduction of nitrate to dinitrogen. A scale model for the organization of the involved genes is displayed for both pathways. Annotations for *ebA1926* through *ebA1938* are provided in Table 4, while those for all other genes have already been described elsewhere (Rabus et al., 2002; Kube et al., 2004). (♦), Consensus sequence [TTA(A/G)GTGTTCGCACCAATTG] in the promoter regions of *bssD*, *bbsA* and *ebA1936* is located 118, 68 and 450 bp upstream of the respective translational starts.

Materials and Methods

Media and cultivation. The denitrifying bacterium strain EbN1 was cultivated under nitrate-reducing conditions as previously described (Rabus and Widdel 1995). Substrates with low solubility in water were provided as dilutions in 2,2,4,4,6,8,8-heptamethylnonan (HMN), rather than directly added to the medium. The used chemicals were of analytical grade and purity of toluene and ethylbenzene was confirmed by GC-analysis.

Mass cultivation was performed to supply sufficient cell material for RNA and protein analysis. Substrate-adapted cells (see below) were used for inoculation. To reduce slime formation, a phosphate-buffered mineral medium supplemented with NaCl (1 g/l) was used (Tschech and Fuchs 1987). For DNA-microarray and 2D DIGE experiments, strain EbN1 was grown with the following substrate concentrations (v/v) in HMN: 1 % toluene, 2.5 % ethylbenzene, a mixture of 0.5 % toluene and 1.25 % ethylbenzene, and 1 % acetophenone. For real-time RT-PCR experiments, cultures with 1 % toluene, 2 % ethylbenzene, and a mixture of 1 % toluene and 2 % ethylbenzene [(v/v) in HMN] were used. The concentration of benzoate was 4 mM. Cultivation was carried out in 500 ml bottles, containing 400 ml medium, 20 ml carrier phase and 80 ml headspace. Up to 20 parallel cultures for each substrate condition were harvested at OD660 around 0.2, corresponding to the mid-exponential growth phase, as described before (Champion et al. 1999). Cells were washed twice with 100 mM Tris-HCl pH 7.5 containing 5 mM $MgCl_2$, and obtained pellets were immediately frozen in liquid nitrogen and stored at -80 °C.

Physiological adaptation experiments. Cells were adapted to growth with toluene, ethylbenzene, a mixture of both alkylbenzenes, or benzoate over at least 5 passages. These four differently adapted subcultures served as inoculum for subsequent cultivation under three different substrate conditions: toluene (0.3 %, v/v), ethylbenzene (0.3 %, v/v) and a mixture of both alkylbenzenes (each 0.15 %, v/v). Chemical analysis of alkylbenzenes (see below) required reduced substrate concentrations in HMN. Controls lacked either organic substrate or inoculum. During incubation samples were withdrawn from the carrier phase (0.2 ml) and the aqueous phase (1 ml) at intervals of about 3 h. To maintain anoxic conditions during sampling, N_2-flushed, sterile syringes were used. Samples from the carrier phase were stored in Teflon-sealed 0.7 ml flange bottles (WiCom, Heppenheim, Germany) and subsequently analyzed by GC to determine substrate consumption (see below). Samples from the aqueous phase were directly used to monitor growth by measuring the optical density at

660 nm (UV-mini 1240, Shimadzu, Duisburg, Germany). The end of incubation was indicated by the depletion of the electron acceptor nitrate and intermediately produced nitrite. This was monitored with Merckoquant Test Strips (Merck, Darmstadt, Germany). Four parallels (based on 2 independent inoculum cultures) were carried out for each tested substrate condition. Parallel cultures yielded highly similar time courses of growth and alkylbenzene utilization.

Chemical analysis. Concentrations of toluene and ethylbenzene in samples from the carrier phase were determined by gas chromatography. The system consisted of a gas chromatograph equipped with a flame ionization detector (Perkin-Elmer Autosystems, Rodgau-Jügesheim, Germany) and an OPTIMA-5 column (0.25 µm × 50 m; Macherey-Nagel, Düren, Germany). Retention times were 4.6 min (toluene) and 7.1 min (ethylbenzene). The dynamic range was between 0.1 and 10 mM in both cases. Undiluted samples (1 µl) of the carrier phase were injected. Separation was achieved with hydrogen as carrier gas at a flow rate of 120 ml min^{-1}, a split of 1:70 and the following temperature gradient: injection port, 250°C; column, 40°C for 2 min, ramping at 4°C min^{-1}, 60°C for 0.1 min, ramping at 20°C min^{-1}, 320°C for 10 min; flame detector, 350°C.

Preparation of mRNA. Total RNA was prepared from substrate-adapted cells using the hot-phenol method described by Oelmüller et al. (1990). To consider biological variations, RNA was prepared from two independent cultures for each substrate condition. After treatment with RNase-free DNase (MBI Fermentas, St. Leon Roth, Germany), the removal of DNA was confirmed by the absence of PCR-amplifiable target genes, RNA quality was controlled as described (Kube et al. 2004), and RNA aliquots were further purified with RNeasy Mini purification columns (Qiagen, Hilden, Germany).

Real-time RT-PCR. Gene-specific primers (Table 1) were designed with the MacVektor program (Accelrys, Munich, Germany). The antisense primers were used for reverse transcription with H minus M-MLuV reverse transcriptase (MBI Fermentas), applied according to the manufacturers instructions and using 2 µg of total RNA. A 40 cycle real-time PCR was performed on a 25-µl scale, using a SYBR Green ready-mix (Eurogentec, Seraing, Belgium) and standard PCR-conditions. For each reaction, 2 µl of the individual RT-reactions served as template. Primerpairs, complementary to the *bcrC*, *bssA* and *ebdA* sequences, were used to amplify gene-specific products (Table 1). Specificity of accumulated products was verified by melting curve analysis and sequencing. Relative expression levels were calculated and PCR-efficiencies determined, as described by Pfaffl (2001; equation 3 therein) and Ramakers et al. (2003). Concurrent substrate concentrations and time points of cell harvesting (see above), as well as highly similar PCR-efficiencies (around 1.9) for the

three target genes under the different growth conditions underline the significance of determined relative expression levels (Fig. 3). For each tested RNA preparation at least 3 independent real-time RT-PCR experiments were conducted.

Table 1. Primer sequences for genes of anaerobic alkylbenzene degradation in denitrifying strain EbN1.

Primer[a]	Target gene	Sequence (5´→3´)	Product length [Nt]
bcrC 537F	*bcrC*	GCTGAAGAAAGTGCTCGC	459
bcrC 995R	*bcrC*	ATACGGATACGGAAGGGG	
bssA 983F	*bssA*	AGAAGGAAGATTCGCTGC	195
bssA 1177R	*bssA*	CCAAGGTCAGGATGAAGAG	
ebdA 2433F	*ebdA*	TGCCCAGTTCTACCTTGAC	496
ebdA 2928R	*ebdA*	TGCTTTCTTGSTGCTTSCC	

[a] Antisense primers (R) were used for reverse transcription. Primer pairs (F and R) were used for PCR amplification.

Probe amplification (DNA-microarray). PCR products were amplified from genomic DNA fragments as described before (Rabus et al. 2002)(Rabus et al. 2002a). Suitable gene specific primers (av. length 23 bp, av. Tm 60-63°C) were designed using the PRIDE plugin (Haas et al. 1998) from the Staden Program Package (Staden et al. 2000). Following amplification, 25 µl of PCR products were concentrated by isopropanol precipitation. The pellets were dried at room temperature, resuspended overnight in 15 µl spotting buffer (3 × SSC, 1.5 M Betain; ref. 13) on a rotary shaker and subsequently transferred to 384-well polystyrene spotting plates (Genetix, New Milton, UK). Size and concentration of the PCR products were verified by agarose gel electrophoresis and spectrophotometric measurements.

DNA-microarray fabrication. Spotting was performed on a modified Genetix Qarray spotting robot using TeleChem CMP4 split pins (TeleChem, Sunnyvale, CA, USA) and TeleChem SuperAmine slides. After incubation for 12 h at 20°C and 55 % relative humidity, the slides were snap-dried on a heating plate (200°C) for 30 sec. DNA was linked to the slide surface by UV radiation in a Stratagene UV-Stratalinker (2 × 1,200 µJ). The slide surface was subsequently blocked as described elsewhere (Diehl et al. 2001)(Diehl et al. 2001). Slides were stored at room temperature protected from light and humidity until further usage.

cDNA synthesis (DNA-microarray). 15 µg of total RNA was reversely transcribed using Cy-labelled dUTP (Perkin-Elmer), SuperScript II RT (Invitrogen, Karlsruhe, Germany) and Random Nonamer primers as described elsewhere (Khodursky et al. 2000). The labelled

cDNAs were purified using Microcon YM-30 spin columns (Millipore Bedford, MA, USA). Quality of cDNA synthesis was verified by agarose gel electrophoresis and fluorescence scanning of the gel on a Fuji FLA-8000 scanner at 532 and 640 nm followed by subsequent staining of the gel with ethidiumbromide.

Hybridization procedure (DNA-microarray). Cy3- and Cy5-labelled reference and test cDNAs were pooled and mixed with 2 µl of a DNA mixture consisting of 2.5 µg/µl Herring Sperm DNA (Invitrogen), 25 ng/µl acetylated BSA (Promega, Mannheim, Germany) and 0.6 µM of unlabelled oligonucleotides. This mixture was added to the labelled samples, concentrated in a SpeedVac (volume of 3 - 5 µl), resuspended in 50 µl DIG Easy Hyb Buffer (Roche Diagnostics, Mannheim, Germany), and denatured at 95°C for 3 min prior to transfer to the slide surface. Hybridization was conducted for 16 h at 42°C in a hybridization chamber (Scienion AG, Berlin, Germany). After washing (once with 0.2 × SSC, 0.1 % [w/v] SDS and twice with 0.2 × SSC for 5 minutes) and drying by centrifugation, the slides were scanned on a GMS 418 microarray scanner (MWG Biotech AG, Ebersberg, Germany). Image processing and grid placement was achieved with the AIDA image analysis software package (Raytest, Straubenhardt, Germany). For each investigated test state (growth with ethylbenzene, toluene or a mixture of both) a total of 7 slides were hybridized with random primed, Cy3- or Cy5-labelled cDNA. Each slide contained 4 identical blocks. For each gene 3 replicate samples were spotted per block. Thus 84 (7 × 4 × 3) replicates were used for each gene and treatment in the final calculations.

Image analysis (DNA-microarray). The quantification was based on the pixel positions resulting from the grid placement with the AIDA software. Intensities of spots and background were calculated and normalized (Steinfath et al. 2001) by applying a median method to three reference genes. For this purpose *bcrC* (γ-subunit of benzoyl-CoA reductase), *mdh* (malate dehydrogenase) and *ssb* (single strand binding protein) were selected, since they were assumed to be constitutively expressed. The median of this reference set (88 replicates per slide) was determined for each single slide and for the whole data set (Steinfath et al. 2001). In the normalized data all replicates of a single gene were averaged resulting in the mean value. The ratios of the means between a test state (adaptation to ethylbenzene, toluene or a mixture of both) and the reference state (adaptation to benzoate) were calculated. Standard error and Welch test indicated significance of differential expression (Sachs 1993). The significance level for the p-value was 0.01.

Reproducibility of the hybridization experiments was as follows. When different RNA preparations from the same test state were used for parallel hybridizations, the correlation coefficients (linear scale) of spot intensities ranged between 0.83 and 0.93. Changing the order of CyDye-labelling between test and reference state resulted in correlation coefficients closer to the lower limit, while constant CyDye-labelling gave values closer to the upper limit. Comparing spot intensities from independent cultures of the same test state gave correlation coefficients in the same range. Correlation coefficients were low (< 0.5), when spot intensities representing different test states were compared.

2-Dimensional difference gel electrophoresis (2D DIGE). Cell disruption with the PlusOne Sample Grinding Kit (Amersham Biosciences, Freiburg, Germany) and preparation of protein extracts were carried out as recently reported (Gade et al. 2003). Protein concentration was determined according to the method described by Bradford (1976).

Isoelectric focussing (IEF) was performed as described before (Gade et al. 2003; Rabus et al. 2002), using the IPGphor system and 24 cm IPG strips with linear pH gradients of 4-7 and 3-10 (Amersham Biosciences). To enhance reproducibility and resolution of IEF in the alkaline range of pH3-10 gradients, the rehydration buffer contained 1.2 % DeStreak reagent (Amersham Biosciences) instead of 0.4 % DTT. The EttanDalt II system (Amersham Biosciences) was used for separation according to molecular weight in 12.5 % Duracryl gels (Genomic Solution, Ann Arbor, Mi, USA) as described (Gade et al. 2003). Low fluorescent glass plates were used for 2D DIGE.

2D DIGE was essentially carried out as described by Gade et al. (2003). 200 pmol CyDye were used for labelling of 50 µg of protein sample. Pre-electrophoretic labelling with different fluorescent dyes allows co-separation 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 5 parallel gels were run per experiment. Protein extracts from benzoate-adapted cultures served as reference state and were labelled with Cy5. Protein extracts from cultures adapted to anaerobic growth with toluene, ethylbenzene, a mixture of both, or acetophenone represented the test states and were each labelled with Cy3. All four test states were related to the same reference state. All performed experiments contained the same preparation of internal standard, which was composed of equal amounts of reference state and all test states and was labelled with Cy2.

2D DIGE gels were scanned immediately after electrophoresis with a Typhoon 9400 scanner (Amersham Biosciences). Analysis of cropped images was performed with the DeCyder software (version 5.0; Amersham Biosciences). Parameters for co-detection of spots

were: (i) detection of 2500 spots in pH 4-7 gels and of 3000 spots in pH 3-10 gels and (ii) exclusion of signals with slope > 1, area < 200, peak height < 190 and volume < 60000. Statistical analysis was based on independent spot maps. Differentially regulated spots were manually controlled and fulfilled the following criteria: average ratio of < -2.5 and > 2.5; ANOVA p-value of < 0.05; t-test value of < 10^{-4}; number of matched gels > 33/45. The pH 4-7 master gel contained 1724 matched protein spots. Total numbers of substrate-specific regulated protein spots were: 215, 182, 239 and 188 up-regulated, and 26, 24, 20 and 73 down-regulated protein spots in cells adapted to toluene, ethylbenzene, a mixture of both, and acetophenone respectively. For better perception the Cy2 channel was omitted in Figs. 4 and 5. A second set of 2D DIGE experiments with samples from independent mass cultures yielded essentially the same results for each of the four investigated test states (data not shown).

In order to analyze regulated proteins by mass spectrometry, separate preparative (500 µg protein load) gels were run and stained with colloidal Coomassie Brilliant Blue (cCBB) according to the method described by Doherty et al. (1998).

Protein identification by mass spectrometry. Selected up-regulated protein spots were excised with an automatic excision workstation (PROTEINEER sp, Bruker Daltonics, Bremen, Germany). Tryptic digest of excised proteins and preparation of the MALDI target with α-cyano-4-hydroxycinnamic acid as matrix was carried out with a PROTEINEER dp digest and sample preparation robot (Bruker Daltonics) as recently described (Kube et al. 2004). Acquisition of MS fingerprints was achieved with an ultraflex TOF/TOF instrument (Bruker Daltonics) as recently described (Kube et al. 2004). Fingerprint information was submitted to a protein database search using the Mascot search engine (Matrix Science, London, UK). The database contained essentially the protein sequences predicted to be involved in the anaerobic degradation of toluene (Kube et al. 2004) and ethylbenzene (Rabus et al. 2002) by denitrifying strain EbN1.

Nucleotide sequence accession numbers. DNA sequences of new genes of strain EbN1 have been deposited at EMBL under the accession numbers CR792447 (*ebA1926*, *ebA1928*, *ebA1929*, *ebA1932*, *ebA1936*, *tnpF* and *ebA1938*), CR792444 (*bcrC*), CR792558 (*mdh*) and CR792561 (*ssb*).

Results

Regulation of the anaerobic degradation pathways for toluene and ethylbenzene in strain EbN1 (Fig. 1) by their respective substrates was investigated *in vivo* by measuring substrate consumption in growth cultures (Fig. 2) and *in vitro* on RNA (Fig. 3, Table 2) and protein level (Figs. 4 and 5, Table 3). The common basis for these experiments was cells of strain EbN1 adapted to one of five different substrate conditions. Compounds used for adaptation were on the one side the key-substrates of the investigated pathways, i.e. toluene and ethylbenzene (supplied as single substrate or as mixture), and acetophenone, and on the other side benzoate, which represents the first common intermediate (Fig. 1). Benzoate-adapted cells served as reference state, since the degradative capacities for the other substrates were not induced in them. To allow optimal comparison, global expression studies (DNA-microarrays or proteomics) were conducted with the same cell batches.

Differential alkylbenzene consumption during growth. Four differently adapted types of cultures of strain EbN1 were studied. In each case, cultures were shifted to either toluene, ethylbenzene or a mixture of both alkylbenzenes as sole source of organic carbon. Due to their toxicity and low water-solubility, the alkylbenzenes were added as solution in an inert carrier phase. During growth, consumption of the alkylbenzenes was monitored in samples withdrawn from the carrier phase. Results are shown in Fig. 2.

When cultures were adapted to benzoate utilization, consumption of toluene and ethylbenzene occurred only after prolonged incubation (Fig. 2A). Regardless of whether the alkylbenzenes were added as single substrates or as mixture, similar induction times of less than 15 h were observed. Interestingly, degradative capacities for both alkylbenzenes were induced simultaneously when provided as mixture. Thus neither of them repressed the onset of the degradation of the other alkylbenzene.

Toluene-adapted cultures (Fig. 2B) utilized toluene, as expected, almost instantly. In contrast, consumption of ethylbenzene required an even longer induction time in toluene-adapted cells, than observed in case of the benzoate-adapted cells. When cells were supplied with a mixture of both alkylbenzenes, toluene utilization again set in early. During the course of toluene consumption, degradation of ethylbenzene already started. This finding indicated that ongoing toluene degradation does not repress the induction of the capacity to degrade ethylbenzene.

In case of ethylbenzene-adapted cultures (Fig. 2C), an analogous pattern of substrate utilization was observed. Ethylbenzene was consumed rather rapidly, whether supplied as single substance or as mixture with toluene. The latter was only utilized after a long induction time, but then also while ethylbenzene was still consumed.

Time courses of toluene and ethylbenzene utilization were similar, when cells were adapted to their mixture (Fig. 2D).

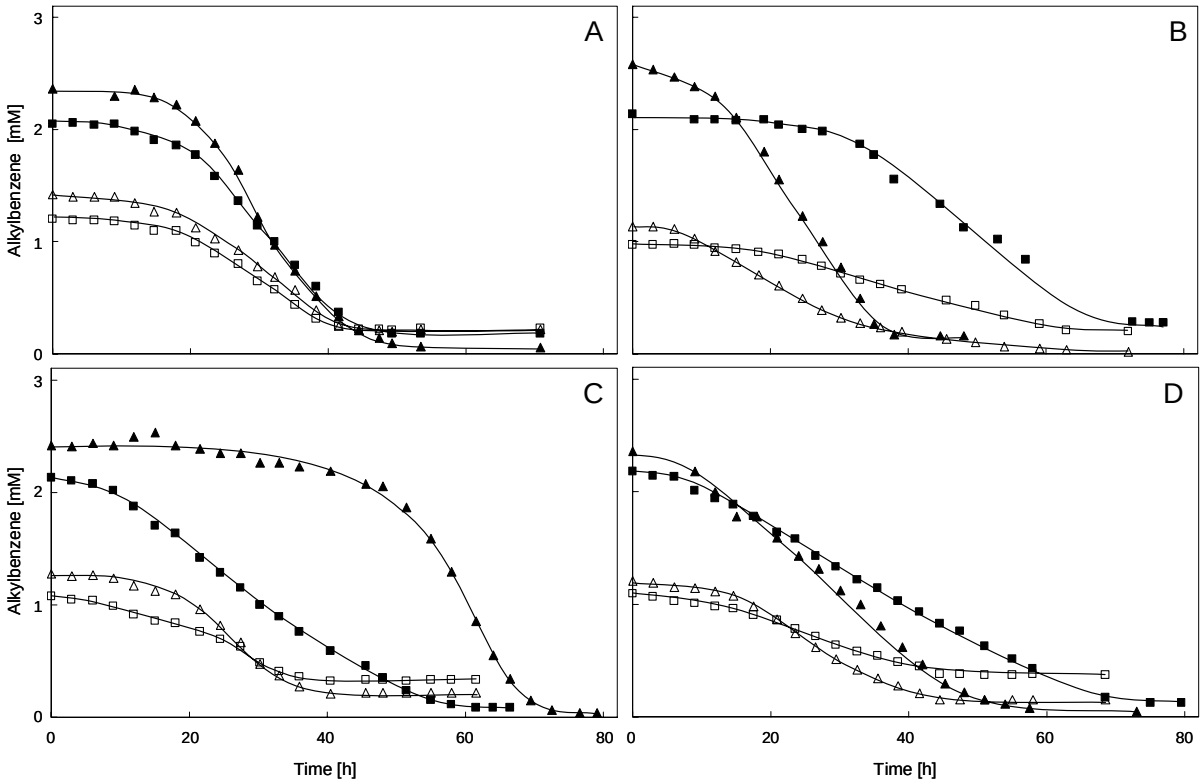

Fig. 2. Consumption of toluene and ethylbenzene during growth of strain EbN1 under nitrate-reducing conditions. Cultures were inoculated with anaerobic substrate-adapted cells. (A) Benzoate-adapted cells. (B) Toluene-adapted cells. (C) Ethylbenzene-adapted cells. (D) Cells were adapted to a mixture of toluene and ethylbenzene. Symbols: ▲, toluene, when provided as single substrate; ■, ethylbenzene, when provided as single substrate; △, toluene, when provided as mixture with ethylbenzene; □, ethylbenzene, when provided as mixture with toluene.

Monitoring *bssA* and *ebdA* expression with real-time RT-PCR. As first step towards the transcriptional analysis of the pathway regulation, a real-time RT-PCR approach was established to monitor expression of the *bssA* and *ebdA* genes (Fig. 3). Both are particularly relevant, since they encode the catalytic subunits of benzylsuccinate synthase and ethylbenzene dehydrogenase, respectively, the initial enzymes of the two pathways. Gene expression was investigated in cells adapted to benzoate, toluene, ethylbenzene, the mixture of both alkylbenzenes, and acetophenone. Benzoate-adapted cells served as reference state. *BcrC* was

selected as reference gene, since it codes for the γ-subunit of benzoyl-CoA reductase, the first common enzyme after convergence of the two pathways. Agreeing with the *in vivo* experiments (Fig. 2A), only basal expression of *bssA* and *ebdA* was observed in benzoate-adapted cells.

In ethylbenzene-adapted cells, expression of *ebdA* was about 550-fold up-regulated, while expression of *bssA* was only marginal. Apparently, the predicted sensor for toluene (TdiS) is not responsive to ethylbenzene. In contrast, cells adapted to growth with toluene displayed an up-regulation of *ebdA* and *bssA* expression of about 100-fold. This unexpected finding may suggest a relaxed specificity of the predicted ethylbenzene sensor (Tcs2), also recognizing toluene.

Agreeing with the *in vivo* behaviour of cells adapted to growth with the alkylbenzene mixture (Fig. 2D), *ebdA* and *bssA* were about 180- and 70-fold up-regulated under these growth conditions. This was in each case about 3-fold lower than observed with cells adapted to toluene or ethylbenzene as single substrates. While the rational behind this observation is currently not understood, it cannot be attributed to differing substrate concentrations, since these were always the same for the individual alkylbenzene, when supplied as single substrate or as mixture.

In acetophenone-adapted cells, the expression of *bssA* and *ebdA* was as low as in benzoate-adapted cells, indicating that neither of the two predicted alkylbenzene sensors (TdiS and Tcs2) recognizes acetophenone. Moreover, this result agrees with the previous hypothesis of a sequential regulation of the ethylbenzene pathway (Rabus et al. 2002).

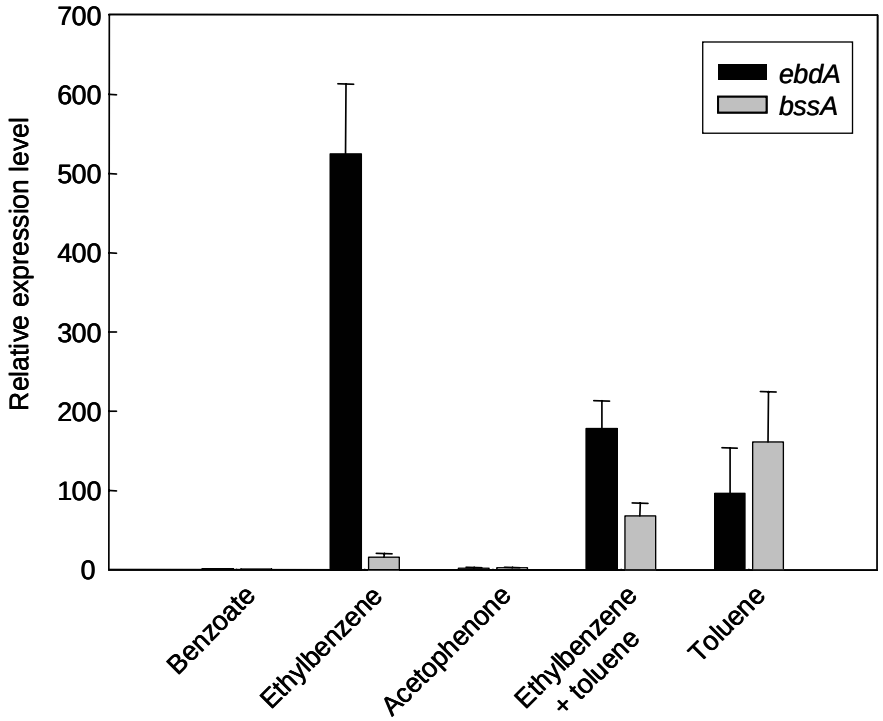

Fig. 3. Expression levels of *bssA* and *ebdA* in substrate-adapted cells of strain EbN1. *bssA* and *ebdA* encode the catalytic subunits of benzylsuccinate synthase and ethylbenzene dehydrogenase, respectively. *bcrC* was used as reference gene and benzoate-adapted cells as reference state. Substrates used for adaptation are indicated. Relative expression levels were determined with real-time RT-PCR.

Transcriptional profiling with DNA-microarray. Following expression analysis of single genes (*bssA* and *ebdA*) by means of real-time RT-PCR, a DNA-microarray was designed for transcriptional profiling of both complete pathways. The DNA-microarray contained probes for all genes predicted to be involved in anaerobic toluene (Kube et al. 2004) and ethylbenzene (Rabus et al. 2002) degradation. In addition, neighbouring genes, mostly coding for proteins of unknown function, were also represented on the DNA-microarray. Labelled cDNA for hybridization experiments was generated from total RNA prepared from substrate-adapted cells. Results from DNA-microarray analysis are summarized in Table 2.

Transcriptional profiling of ethylbenzene-adapted cells revealed a specific up-regulation of all genes previously predicted to be involved in anaerobic ethylbenzene degradation (Rabus et al. 2002). Highest average ratios were observed for *ebdC* (21.1-fold; encoding γ-subunit of ethylbenzene dehydrogenase), *ped* (24.5-fold; encoding (*S*)-1-phenylethanol dehydrogenase) and *apc4* (22.2-fold; encoding a subunit of acetophenone carboxylase). The previous suggestion of *bal* encoding benzoylacetate CoA-ligase was supported by its specific 16.1-fold up-regulation; biochemical characterization will provide final proof. Interestingly, *orf92*, *orf90*, *orf87* and *orf84*, which are located directly downstream of the *apc*/*bal* gene cluster, were also specifically up-regulated (9.1-, 15.3-, 22.1- and 6.8-fold). An unexpected finding was the strong up-regulation of *orf68* and *orf57* (22.5- and 14.9-fold). These orfs frame the *ebd*-operon and their products are of unknown function (Rabus et al. 2002). As observed during the real-time RT-PCR experiment with *bssA* (see above), genes involved in anaerobic toluene degradation (Kube et al. 2004) were not up-regulated in ethylbenzene-adapted cells. The 17 selected toluene-related genes displayed average ratios ranging between 1.1 and 2.0 (median at 1.4). These results further corroborated the aforementioned specificity of the suggested toluene sensor (TdiS) and also demonstrated the suitability of the used DNA-microarray design to study differential regulation of the two pathways.

When cultures of strain EbN1 were adapted to anaerobic growth with toluene, all 17 genes assigned to the *bss* and *bbs* operons were up-regulated, agreeing with the growth physiology of the investigated cells. Highest expression levels were observed for *bssA*, *bssB* and *bbsG* (28.8-, 18.1- and 17.4-fold). Several proteins of unknown function are encoded directly downstream of the *bss*-operon by the *c2B001*, *c2A200*, *c2A203*, *c2A204* and *c2A308* genes. These genes appeared to be up-regulated during anaerobic growth with toluene (average ratios of 3.2-, 2.5-, 4.8-, 3.4-and 3.6-fold). Specificity of expression is confirmed by only marginal expression during anaerobic growth with ethylbenzene (average ratios around 1.1).

Table 2. Average ratios[a] of expression of genes involved in anaerobic alkylbenzene degradation, as determined by DNA-microarray analysis of substrate-adapted cells of strain EbN1.

Gene[b]	Avg ratios (± SD) Cells adapted to anaerobic growth with		
	Ethylbenzene	**Toluene**	**Ethylbenzene + toluene**
Toluene-related genes			
bssD	1.1 ± 0.1	6.4 ± 0.8	3.2 ± 0.3
bssC	1.4 ± 0.1	12.8 ± 1.7	6.2 ± 0.8
bssA	1.6 ± 0.2	28.8 ± 4.4	13.6 ± 1.7
bssB	1.5 ± 0.2	18.1 ± 2.3	8.8 ± 0.9
bssE	1.2 ± 0.1	9.2 ± 1.1	5.0 ± 0.5
bssF	1.2 ± 0.2	4.6 ± 0.5	2.7 ± 0.3
bssG	1.3 ± 0.1	3.7 ± 0.3	2.1 ± 0.2
bssH	1.1 ± 0.1	3.5 ± 0.3	1.7 ± 0.1
c2B001	1.3 ± 0.1	3.2 ± 0.3	1.5 ± 0.1
c2A200	0.8 ± 0.1	2.5 ± 0.4	1.2 ± 0.2
c2A203	1.1 ± 0.1	4.8 ± 0.4	1.9 ± 0.1
c2A204	1.1 ± 0.1	3.4 ± 0.3	1.5 ± 0.1
c2A308	1.1 ± 0.1	3.6 ± 0.4	1.6 ± 0.2
bbsH	1.1 ± 0.1	11.1 ± 1.4	3.9 ± 0.4
bbsG	1.4 ± 0.2	17.4 ± 2.2	9.1 ± 0.9
bbsJ	1.8 ± 0.2	13.9 ± 1.8	7.5 ± 1.0
bbsF	1.2 ± 0.1	7.8 ± 0.9	3.9 ± 0.4
bbsE	1.4 ± 0.1	4.5 ± 0.5	2.8 ± 0.2
bbsD	1.3 ± 0.1	4.5 ± 0.5	3.8 ± 0.4
bbsC	1.7 ± 0.3	5.2 ± 0.7	4.0 ± 0.6
bbsB	2.0 ± 0.2	4.3 ± 0.5	3.2 ± 0.3
bbsA	1.3 ± 0.1	2.3 ± 0.2	1.7 ± 0.1
Ethylbenzene-related genes			
orf57	14.9 ± 1.9	5.1 ± 0.6	5.8 ± 0.7
ped	24.5 ± 3.6	5.7 ± 0.8	5.7 ± 0.8
ebdD	11.4 ± 1.5	5.1 ± 0.7	3.6 ± 0.5
ebdC	21.1 ± 2.2	7.8 ± 0.8	4.7 ± 0.5
ebdB	15.8 ± 1.9	6.2 ± 0.8	4.3 ± 0.5
ebdA	12.0 ± 1.4	5.7 ± 0.6	2.6 ± 0.3
orf68	22.5 ± 4.1	12.0 ± 2.2	4.7 ± 0.8
orf84	6.8 ± 0.7	2.8 ± 0.3	3.6 ± 0.3
orf87	22.1 ± 2.3	7.4 ± 0.8	14.3 ± 1.5
orf90	15.3 ± 1.8	5.0 ± 0.6	6.7 ± 0.8
orf92	9.1 ± 1.1	3.7 ± 0.4	5.2 ± 0.5
bal	16.1 ± 1.9	3.8 ± 0.4	6.6 ± 0.8
apc5	14.1 ± 2.0	3.7 ± 0.5	6.4 ± 0.7
apc4	22.2 ± 2.7	4.5 ± 0.5	7.6 ± 0.9
apc3	12.5 ± 1.6	2.7 ± 0.3	3.4 ± 0.4
apc2	11.8 ± 1.4	2.0 ± 0.2	2.3 ± 0.3
apc1	10.9 ± 1.5	2.0 ± 0.3	2.3 ± 0.3
Other genes			
orf206	1.8 ± 0.2	2.3 ± 0.2	1.6 ± 0.1
fadB	1.6 ± 0.2	2.4 ± 0.2	1.5 ± 0.2
fadA	2.3 ± 0.2	2.7 ± 0.2	2.0 ± 0.2
fadE	1.5 ± 0.2	2.1 ± 0.2	1.6 ± 0.2

[a] Average ratios were calculated with benzoate-grown cells as reference as described in the Materials and Methods section. Average ratios are provided with standard deviation.

[b] Organization and names of genes are as described in Fig. 1; except for the *fad* genes and *orf206* which are located in 15 kb distance from the gene cluster of the ethylbenzene pathway (42).

Previous genomic reconstruction of the toluene pathway in strain EbN1 also indicated the presence of the *bbsJ* gene within the *bbs*-operon. Only fragments of this gene are detectable in the *bbs*-operon of well-studied *T. aromatica* strain K172 (Kube et al. 2004). The *bbsJ* gene was specifically up-regulated by 13.6-fold in toluene grown cells, while the expression level in ethylbenzene-grown cells was only 1.8. In analogy to the above described results from the real-time RT-PCR experiment, genes involved in the ethylbenzene pathway were also up-regulated. However, expression levels for *ebd* and *apc* genes were 2-3 and 4-5-fold, respectively, lower than in ethylbenzene-adapted cells.

Genes for both pathways were clearly up-regulated in cells adapted to simultaneous utilization of ethylbenzene and toluene. In both cases the determined expression levels were about 3- to 4-fold lower than in cells grown with the individual alkylbenzene, agreeing with the results from the real-time RT-PCR experiment.

In the present study, the average ratios for most highly up-regulated genes ranged between 20 and 30. These values are considerably lower than relative expression levels determined with the real-time RT-PCR approach. This agrees with the observation from several laboratories that DNA-microarray analyses generate about 10-fold lower expression levels than other generally applied methods for RNA analysis (Conway and Schoolnik 2003).

Analysis of relative protein abundances with 2D DIGE. Soluble proteins extracted from the differently adapted cells were electrophoretically separated in a pH range from 3 to 10. Expanding IEF to alkaline pH was required, since some of the predicted proteins displayed a theoretical isoelectric point in this range (e.g. EbdC with pI 9.16). More than 150 protein spots were detected by 2D DIGE analysis having increased abundances in response to anaerobic growth with toluene or ethylbenzene (see Materials and Methods). For identification of the abundant ones among them, corresponding spots were excised from cCBB-stained gels. Mass-spectrometric analysis yielded average MASCOT scores of at least 100, based on at least 3 independent analyses. With this procedure, 8 out of the 17 predicted gene products of anaerobic toluene degradation (Kube et al. 2004) and 7 out of the 11 predicted gene products of anaerobic ethylbenzene degradation (Rabus et al. 2002) were identified. Several other abundant, differential proteins could not be identified at present. Results from 2D DIGE analysis and mass-spectrometric identification are summarized for alkylbenzene-adapted cells in Fig. 4 and Table 3 and for acetophenone-adapted cells in Fig. 5.

In toluene-adapted cells (Fig. 4A) the 8 identified toluene-specific proteins were 4- to 155-fold up-regulated, e.g. BbsH by a 155-fold. In accordance with the transcriptional analysis also gene products from the ethylbenzene pathway were up-regulated in toluene-adapted

cells, even though to a lesser extent than the toluene-specific gene products. Further proteins, which are not encoded in the *bss* and *bbs* operons, but nevertheless specifically up-regulated in toluene-adapted cells, were EbA1932 and EbA1936 (exclusively discovered by the global proteomic approach). Their identified coding genes are organized in an operon-like structure and located 7.2 kb upstream of the *bbs* operon (Fig. 1, Table 4). Interestingly, the same consensus sequence as previously described for the promoter regions of the *bss* and *bbs* operons (legend to Fig. 1A; ref. 31) is present upstream of *ebA1936*, agreeing well with the toluene-specific co-induction.

Table 3. 2D DIGE determined average ratios of amounts of mass-spectrometrically identified proteins of anaerobic alkylbenzene degradation in substrate-adaptated cells of strain EbN1.

Protein[a]	MS-identification[b]		Avg ratios in cells adapted to anaerobic growth with[e]		
	No. of repeats[c]	Avg score[d]	Toluene	Ethylbenzene	Toluene + ethylbenzene
Toluene pathway					
BssD	4	226	9	1	9
BssE	4	383	14	1	13
BssG	4	163	nd	nd	nd
BbsH	4	278	155	2	138
BbsG	3	176	4	1	3
BbsF	4	180	9	1	7
BbsD	3	280	34	2	27
BbsC	4	149	77	-2	65
BbsB	3	117	25	-2	19
BbsA	4	231	27	1	24
EbA1932	3	246	54	1	44
EbA1936	3	271	46	1	45
Ethylbenzene pathway (upper part)					
EbdD	4	391	3	14	8
EbdC	4	114	40	242	92
Orf68	11	222	163[f]	281[f]	63[f]
Ethylbenzene pathway (lower part)					
Orf84	3	216	6	28	15
Bal	3	425	2	4	3
Apc5	3	249	5	34	17
Apc4	4	466	13	88	32
Apc3	4	188	4	13	9
Apc1	4	423	3	8	5

[a] Order and names of proteins as described for the corresponding genes in Fig. 1.
[b] A MASCOT-score of >100 was used as threshold for positive identification by PMF.
[c] Regulated proteins were identified under each growth condition at least three times.
[d] Average MASCOT score was calculated from the repetitive identification experiments.
[e] Average ratios were determined with 5 parallel gels; benzoate-grown cells were used as reference. For details see Materials and Methods. nd, not detected in 2D DIGE gels, even though detected in CBB/silver stained gels. Possibly, the presence of only one lysine residue in BssG rendered this protein unsuitable for labeling with CyDyes under the applied conditions.
[f] Protein species that are separated as two spots with different pI and similar M*r* on the 2DE gel.

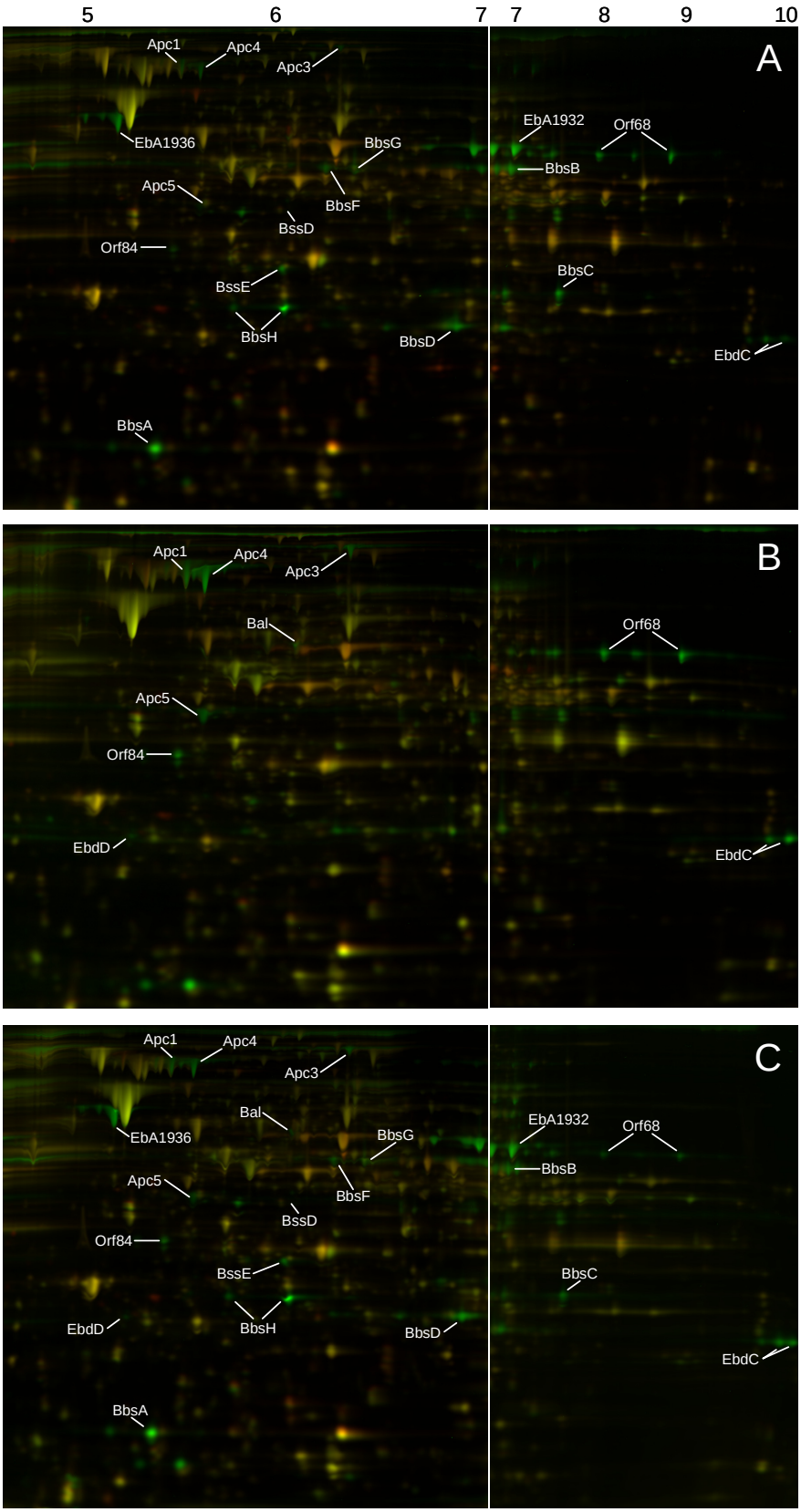

Fig. 4. Formation of pathway-specific proteins in substrate-adapted cells of strain EbN1 as determined by 2D DIGE. (A) Toluene-adapted cells. (B) Ethylbenzene-adapted cells. (C) Cells adapted to a mixture of toluene and ethylbenzene. Enzyme abbreviations are as described in Fig. 1. Mass-spectrometric identification and relative abundances of marked proteins are shown in Table 3.

When cells were adapted to anaerobic ethylbenzene utilization (Fig. 4B), the 7 identified ethylbenzene-specific proteins were strongly up-regulated (4- to 242-fold). In contrast, the electrophoretically detectable proteins of the toluene pathway appeared to be absent. Thus also the proteomic results support the view that toluene and ethylbenzene sensors of strain EbN1 exhibit different degrees of specificity towards their target molecules. In addition to the previously described gene products of the ethylbenzene pathway (Rabus et al. 2002), also the products of *orf68* and *orf84* were found as most strongly up-regulated in response to ethylbenzene degradation (281- and 28-fold, respectively). In fact, the *orf68* product belongs to the abundant proteins detected on the 2DE gels. Specific up-regulation of *orf68* and *orf84* was also found by DNA-microarray analysis (Table 2).

When cells were anaerobically grown with a mixture of toluene and ethylbenzene (Fig. 4C), both sets of proteins were up-regulated. Relative to the cultures adapted to a single alkylbenzene, here the relative protein abundances were lower.

Acetophenone-adapted cells (Fig. 5) are characterized by the absence of EbdC (γ-subunit of ethylbenzene dehydrogenase) and EbdD (predicted chaperone for maturation of ethylbenzene dehydrogenase). This agrees well with the absence of *ebdA* expression (α-subunit of ethylbenzene dehydrogenase) in acetophenone-adapted cells as revealed by the aforementioned real-time RT-PCR experiments (Fig. 3). In contrast, several subunits of acetophenone carboxylase (Apc1, 72-fold; Apc3, 36-fold; Apc4, 104-fold; Apc5, 6-fold) and the predicted benzoylacetate CoA-ligase (Bal, 11-fold) were strongly up-regulated in the same cells (Fig. 5). Apparently, the adaptation to acetophenone as sole organic substrate resulted in the specific formation of only those enzymes, which are involved in its catabolism.

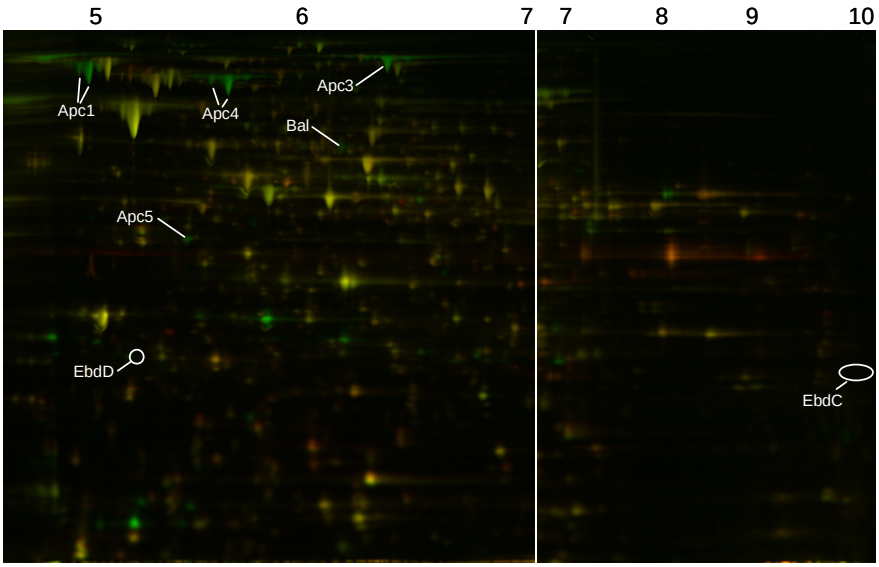

Fig. 5. Formation of pathway-specific proteins in acetophenone-adapted cells of strain EbN1 as determined by 2D DIGE. Enzyme abbreviations are as described in Fig. 1. Identification of annotated spots was based on mass-spectrometric analyses. Spot coordinates of Apc proteins deviate for presently unknown reasons from those in alkylbenzene-adapted cells, while about 800 other protein spots could be mapped.

Discussion

Simultaneous toluene and ethylbenzene utilization. The parallel utilization of toluene and ethylbenzene in growth cultures was reflected in a simultaneous expression of genes from both degradation pathways. Therefore, none of the two alkylbenzenes is regarded as a preferred substrate, precluding a regulatory process such as catabolite repression at this level. This regulatory behaviour of strain EbN1 might reflect an adaptation to low concentrations of the rather insoluble alkylbenzenes, as usually encountered in the natural environment. Repression of these pathways by substrates like succinate might occur, since this is known from aerobic aromatic degradation (Dahl et al. 2002; Duetz et al. 1996; Galan et al. 2001; Torres et al. 2003).

Sequential regulation of ethylbenzene pathway. The upper and lower parts of the pathway for anaerobic ethylbenzene degradation were previously suggested to be sequentially induced by their respective substrates, ethylbenzene and acetophenone (Rabus et al. 2002). In fact strain EbN1 can utilize both compounds as growth substrates (Rabus and Widdel 1995). Such a regulatory scenario is supported by the absence of *ebdA* expression (Fig. 3) and EbdCD formation (Fig. 5) in acetophenone-adapted cells, as well as by the concomitant presence of several subunits of acetophenone carboxylase and of putative benzoylacetate CoA-ligase (Fig. 5). A sequential mode of regulation could be of economic benefit, considering that strain EbN1 probably encounters acetophenone (a plant metabolite) as substrate in the natural soil habitat. Similarly, in *Pseudomonas putida*, the pathway for aerobic degradation of toluene/xylenes is also divided into two sequentially regulated parts (Ramos et al. 1997).

Substrate-specificity of pathway regulation. Substrate-dependent regulation of aromatic catabolism usually occurs on the transcriptional level involving a large variety of transcriptional regulators (Diaz and Prieto 2000; Gerischer 2002). Two different two-component regulatory systems were previously suggested to trigger expression of genes involved in anaerobic toluene (TdiSR system) and ethylbenzene (Tcs2/Tcr2 system) degradation in strain EbN1. Toluene recognition by the TdiSR system was suggested due to the close neighborhood of its coding genes to the *bss* operon and the high similarity to other two-component regulatory systems for toluene degradation in related strains (Kube et al. 2004). Even though the Tcs2/Tcr2 and TdiSR systems are also highly similar to each other, their sensor components differ markedly in one of the PAS domains implicated in substrate sensing. This finding together with the close proximity of the *tcs2*/*tcr2* genes to the catabolic operons of the

ethylbenzene pathway suggested that Tcs2 recognizes ethylbenzene (Kube et al. 2004). In addition, a third two-component system (Tcs1/Tcr1 system) was suggested to be implicated in recognition of acetophenone, due to its similarity to described *p*-hydroxyacetophenone sensing systems of plant pathogenic bacteria (Kube et al. 2004). The present study suggests a strict specificity of the predicted toluene sensor, which is contrasted by relaxed specificities of the predicted ethylbenzene (Tcs2) and acetophenone (Tcs1) sensors. One may speculate that the sensory pocket receiving toluene in the TdiS-protein cannot accommodate the larger ethylbenzene molecule. In contrast, toluene may access the sensory pockets of Tcs1 and Tcs2. Alternatively, a cross-regulation between TdiSR and the other two systems may occur. The relaxed specificity of the ethylbenzene and acetophenone sensors could explain the low-level expression of all "ethylbenzene"-genes in cells grown with toluene, suggesting that toluene acts as gratuitous inducer for the ethylbenzene-specific operons. The *in vivo* experiments with toluene-adapted cells (Fig. 2B) indicated that the capacity to degrade ethylbenzene had to be induced under these conditions. Thus the low-level expression of "ethylbenzene"-genes may facilitate metabolic adaptation when ethylbenzene becomes available. Indeed, the maximal consumption rate of ethylbenzene was reached earlier in toluene-adapted cells (around 20 h; Fig. 2B) than that of toluene in ethylbenzene-adapted cells (>30 h; Fig. 2C). Future investigations might aim at defining the sensory domain and molecular mechanism that are responsible for alkylbenzene recognition. In this respect, it should be noted, that benzylsuccinate rather than toluene was only recently suggested as the true inducer of *tut* (corresponds to *bss*) expression in *T. aromatica* strain T1, since cells grown with pyruvate expressed *tut* genes upon addition of benzylsuccinate (Coshigano and Bishop 2004). Such a regulatory scenario appears unlikely in strain EbN1, since an upregulation of the *bssA* expression (real-time RT-PCR) or formation of Bss/Bbs proteins (proteomics) was not observed in cells grown with a mixture of pyruvate (10 mM) and benzylsuccinate (1 mM) (data not shown).

Further gene products involved in anaerobic alkylbenzene degradation. Another valuable outcome of the DNA-microarray/proteomic approach was that due to their differential expression further genes could be related to either of the two investigated pathways. The genes *orf68* and *orf57* are located directly up- and downstream of the *ebdA* and *ped* genes, respectively (Fig. 1B). The rather large intergenic regions between *orf68* and *ebdA* (267 bp), and *ped* and *orf57* (150 bp), indicate that *orf68* and *orf57* may not be part of the *ebd* operon, which was previously suggested to consist of the *ebdABCD* and *ped* genes (Rabus et al. 2002). Nevertheless, they are likely to be also involved in anaerobic

ethylbenzene degradation, since their expression was highest up-regulated in cells adapted to ethylbenzene (in case of *orf68* by 22.5- and >200-fold, according to DNA-microarray and 2D DIGE analysis, respectively). The predicted *apc* operon of the lower part of the ethylbenzene pathway probably extends beyond *bal*, since adjacent genes *orf92*, *orf90*, *orf87* and *orf84* were highest up-regulated in response to growth with ethylbenzene. However, their function remains elusive so far. Since selected genes of a β-oxidation complex (*fadABE*) and a thioesterase (*orf206*) encoded in 15 kb distance to the *ebd* operon were not up-regulated during growth with ethylbenzene (Table 2), the missing benzoylacetyl-CoA thiolase remains unaccounted for. In toluene-adapted cells 5 genes (*c2B001*, *c2A200*, *c2A203*, *c2A204* and *c2A308*) were specifically up-regulated according to DNA-microarray analysis. These genes are located directly downstream of *bssH* and the short intergenic distances (0-22 bp) of *c2B001-c2A204* already suggested them to be part of the *bss* operon (Kube et al. 2004). Considering the specific expression of *bbsJ*, *ebA1936* and *ebA1932* anaerobic toluene degradation might also involve more gene products than previously thought. EbA1932 and EbA1936 are highly similar to the products of *orf1* and *orf2* from *T. aromatica* K172 where they are located adjacent to the *tdiSR* genes. Due to the presence of transposase genes (*istAB*) and a transposon gene fragment (*tnpF4*) downstream of *ebA1932* and another transposon gene fragment (*tnpF*) upstream of *ebA1936* (Fig. 1A, Table 4), one may speculate that these genes have been translocated in the genome of strain EbN1, as compared to the organization in *T. aromatica* K172. Since the products of both genes belong to the most abundant proteins on the 2DE gel, they are probably involved in anaerobic toluene degradation; however an erroneous formation cannot be excluded. The close proximity of an RND-type transporter encoding gene (*ebA1928*) may point to an involvement in coping with toxic toluene concentrations, as recently suggested for BssH (Kube et al. 2004). Results also indicate an involvement of more than two transcriptional units in case of both pathways.

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Response of denitrifying strain EbN1 to sublethal concentrations of alkylbenzene growth substrates

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Abstract

The denitrifying Betaproteobacterium strain EbN1 is unique for its capacity to degrade toluene and ethylbenzene under anoxic conditions. Alkylbenzenes are unusual substrates, since their extraordinary chemical stability necessitates various novel degradation reactions and their toxic properties challenge cellular viability. To study the toxic effects, strain EbN1 was grown with standard to sublethal (almost growth preventing) concentrations of toluene (80, 240 and 740 µM) and ethylbenzene (80, 210 and 315 µM). Growth was similar at the low and intermediate concentrations. However, at the high (sublethal) concentration, doubling times increased from 8 to 40 h, intermediate excretion of nitrite was markedly reduced and phosphatidylglycerol (PG) increased at the expense of phosphatidylethanolamine (PE). These physiological responses were paralleled by multiple changes in the protein composition, as revealed by 2D DIGE and mass spectrometric analysis. Overall, 158 proteins display changes in their relative abundances ranging from -37 to 66-fold. Some of these proteins were involved in the *(i)* stress response (SodB, BetB), *(ii)* storage compound formation (PhbB, PhaC, phasins) and *(iii)* respiration (NirS) all of which were up-regulated at sublethal concentrations of toluene and ethylbenzene. Thus, these conditions affect the overall physiology of strain EbN1, while the abundances of enzymes involved in alkylbenzene degradation are not altered.

Introduction

Alkylbenzenes are major constituents of crude oil (up to 4 % [v/v]; (Tissot and Welte 1984) and fuels and are widely used as solvents and starting compounds in industrial chemical synthesis. Accidents during transport and storage, e.g. leaking underground fuel storage tanks (EPA 1986), result in an increased release of alkylbenzenes and other hazardous oil-derived hydrocarbons into the environment where mostly oxygen-limited or anoxic conditions prevail. Thus biodegradation of these compounds is of long-standing ecological and microbiological interest, in particular or when considering the toxic properties of BTEX (benzene, toluene, ethylbenzene, xylenes) (Dean 1985).

First evidence for on the principle possibility of an anaerobic degradation of alkylbenzenes was derived from laboratory column experiments (Kuhn et al. 1988). Subsequently numerous pure cultures with the respective degradation capacities were isolated (for overview see: Spormann and Widdel 2000; Widdel and Rabus 2001) . The majority of denitrifying alkylbenzene degraders (incl. strain EbN1) forms a distinct cluster within *Betaproteobacteria*, with the *Azoarcus* and *Thauera* genera as the closest relatives. This cluster will be described as a new genus.

Investigations of anaerobic alkylbenzene degradation in denitrifiers revealed many novel, oxygen-independent reactions. Toluene is initially degraded via a radical-addition to fumarate forming (*R*)-benzylsuccinate (Leuthner et al. 1998), a reaction that is widespread among anaerobic toluene degraders (Rabus 2005). Subsequent degradation to benzoyl-CoA proceeds via β-oxidation-like reaction sequence (Leuthner and Heider 2000). In contrast, ethylbenzene is directly oxidized at the methylene-carbon via (*S*)-1-phenylethanol (Johnson et al. 2001; Kniemeyer and Heider 2001a) to acetophenone (Kniemeyer and Heider 2001b). Further degradation presumably involves carboxylation of acetophenone (Champion et al. 1999) and formation of benzoyl-CoA via thiolytic removal of an acetyl-CoA moiety. Degradation of the central intermediate benzoyl-CoA proceeds via reductive dearomatization, hydrolytic ring cleavage and β-oxidation to acetyl-CoA units (Boll et al. 2002), which are terminally oxidized to CO_2 via the TCA-cycle. In case of strain EbN1 the complete genetic blueprints for the peripheral toluene and ethylbenzene degradation pathways have been deciphered (Rabus et al. 2002b; Kube et al. 2004) and their coordinated (toluene) versus sequential (ethylbenzene) regulation in response to the respective substrates has been revealed (Kühner

et al. 2005). Only recently the complete genome sequence of strain EbN1 was determined (Rabus et al. 2005).

Toluene-amended microcosms from oil-contaminated and pristine sites displayed a rapid consumtion of nitrate (Pelz et al. 2001) and contained high numbers of bacteria related to the phylogenetic cluster of strain EbN1 (Hess et al. 1997; Pelz et al. 2001). In addition, expression of the *bssA*-gene (coding for benzylsuccinate synthase of initial toluene degradation) was detected in contaminated aquifers (Beller 2002). Thus, these bacteria and their degradation capacities could play an important role in biodegradation *in situ*. Depending on the geological properties of the contaminated site and the extent and nature of a spill, local *in situ* concentrations of contaminants can vary drastically. E.g. concentrations of up to 30 mg/l BTEX can be reached in the groundwater (Aronson and Howard 1997).

Despite of the described insight obtained during the last decade on organisms, biochemical reactions, genes and regulation of anaerobic alkylbenzene degradation only little is known about how the novel isolates deal with the toxic properties of these hydrocarbon compounds. Earlier studies with aerobic microorganisms revealed that adaptation towards solvents mainly involved modifications of the membrane fatty acids or the polar headgroup of phospholipids, and deployment of solvent efflux pumps and heat shock proteins. Biotechnologically relevant strains were even shown to thrive in toluene-saturated medium. Here we investigated physiological and molecular responses towards sublethal concentrations of toluene and ethylbenzene for the first time with an anaerobe degrader, the denitrifying strain EbN1.

Materials and methods

Medium and cultivation. The denitrifying bacterium strain EbN1 was cultivated under nitrate-reducing conditions as previously described (Rabus and Widdel 1995a). Water soluble substrates were added directly to the medium, alkylbenzene substrates were provided as dilutions in deaerated 2,2,4,4,6,8,8-heptamethylnonan (HMN) as inert carrier phase. The used chemicals were of analytical grade. Cells were adapted for at least 5 passages to the 'standard' substrate concentrations of 2 % toluene or 5 % ethylbenzene [(v/v) in HMN]. These two types of subcultures served as inoculum for subsequent cultivation at low, standard and sublethal (here defined as inhibitory but not growth arresting) substrate concentrations. These were in case of toluene 0.5, 2, and 6 % [(v/v) in HMN] and in case of ethylbenzene 2, 5, and 8 % [(v/v) in HMN]. Cultivation was carried out in 500 ml flat glass bottles, which contained 400 ml medium, 20 ml carrier phase and were anoxically sealed under an N_2/CO_2 (90/10 [v/v]) atmosphere. Additionally, cells were cultivated with 4 mM benzoate and 5 mM succinate.

Physiological adaptation experiments. Samples (2.5 ml) for determination of alkylbenzene- and nitrate/nitrite- concentrations and optical density were taken from the aqueous phase of the cultures using N_2-flushed, sterile syringes. From these samples, two subsamples were processed as follows: For HPLC-analysis of alkylbenzenes a 1 ml subsample was diluted with 1 ml methanol (HPLC-grade), filtrated (Spartan 13/0.2 RC, Schleicher & Schuell, Dassel, Germany) and stored in teflon-sealed, 1.5 ml vials at 4°C. For HPLC-analysis of nitrate and nitrite, a 1 ml subsample was filtrated (Spartan 13/0.2 RC), stored at 4°C and diluted 1 to 10 in water (HPLC-grade) prior to analysis. Growth was determined by measuring the optical density at 660 nm (UV-mini 1240, Shimadzu, Duisburg, Germany). The end of incubation was indicated by the depletion of the electron acceptor nitrate and intermediately produced nitrite. This was determined with Merckoquant Test Strips (Merck, Darmstadt, Germany). Six cultures (based on 2 independent inoculum cultures) were analyzed for each tested substrate concentration. Parallel cultures yielded highly similar time courses of growth, alkylbenzene and nitrate/nitrite consumption. Controls lacked either organic substrate or inoculum.

Mass cultivation. Mass cultivation was performed to supply sufficient cell material for protein and phospholipid analysis. To reduce slime formation, a phosphate-buffered mineral medium supplemented with NaCl (1 g/l) was used (Tschech and Fuchs 1987). Twelve parallel cultures for each of the three different concentrations of toluene and ethylbenzene were inoculated with the respectively adapted precultures, for cultures grown with succinate and benzoate respectively adapted cells were used. All cultures were harvested at an OD_{660} around 0.2, corresponding to the half-maximal optical density during growth with 8 % [v/v] ethylbenzene. The latter gave rise to the lowest maximal optical density among the six applied alkylbenzene concentrations. Cells were harvested by centrifugation (8900 g for 20 min. at 4°C with a J2-MC centrifuge, Beckman, Fullerton) washed twice with 100 mM Tris-HCl pH 7.5 containing 5 mM $MgCl_2$, immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Chemical analysis. Alkylbenzene concentrations were determined with an HPLC system (Sykam, Fürstenfeldbruck, Germany) as previously described (Rabus and Widdel 1995a) separation was achived with a C18 column (Grom-Sil 100 ODS-2 Fe, 250×4.6 mm, Grom, Rottenburg, Germany) temperature controlled at 25°C. The eluent was composed of 80 % (v/v) acetonitrile and 0.75 mM phosphoric acid, and the flow rate was 1 ml/min. Toluene and ethylbenzene were detected with a UV-detector at 215 nm and had retention times of 4.5 and 5.1 min, respectively. Using a method developed by (Hubaux and Vos 1979), the detection limits for toluene and ethylbenzene were estimated to be 2.2 and 5.0 µM, respectively.
Nitrate and nitrite were analyzed with an HPLC-system (Sykam) as previously described (Rabus and Widdel 1995a). Separation was performed with an anion exchange column (4×60 mm, IBJ A3, Sykam) and temperature-controlled at 50°C. The eluent was composed of 45 % ethanol with 20 mM NaCl, the flow rate was 1 ml/min, and nitrate and nitrite were detected at 220 nm.

2-Dimensional difference gel electrophoresis (2D DIGE). Cells were disrupted with the PlusOne Sample Grinding Kit (Amersham Biosciences, Freiburg, Germany) and preparation of protein extracts was carried out as recently reported (Gade et al. 2003). Protein concentration was determined according to the method described by Bradford (Bradford 1976). Isoelectric focussing (IEF) was performed as described before (Gade et al. 2003; Kühner et al. 2005), using the IPGphor system and 24 cm IPG strips with a nonlinear pH gradient of 3-10 (Amersham Biosciences). The EttanDalt II system (Amersham Biosciences)

was used for separation according to molecular weight in 12.5 % Duracryl gels (Proteomic Solution, Ann Arbor, Mi, USA). Low fluorescent glass plates (Amersham Biosciences) were used for 2D DIGE.

2D DIGE was essentially carried out as described before (Gade et al. 2003; Kühner et al. 2005). For labeling 200 pmol CyDye were used per 50 µg of protein sample. Pre-electrophoretic labeling with different fluorescent dyes allows co-separation of 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 5 parallel gels were run per experiment. Protein extracts from cultures grown with 240 µM toluene and 210 µM ethylbenzene, respectively, served as reference states and were each labeled with Cy5. Protein extracts from cultures adapted to 70 or 740 µM toluene and 80 or 315 µM ethylbenzene represented the four test states and were each labeled with Cy3, each of the two groups of test states was related to its respective reference state. All performed experiments contained the same preparation of internal standard, which was composed of equal amounts of all reference and all test states and was labeled with Cy2.

2D DIGE gels were scanned immediately after electrophoresis with a Typhoon 9400 scanner (Amersham Biosciences). Analysis of cropped images was performed with the DeCyder software (version 5.0; Amersham Biosciences). Parameters for co-detection of spots were: (i) detection of 3000 spots and (ii) exclusion of signals with slope > 1, area < 200, peak height < 190 and volume < 60000. Statistical analysis was based on independent spot maps. Differentially regulated spots were manually controlled to fulfill the following criteria: average ratio of < -2.5 and > 2.5, ANOVA p-value of < 0.05, t-test value of < 10^{-5}, number of matched gels > 45/60. The master gel contained 1921 matched protein spots. For better perception the Cy2 channel was omitted in Fig. 2.

Fig. 3 gives an overview of the number of regulated proteins related to their relative spot volumes and –fold changes in abundance. The total sum of all spot volumes was determined from the Cy3 images. The share of each regulated spot from that sum was calculated and plotted against the -fold change. In case of identified proteins represented by more than one spot on the gels, the respective volumes were added and the -fold changes averaged.

2D DIGE is a minimal labeling technique, where approximately one lysine in 3 % of a protein species is labeled. This allowed subsequent staining with colloidal Coomassie Brilliant Blue (cCBB) (Doherty et al. 1998) to manually excise the differentially regulated proteins from the gels and identify them by mass spectrometry.

Protein identification by mass spectrometry. Tryptic digest of excised proteins was performed as described before (Jeno et al. 1995). Peptide masses were determined by MALDI-TOF mass spectrometry (Corthals et al. 2000). Protein identification and genome analysis were based on the published list of annotated genes from the genomic sequence of strain EbN1 (Rabus et al. 2005). Peptide mass fingerprints were mapped to the coding genes by using the MS-Digest program (Clauser et al. 1999).

Phospholipid analysis. Phospholipid (PL) and phospholipid fatty acid (PLFA) analysis of strain EbN1 was carried out as previously described (Zink and Mangelsdorf 2004). The lipid fraction was directly extracted from culture samples (0.04 to 0.1 g) by flow-blending (Radke et al. 1978) using methanol:dichloromethane (DCM; 1:2, v/v). A distearoyl-D_{70}-phosphatidyl-choline (MW 860.6 D) and a palmitoyl-D_{31}-lysophosphatidylcholine (MW 526.82 D) were added as internal standards. For phase separation and removal of water soluble compounds, dichloromethane, methanol and water were added (1:1:0.9, v/v). Total lipids were obtained from the lower organic phase and the upper aqueous phase was re-extracted twice with dichloromethane. Afterwards the organic phases were combined and evaporated to dryness.

Treatment of the dried extract according to the method described by Zink and Mangelsdorf (2004) yielded four fractions of different polarity. The methanol fraction contained the entire complement of intact phospholipids. Half of this fraction was used for intact PL analysis by LC-MS(MS). Analyses were performed using a Shimadzu SCL-10a VP HPLC system linked via an electrospray ionisation (ESI) interface to a Finnigan TSQ 7000 triple-quadrupole mass spectrometer. Separation of PL classes by their head groups was achieved with normal phase HPLC on a pure silica gel column, 125 × 2.1 mm, Hypersil100 Si-5µ. The LC was equipped with a 5 µl sample loop, the flow rate was set to 150 µl/min with a gradient solvent system of chloroform (A), methanol (B), and ammonium acetate buffer (C; 25 mM, pH 7.5). The initial mobile phase composition was 30% A, 65% B, 5% C. After 3 min B was decreased to 55%, and C increased to 15%; after 8 min the mobile phase was reset to initial conditions and was held isocratically for 7 min (modified after Carrier et al. 2000). Full scan mass spectra in the negative ion mode were recorded using a spray voltage of –4 kV and a capillary column temperature of 220 °C. Collisionally activated dissociation (CAD) MSMS experiments were performed over an m/z range of 100 to 1000 at a scan rate of 1 scan/s. During CAD the collision energy was set to 30 eV and argon was used as collision gas.

The second aliquot was used for mild alkaline hydrolysis (White et al. 1979) to obtain PLFA, the methyl esters of which were analysed by GC-MS. Separation was achieved with a BPX 5 column using the following temperature program: initial temperature 50°C, heating rate 4°C/min to 310°C, held isothermal for 15 min. An Agilent 6890 Series gas chromatograph coupled with a Finnigan MAT 95 XL mass spectrometer operating in the electronic impact mode at 70 eV was used for compound identification. Helium was used as the carrier gas. Full scan mass spectra were recorded from m/z 50 to 650 amu at a scan rate of 1 s per decade and an inter scan delay of 0.2 s, resulting in a scan cycle time of 1.3 s.

Analysis of PHA. PHAs were analyzed in whole-cell samples or after extraction with chloroform and purification by repeated precipitation from a chloroform solution with ethanol (Steinbüchel and Wiese 1992). The PHA content and composition were determined by subjecting 5 to 8 mg of lyophilized cells and 1 to 2 mg of isolated PHAs, respectively, to methanolysis, which was done in a mixture of chloroform and methanol containing 15% (v/v) sulfuric acid (Steinbüchel and Wiese 1992). The resulting hydroxyacyl-methylesters were analyzed with a Hewlett-Packard type GC6850 gas chromatograph (Brandl et al. 1988; Timm et al. 1990). The initial structural assignments of the methylesters analyzed were based on their retention times compared to those of authentic standards. Final confirmation of structures was performed by using a model GC6890 gas chromatograph coupled to a model 5973 mass selective detector (Hewlett-Packard).

Results

Initial growth experiments of strain EbN1 with toluene and ethylbenzene (each supplied in a concentration range of 0.5 to 10 %, [v/v] in HMN) revealed sublethal (inhibitory but not growth arresting) concentrations of 6 % and 8 %, respectively. In subsequent experiments strain EbN1 was grown with three different concentrations of toluene (0.5, 2 and 6 %) and ethylbenzene (2, 5 and 8 %), with 2 % toluene and 5 % ethylbenzene representing routine cultivation conditions. The adaptive response to these different alkylbenzene concentrations was analyzed *in vivo* by measuring growth at 660 nm and determining alkylbenzene and nitrate/nitrite concentrations in the medium (Fig. 1 and Table 1) and *in vitro* on the level of proteins (Figs.1 and 3, Table 2) and phospholipids (Table 3).

Growth experiments with toluene. The time courses of growth, and toluene and nitrate/nitrite concentrations in the medium are shown in Fig. 1 A-C for the three different toluene concentrations (0.5, 2 and 6 %, [v/v] in the carrier phase). With 0.5 % and 2 % toluene highly similar doubling times of 10 h and 9.5 h, respectively, were observed (Fig. 1A). In contrast, the doubling time was prolonged to 44 h in case of 6 % toluene. Despite of these differences, similar maximal optical densities (around 0.35) were reached with all three concentrations of toluene.

The toluene concentrations in the aqueous phase differed significantly by about one order of magnitude (Fig. 1B). With 0.5 % (v/v) toluene in the carrier phase, the concentrations in the medium displayed an initial maximum at 70 µM, decreased below the detection limit of 2.2 µM prior to entry into stationary growth phase and then resumed to about 30 µM (at the end of growth). With 2 % or 6 % (v/v) toluene in the carrier phase, the equilibrium concentrations in the medium increased to 240 and 740 µM, respectively. An intermediary decrease to 160 µM was only observed in the former case.

The profiles of nitrate consumption and intermediate formation of nitrite were very similar in cultures with 70 or 240 µM toluene (Fig. 1C), agreeing with the similar growth curves (Fig. 1A). Corresponding to the prolonged doubling times in the cultures with 740 µM, also the consumption of nitrate was markedly decelerated and the maximal concentration of the intermediately excreted nitrite was halved.

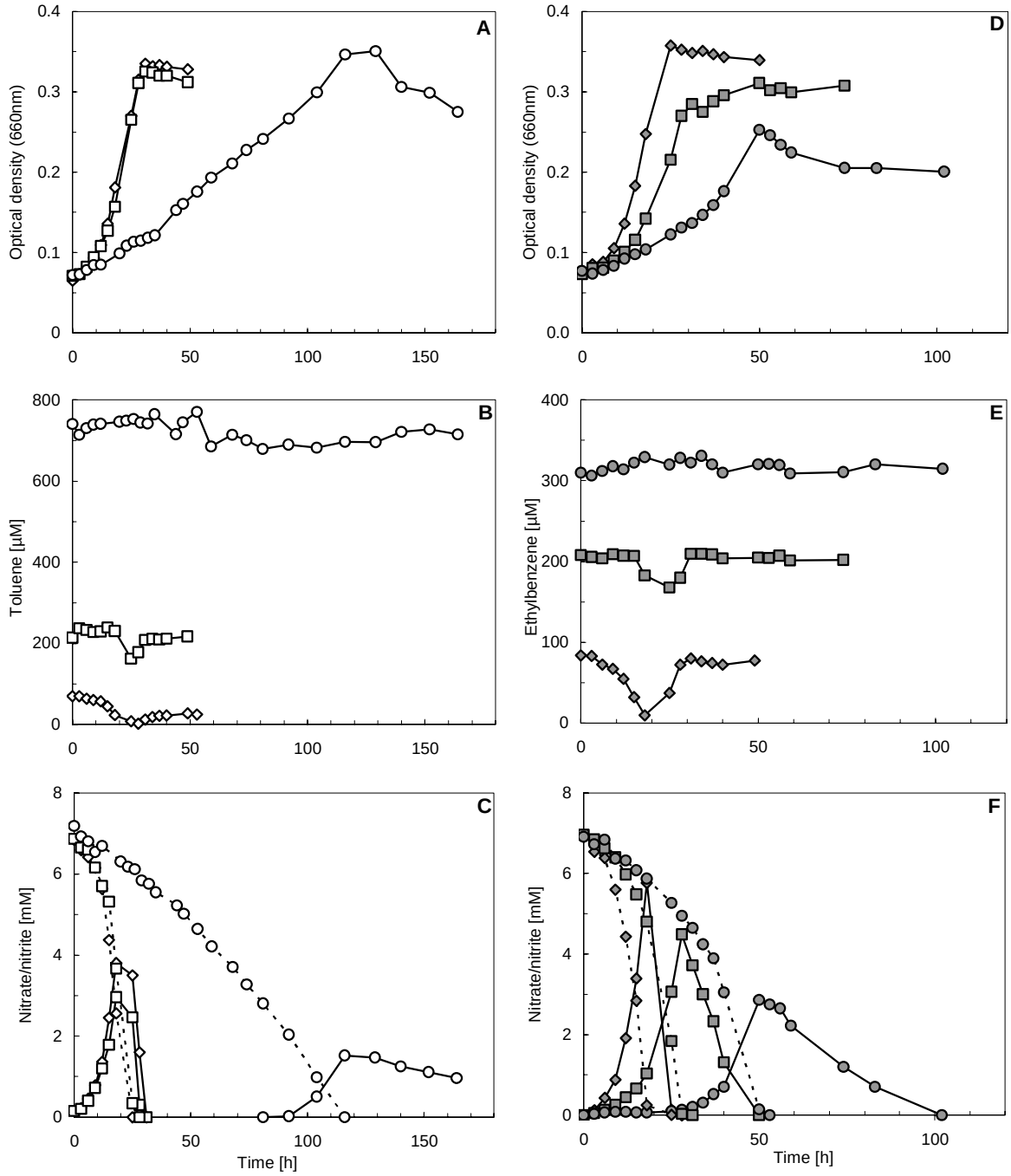


Fig. 1. Anaerobic growth of strain EbN1 with different toluene (A-C) and ethylbenzene (D-F) concentrations. ◇, □ and ○: 70, 240 and 740 µM toluene; ◆, ■ and ●: 80, 210 and 315 µM ethylbenzene. Concentrations refer to the equilibrium concentration of the alkylbenzenes in the aqueous medium when supplied as dilution in the inert carrier phase (HMN). A and D, optical density; B and E, concentration of toluene and ethylbenzene in the medium; C and F concentration of nitrate (dashed lines) and nitrite (full lines).

Table 1. Anaerobic growth of strain EbN1 with different alkylbenzene concentrations.

Alkylbenzene	conc. in HMN		conc. in medium	t_D	max. OD	NO_3^-- consumption
	[%, v/v]	[mM]	[µM]	[h]		[µM/h]
Toluene	0.5	45	70	10	0.335	600
	2	190	240	9.5	0.328	550
	6	570	740	44	0.350	65
Ethylbenzene	2	165	80	7.5	0.357	860
	5	410	210	10	0.311	600
	8	660	315	50	0.253	80

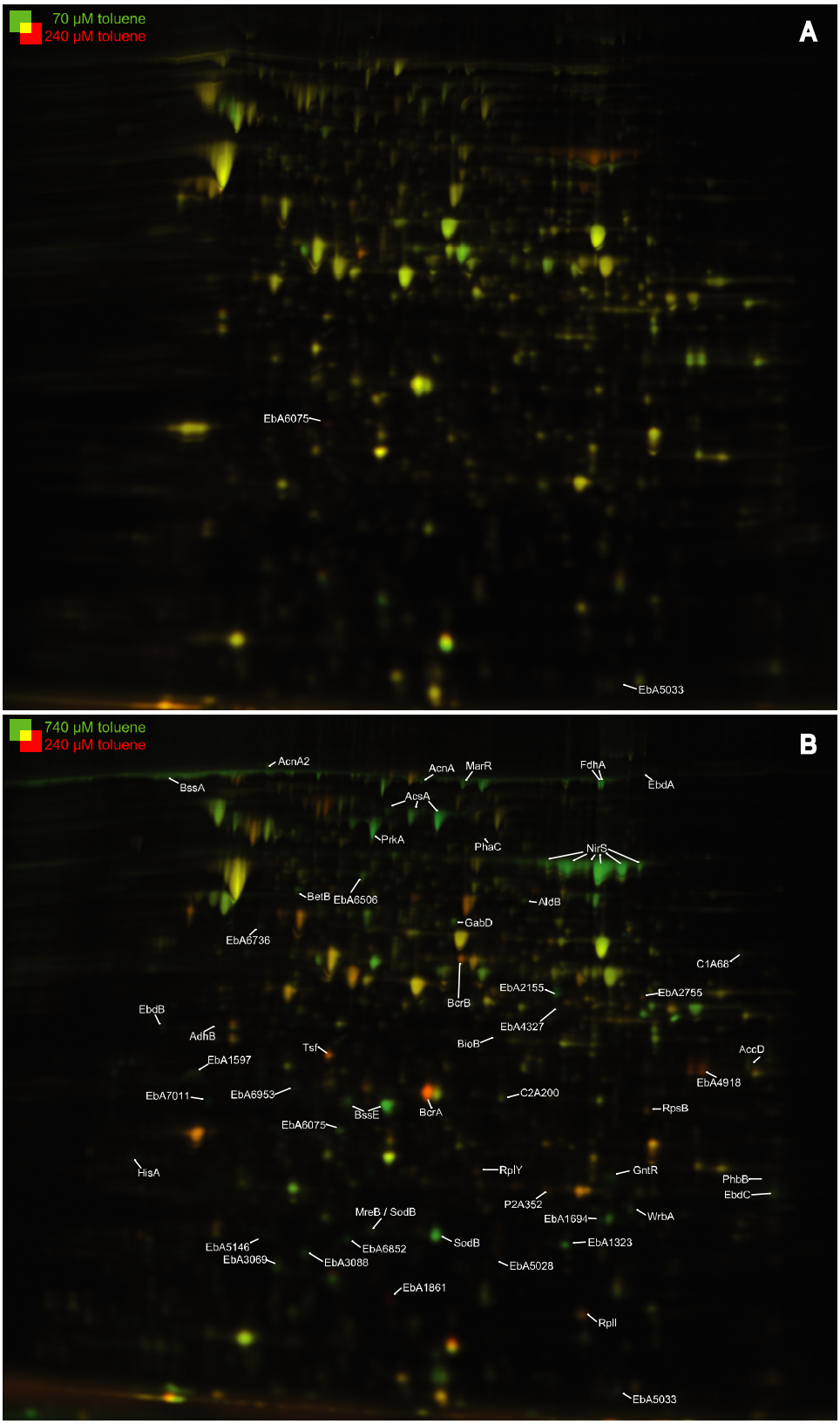
t_D, doubling time; OD, optical density

Growth experiments with ethylbenzene. Growth parameters for the cultures with the three different ethylbenzene concentrations (2, 5 and 8 %, [v/v] in the carrier phase) are shown in Fig. 1 D-F. With increasing ethylbenzene concentrations the doubling times also increased from 7.5 to 10 and 50 h while the maximal optical densities decreased (Fig. 1D). The latter is in contrast to the above described observations with toluene-grown cultures.

The ethylbenzene concentrations in the aqueous phase varied by about a 3-fold (Fig. 1E). With 2, 5 and 8 % (v/v) ethylbenzene in the carrier phase, respectively, the equilibrium concentrations in the medium were 80, 210 and 315 µM, respectively. Intermediate decreases to 10 and 170 µM, respectively, were only observed in the former two cases. This is reminiscent of the cultures growing with 70 and 240 µM toluene, respectively.

Nitrate consumption decelerated and intermediate nitrite formation (Fig. 1F) decreased with rising ethylbenzene concentration as observed for the doubling times.

Proteomic analysis. The effect of alkylbenzene concentrations on the protein composition of strain EbN1 was quantitatively studied with 2D DIGE. Thereby 158 protein spots were detected, the abundance of which changed more than a |2.5| -fold in response to at least one of the tested alkylbenzene concentrations, with 240 µM toluene and 210 µM ethylbenzene cultures used as the respective reference states. Thus, only a small fraction of the total proteins of strain EbN1 were affected by varying alkylbenzene concentrations, considering that the master gel was composed of almost 2000 protein spots. Fiftysix regulated proteins were subsequently identified from cCBB-stained 2D DIGE gels by mass-spectrometric analysis (MASCOT-score >50). Results from the proteomic analysis are shown in Figs. 2 and 3, and in Table 2.


70 µM toluene
240 µM toluene
A
EbA6075
EbA5033
740 µM toluene
240 µM toluene
B
AcnA2
BssA
AcnA
MarR
FdhA
EbdA
AcsA
PrkA
PhaC
NirS
BetB
EbA6506
AldB
GabD
EbA6736
C1A68
EbA2155
EbA2755
BcrB
EbdB
EbA4327
AdhB
BioB
Tsf
AccD
EbA1597
EbA4918
EbA7011
EbA6953
C2A200
BssE
BcrA
RpsB
EbA6075
HisA
RplY
GntR
PhbB
EbdC
P2A352
MreB / SodB
EbA1694
WrbA
EbA5146
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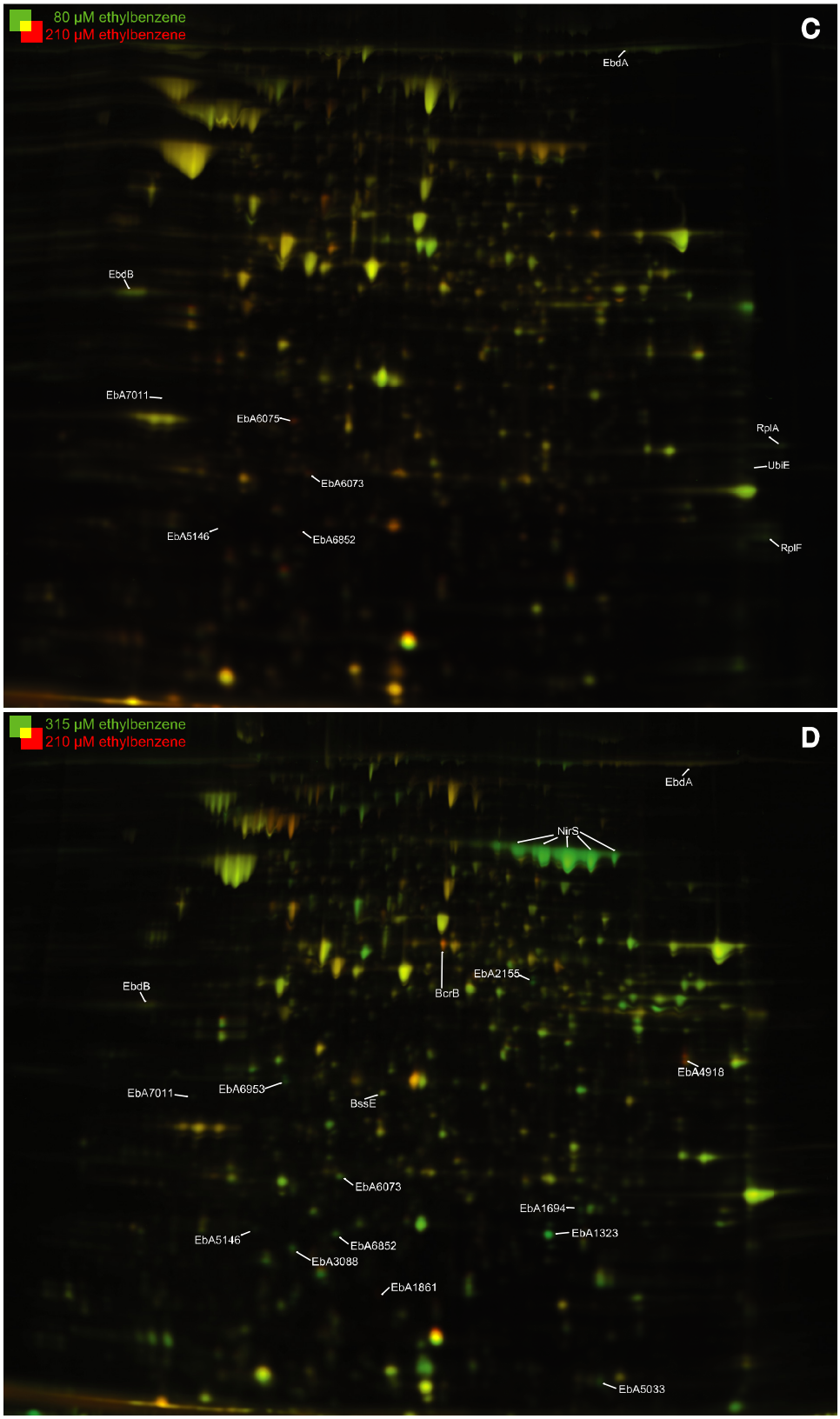

Fig. 2. Changes in protein profiles in response to anaerobic growth with different alkylbenzene concentrations as determined by 2D DIGE. A, 70 µM toluene; B, 740 µM toluene; C, 80 µM ethylbenzene; D, 315 µM ethylbenzene. Reference states were extracts from cultures grown with 240 µM toluene for A and B, and with 210 µM ethylbenzene for C and D. Proteins with increased and decreased abundances, respectively, appear as green and red spots, respectively. Mass spectrometrically identified proteins regulated more than |2.5|-fold are marked. Fold changes of their abundances are given in Fig. 3 and Table 2.

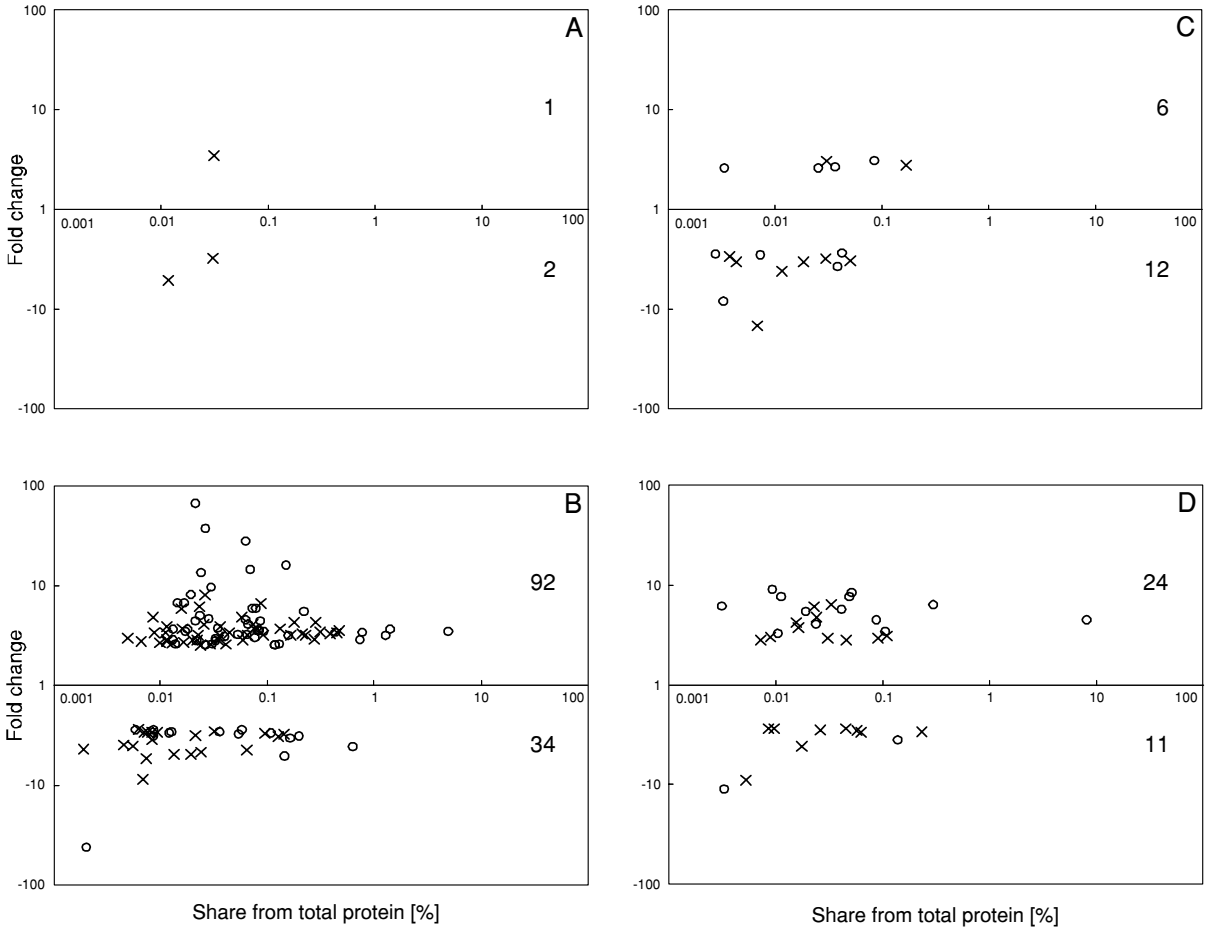

Fig. 3. Distribution of regulated proteins with respect to fold change in abundance and share from total cellular protein. A, 70 µM toluene; B, 740 µM toluene; C, 80 µM ethylbenzene; D, 315 µM ethylbenzene. Symbols: ○, identified proteins; ×, not identified proteins. In case of proteins separated as more than one spot on the 2D gel (e.g. NirS), the corresponding spot volumes were added up and the fold changes were averaged. Total numbers of up- and down-regulated proteins are indicated for each alkylbenzene concentration.

The most extensive changes in protein abundance were observed at the sublethal concentrations of toluene and ethylbenzene. E.g. during growth with 740 µM toluene 110 up- and 34 down-regulated proteins were detected relative to growth with 240 µM toluene. The most strongly regulated proteins were the hypothetical proteins EbA7011 and EbA1861 with fold-changes of 66 and -37, respectively. During growth with 315 µM ethylbenzene, 40 up- and 15 down-regulated proteins were detected, all of which were remarkably also regulated during growth with 740 µM toluene. In the following, the identified, regulated proteins are grouped and described according to their predicted functions.

Toluene and ethylbenzene degradation. Based on the present and previous work (Kühner et al. 2005), 12 and 11 protein spots on the DIGE-gels, respectively, could be correlated to the pathways of anaerobic toluene and ethylbenzene degradation, respectively. The abundance of these proteins was apparently not influenced by the actual toluene or ethylbenzene

concentration (Table 2). The only exceptions were BssA (catalytic subunit of benzylsuccinate synthase), BssE (chaperone of benzylsuccinate synthase) and C2A200 (hypothetical protein), which were 2.8 to 3.3 fold up-regulated in cultures growing with 740 µM toluene. Conversely, subunits of ethylbenzene dehydrogenase displayed unchanged abundances in 315 µM ethylbenzene cultures. Interestingly, the abundance of BcrAB subunits of benzoyl-CoA reductase (the first common enzyme of both pathways) was decreased at 740 µM toluene and 315 µM ethylbenzene.

Polyhydroxyalkanoate (PHA) synthesis. Acetoacetyl-CoA reductase (PhbB) and PHA-synthase (PhaC) showed about 3-fold increased abundance in cultures with 740 µM toluene. Both enzymes are involved in biosynthesis of polyhydroxyalkanoates from acetyl-CoA. In addition, the phasin-like proteins EbA1323, EbA6852 and EbA5033 were up-regulated in 740 µM toluene (5.5- to 27.4-fold) and 315 µM ethylbenzene (4.0- to 5.7-fold) cultures.

Stress-related proteins. Two proteins which are directly related to stress were found to be of increased abundance (1.5- to 3.5-fold) in the presence of 740 µM toluene and 315 µM ethylbenzene: SodB, a superoxide dismutase with an iron co-factor and BetB, the betaine aldehyde dehydrogenase, catalyzing the last reaction of the betaine biosynthesis.
In addition, two conserved hypothetical proteins EbA1861 and EbA4918 displayed strongly decreased abundances (-2.6 to -37.3-fold) under these conditions. Both proteins are probable periplasmatic binding proteins involved in Fe^{2+}-uptake. EbA1861 is related to the small COG 3470 group, which comprises uncharacterized proteins assumed to function in high-affinity Fe^{2+}-uptake. The coding gene *ebA1861* is located in close proximity of *ebA1866* encoding a high affinity iron permease. EbA4918 is similar to the multi-member COG 1840 group of periplasmatic components of ABC-type Fe^{3+}-transporters.

One may speculate that the down-regulation of these two proteins results in a reduced intracellular iron availability. The latter would agree with the upregulation of aconitase A (4.5 to 6-fold with 740 µM toluene). In *Escherichia coli* aconitase occurrs as the two isoenzymes A and B, the latter represents the primary housekeeping protein, AcnA is expressed under conditions of oxidative stress and iron limitation (Varghese et al. 2003).

The tryptophane repressor binding protein WrbA was 2.4- to 5.8 -fold up-regulated under sublethal alkylbenzene concentrations. In E. coli *wrbA* is under control of the general stress response sigma factor RpoS and is known to enhance activity of the repressor TrpR (Yang et al. 1993).

A putative aldehyde dehydrogenase (AldB) and an alcohol dehydrogenase II (AdhB) were about 4-fold up-regulated in the presence of 740 µM toluene. Their coding genes are separated by only 83 bp. Both display 75 % similarity to the orthologs of *E. coli* K12, which are also similarly arranged. Expression of the gene for AldB (b3588) in *E. coli* is controlled by RpoS (Xu and Johnson 1995). AldB is a predicted 4-aminobutyraldehyde dehydrogenase (Reed et al. 2003) which is part of the putrescine degradation pathway. The AdhB ortholog YiaY of *E. coli* is a unknown protein. The RpoS-dependent succinate semialdehyde dehydrogenase GabD (2.6-fold increased with 740 µm toluene) links this pathway to the TCA cycle (Metzner et al. 2004).

Respiration. The periplasmatic nitrite reductase (NirS) was about 3-fold up-regulated in cultures with 740 µM toluene and 315 µM ethylbenzene. This protein was represented by 6 neighbouring spots which comprise about 6 % of the total protein separated on the 2D-gels. Also FdhA, the α-subunit of periplasmatic formate dehydrogenase, was found to be up-regulated (4-fold) in the presence of 740 µM toluene.

Metabolism, fatty acid synthesis and genetic information processing. The abundance of several dehydrogenases specifically increased in cultures with 740 µM toluene. In particular acetyl-CoA dehydrogenase EbA4327 was 13.4-fold up-regulated. Directly upstream of its coding gene are genes for a putative acyl carrier protein and an acyl-CoA synthase, indicating an involvement of EbA4327 in fatty acid metabolism. Conversely, AccD and BioB are down-regulated. AccD is a subunit of the biotin-dependent acetyl-CoA carboxylase, which carboxylates acetyl-CoA to malonyl-CoA as the initial steps of fatty acid biosynthesis. The biotine synthase BioB catalyzes the last step of the biotin pathway.

At 740 µM toluene and 315 µM ethylbenzene the abundance of several identified ribosomal proteins was decreased up to 2.7-fold, agreeing with the overall reduced metabolism, viz. profoundly reduced doubling times. In contrast, a GntR-like transcriptional regulator and a protein kinase (Eba4966) are up to 3.5-fold up-regulated possibly indicating their involvement in stress-related regulatory circuits. E.g. the gene for PrkA (79 % similarity to Eba4966) of *Sinorhizobium meliloti* was up-regulated in situations of phosphate- and carbon-limitation or with nitrate instead of ammonium as nitrogen source (Summers et al. 1998).

Table 2. Fold changes of the abundance of identified proteins during anaerobic growth of strain EbN1 with different concentrations of toluene and ethylbenzene.

Protein name[a]	Predicted function[b]	coverage[c]	score[d]	Toluene		Ethylbenzene	
				70[e]	740[e]	80[e]	315[e]
	Toluene degradation pathway						
BssA	Benzylsuccinate synthase, α-subunit	34	297	1.03	3.32	1.69	1.64
BssE	Benzylsuccinat synthase, chaperone	72	237	-1.36	3.08	1.15	2.04
C2A200	Hypothetical protein, related to toluene degradation	77	239	1.01	2.81	1.16	1.76
BbsH	Phenylitaconyl-CoA hydratase	nd	nd	-1.34	-1.09	1.02	-1.32
BbsG	(*R*)-Benzylsuccinyl-CoA dehydrogenase	nd	nd	1.11	1.46	1.15	-1.02
BbsF	Succinyl-CoA:(*R*)-benzylsuccinat CoA-transferase, subunit	nd	nd	1.04	1.71	1.93	2.03
BbsD	2-[Hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase, subunit	nd	nd	-1.3	-2.38	-1.06	-1.25
BbsC	2-[Hydroxy(phenyl)methyl]-succinyl-CoA dehydrogenase, subunit	nd	nd	-1.15	-2.2	1.35	-1.66
BbsB	Benzoylsuccinyl-CoA thiolase, β-subunit	nd	nd	-1.25	-1.12	1.23	1.07
BbsA	Benzoylsuccinyl-CoA thiolase, α-subunit	nd	nd	-1.24	-1.07	-1.1	1.04
EbA1932	Hypothetical protein, related to toluene degradation	nd	nd	-1.05	-1.14	1.39	-1.03
EbA1936	Hypothetical protein, related to toluene degradation	nd	nd	-1.49	1.22	1.19	1.6
	Ethylbenzene degradation pathway						
EbdA	Ethylbenzene dehydrogenase, α-subunit	43	231	1.09	2.68	2.11	-1.34
EbdB	Ethylbenzene dehydrogenase, β-subunit	75	285	1.27	3.63	1.43	-1.05
EbdC	Ethylbenzene dehydrogenase, γ-subunit	89	164	-1.12	2.81	1.19	1.07
EbdD	Ethylbenzene dehydrogenase, chaperone	nd	nd	-1.25	1.29	-1.36	-1.84
C1A68	Hypothetical protein, related to ethylbenzene degradation	56	292	1.04	2.95	1.24	-1.08
C1A84	Hypothetical protein, related to ethylbenzene degradation	nd	nd	-1.3	3.59	-2.1	1.6
Bal	Benzoylacetate CoA-ligase	nd	nd	-1.06	1.84	1.1	-2.33
Apc1	Acetophenone carboxylase, subunit 1	nd	nd	-1	1.69	-1.07	-2.42
Apc3	Acetophenone carboxylase, subunit 3	nd	nd	-1.08	1.57	1.1	-1.88
Apc4	Acetophenone carboxylase, subunit 4	nd	nd	1.48	1.75	-1.06	-1.59
Apc5	Acetophenone carboxylase, subunit 5	nd	nd	-1.21	1.95	-1.55	-1.42
	Benzoyl-CoA degradation pathway						
BcrA	Benzoyl-CoA reductase, α-subunit	90	275	-1.15	-3.7	1.28	-1.66
BcrB	Benzoyl CoA reductase, β-subunit	69	369	1.01	-2.89	1.45	-3.11
	Polyhydroxyalkanoate synthesis						
PhbB	Acetoacetyl-CoA reductase	57	128	1.22	3.05	1.25	1.26
PhaC	Putative poly-β-hydroxyalkanoate synthase	35	121	1.52	3.46	1.05	1.04
EbA1323	Conserved hypothetical protein, predicted phasin family	84	155	-1.53	5.46	-2.07	5.72
EbA6852	Probable phasin	91	126	-1.24	27.37	-7.56	3.95
EbA5033	Hypothetical protein, probable phasin	72	87	2.56	6.61	2.42	5.36
	Stress related proteins						
BetB	Putative betaine aldehyde dehydrogenase (BADH) oxidoreductase protein	46	190	-1.66	3.52	-1.99	2.26
SodB	Superoxide dismutase (Fe)	54	95	1.09	2.83	-2.17	1.57
EbA1861	Similar to uncharacterized protein probably involved in high affinity Fe^{2+} transport	54	71	2.15	-37.3	1.34	-9.94
EbA4918	Putative iron binding protein component of ABC iron transporter (periplasmic component, Fe^{3+} transport)	74	275	1.32	-4.54	1.09	-2.64
AcnA	Aconitase A	40	194	1.08	5.93	1.02	2.07
AcnA2	Aconitase A	40	222	-1.07	4.48	-1.29	1.78
WrbA	Flavoprotein[f], TrpR enhancer	63	85	-1.08	5.83	-1.8	2.37
AldB	Putative aldehyde dehydrogenase	32	73	1.22	4.03	1.08	-1.01
AdhB	Alcohol dehydrogenase II	48	96	-1	4.91	1.04	1.05
GabD	Succinate-semialdehyde dehydrogenase	47	146	1.26	2.55	-1.28	1.08

Protein name[a]	Predicted function[b]	coverage[c]	score[d]	Toluene		Ethylbenzene	
				70[e]	740[e]	80[e]	315[e]
	Respiration						
NirS	Cytochrome cd_1 nitrite reductase precursor (means)	67	328	-2.28	3.24	-1.34	2.99
FdhA	Formate dehydrogenase, α-subunit[g]	26	117	1.07	3.97	1.2	1.6
UbiE	Ubiquinone/menaquinone biosynthesis methyltransferase	47	90	1.24	1.38	2.5	1.08
	Fatty acid synthesis						
AccD	Putative acetyl-CoA carboxylase carboxyl transferase, β-subunit	50	158	1.07	-2.53	-1.09	-1.33
BioB	Biotin synthase protein	44	107	-1.1	-2.88	1.04	-1.55
EbA4327	Acyl-CoA dehydrogenase	47	93	-1.31	13.42	-1.17	2.89
	Metabolism						
AcsA	Acetyl-CoA synthase	59	299	-1.1	4.59	1.36	2
EbA6506	Acyl-CoA dehydrogenase	69	386	-1.17	2.5	-1.28	1.44
EbA6075	Predicted short chain dehydrogenase	67	176	-2.82	2.36	-3.45	1.68
EbA6736	Probable oxidoreductase	50	145	-1.17	4.57	-1.28	2.44
EbA2755	ABC-transporter, periplasmatic component	45	145	-1.06	-2.6	1.06	-1.02
	Amino acid synthesis						
HisA	1-5-(Phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase	48	87	1.22	2.51	-1.11	1.83
	Transcription, translation, regulation						
RplI	50S ribosomal protein L9	92	193	-1.13	-2.67	1.2	-1.59
RplY	50S ribosomal protein	52	89	-1.16	-2.7	-1.03	-1.76
RplA	50S ribosomal protein	92	276	1.16	1	2.59	-1.41
RplF	50S ribosomal protein	75	209	1.32	-1.91	2.95	-1.2
RpsB	30S ribosomal protein S2	59	154	-1.09	-2.5	1.32	-1.54
Tsf	Elongation factor Ts	86	288	-1.21	-3.06	-1.07	-1.7
PrkA	Protein kinase	40	254	-1.05	3.38	-1.77	1.71
GntR	Transcriptional regulator	54	84	-1.06	3.49	-1.1	1.63
Tnp17	Transposase IS4 family	33	81	1.13	2.6	2.24	-1.33
P2A352	Probable pyridoxamin 5'-phosphate oxidase	74	123	1.08	-2.75	1.03	-1.38
	Unknown, conserved proteins						
EbA1694	Conserved hypothetical protein	47	56	-1.23	14.26	-2.24	5.07
EbA5146	Conserved hypothetical protein	78	124	-1.34	6.58	-2.54	3.61
EbA3069	Conserved hypothetical protein	69	96	-1.03	2.49	-1.46	2.07
EbA6073	Conserved hypothetical protein	61	141	-2.05	1.75	-2.55	1.54
EbA5028	Conserved hypothetical protein	67	136	-1.14	-2.48	1.05	-1.84
	Unknown, unique proteins						
EbA7011	Hypothetical protein	55	107	-1.01	66.42	-2.64	6.73
EbA2155	Hypothetical protein	44	116	-1.04	15.98	1.13	6.69
EbA6953	Hypothetical protein, probable apolipoprotein A/E/C3	61	288	-1.13	9.5	-1.64	7.38
EbA3088	Hypothetical protein	63	108	-1.12	4.38	-1.06	2.9
EbA1597	Hypothetical protein	36	52	1.07	2.8	-1.76	1.67

[a] Protein name as published, http://www.micro-genomes.mpg.de/ebn1 (Rabus et al. 2005).
[b] Predicted function as published (Rabus et al. 2005). Proteins may fit in more than one group.
[c] Coverage [%] of the mass-spectrometrically determined peptides relative to the total amino acid sequence of the identified proteins.
[d] Score of MS-based identification as calculated using MASCOT.
[e] Concentration [µM] of alkylbenzene in the medium.
[f] EbA2303
[g] EbA2936
nd, not determined in this study. Protein-assignment was based on co-detection of previously identified proteins (Kühner et al. 2005).

Phospholipid analysis. To study the effect of different alkylbenzenes concentrations on the phospholipid composition intact PL and PLFA were determined. The main cellular phospholipids were phosphatidyldiacylethanolamines (PE) and phosphatidyldiacylglycerols (PG), two minor groups were phosphatidyldiacylcholines (PC) and a second PE group with an extended head group eluting together with the common PE and showing synchronous PLFA distribution patterns. Notably, an additional methylcyclohexane residue linked to the PE head group (abbreviation MCH-PE) was tentatively identified for the second PE group; this was further corroborated by LC-MSMS analysis. The fatty acyl side-chain composition corresponds to that of normal PE. With increasing concentration of toluene (70, 240 and 740 µM) the share of PG first decreased from 30.1 to 22.6 % and then increased again to 25.4 %, while changes in PE concentration behaved oppositely (Table 3). The MCH-PE concentration only increase at highest toluene concentration. Remarkable is the significant increase of PC up to 3.5 % at 740 µM toluene (Table 3). The three concentrations of ethylbenzene (80, 210 and 315 µM) had a different effect on the PL distribution. The PG share remained constant at 80 and 210 µM ethylbenzene and increased to about 10.6 % at 315 µM ethylbenzene. In contrast, the PE portion showed the opposite trend (Table 3). PC are already detectable at 80 µM ethylbenzene and displayed increased shares at the higher ethylbenzene concentrations. Cells of strain EbN1 grown with benzoate or succinate had similar phospholipid distributions, differing only slightly from those of the alkylbenzene grown cultures. Under the former two growth conditions PC were not detected and the proportions of MCH-PE/PE corresponded to a great extent with that observed in cultures grown with 80 µM ethylbenzene and 240 µM toluene, respectively.

The trends of compositional changes within individual intact phospholipids (Table 3) reflect in more detail the respective adaptation process within membrane lipids. Firstly, both main PL groups (PE, PG) showed a synchronous fatty acyl side-chain combination pattern with 16:0/16:1 (pos-1/pos-2) as the predominant combination, followed by 16:1/16:1, and 18:1/16:1. In addition both groups (PG and PE) showed the same changes of their individual side-chain combinations in response to elevated alkylbenzene concentrations. Interestingly, more obvious changes were seen for the toluene-grown cultures. For instance, individual PL containing a combination of saturated and monounsaturated fatty acids increased with rising toluene concentrations, whereas those with two monounsaturated side-chains were diminished (Table 3). A slight decrease for PL with two monounsaturated chains was also observed in cultures grown with ethylbenzene. Combinations of two saturated side-chains stayed more or less constant. All in all a decrease of unsaturation versus saturation could be deduced from

the compositional changes of phospholipid side-chains. A slight shift to shorter chains was evident from the ratios of the main compounds within each PL group (e.g. PG 719/745 and PE 688/714) or the ratio PE 688/716. In addition, the proportions of PG 717 and PE 686, both containing the combination 16:1/16:1, were in general higher for ethylbenzene-grown cells, but not influenced by changing solvent concentration.

The distribution of the single phospholipid fatty acids was also determined by GC-MS. The PLFA comprised a range of chain lengths from C_{14} to C_{18}; most abundant were saturated and monounsaturated C_{16} (Table 3). In addition, cy$C_{17:0}$ (or $C_{17:1}$) and $C_{18:1}$ PLFA were identified as minor components. The PLFA distribution determined by GC-MS analysis was generally agreeing with the fatty acyl side-chain composition of intact phospholipids determined by LC-MSMS, although 18:0, identified by LC-MSMS, showed only trace amounts during GC-MS analysis. This slight discrimination was frequently observed during comparison of LC-MSMS and GC-MS PLFA data (Zink and Mangelsdorf 2004).

The increase in toluene concentration resulted in a decrease of the dominant fatty acid $C_{16:1}$ from 61.6 % to 49.6 %, whereas the saturated C_{16} fatty acid increased by approx. 10 %. In contrast no significant changes were observed in cultures grown with different concentrations of ethylbenzene (Table 3).

PHA analysis. Presence of PHA as suggested by proteomic data (up-regulation of PhbB, PhaC and phasins) was analyzed in actively growing cultures. Cells grown with 80 and 240 µM toluene or with 80 µM ethylbenzene did not contain detectable amounts of PHAs. However, in the presence of 740 µM toluene and 210 and 315 µM ethylbenzene poly-β-hydroxybutyrate (PHB) accumulate up to 5.2, 2.0 and 10.3 % of dry weight, respectively. PHAs other than PHB were not observed.

Table 3. Phospholipid headgroup and fatty acid residue composition of strain EbN1 grown anaerobically at different alkylbenzene concentrations, benzoate or succinate.

Parameter [%]	Growth substrate [µM]							
	Toluene			Ethylbenzene			Benzoate	Succinate
	70	240	740	80	210	315	4000	5000
Fatty acid residues (FA)								
14:0	0.7	0.3	1.0	0.4	0.6	0.8	0.3	0.8
16:1	61.6	54.9	49.6	58.0	58.2	58.3	56.6	55.9
16:0	35.1	41.0	45.1	37.3	36.6	37.5	37.2	34.8
cyc.17	<DL	<DL	1.1	<DL	<DL	0.3	<DL	<DL
18:1	2.6	3.8	3.2	4.3	4.6	3.1	5.8	8.5
Phospholipid headgroups								
Phosphatidylethanolamine (PE)	69.8	76.8	71.2	77.9	76.9	65.0	75.9	77.3
Phosphatidylglycerine (PG)	30.1	22.6	25.4	21.5	21.6	32.0	24.1	22.7
Phosphatidylcholine (PC)	<DL	0.6	3.5	0.6	1.4	3.0	<DL	<DL
FA of intact phospholipids								
Phosphatidylethanolamine (PE)								
30:1 (14:0/16:1)	1.0	1.3	2.7	1.2	1.7	2.6	0.9	0.9
32:2 (16:1/16:1)	17.1	15.5	11.2	22.5	21.2	22.8	20.0	18.3
32:1 (16:0/16:1)	53.5	53.0	57.0	48.7	50.1	51.6	46.5	48.9
32:0 (16:0/16:0)	5.9	5.6	6.5	5.0	5.3	5.6	5.1	5.6
34:2 (18:1/16:1)	14.5	13.0	8.0	13.8	12.2	10.7	16.6	16.5
34:1 (18:1/16:0, 18:0/16:1)	2.0	1.9	1.2	2.0	1.8	1.6	2.2	2.5
Phosphatidylglycerine (PG)								
30:1 (14:0/16:1)	0.8	0.7	6.4	0.9	1.0	1.0	0.8	0.8
32:2 (16:1/16:1)	16.0	14.2	10.6	18.3	19.6	18.7	18.4	17.5
32:1 (16:0/16:1)	58.7	58.0	61.5	55.2	53.8	58.5	50.5	54.2
32:0 (16:0/16:0)	7.0	7.5	8.1	6.6	7.0	6.8	7.4	6.5
34:2 (18:1/16:1)	14.6	14.8	8.8	15.7	14.7	11.6	18.6	18.0
34:1 (18:1/16:0, 18:0/16:1)	2.3	3.4	2.8	2.8	2.2	2.8	3.3	2.5
36:0 (18:0/18:0)	0.7	1.4	1.7	0.5	1.6	0.5	1.3	0.5
Phosphatidylcholine (PC)								
32:2 (16:1/16:1)	0	42.1	29.7	41.6	55.1	52.6	0	0
32:1 (16:0/16:1)	0	37.3	54.5	40.7	20.8	29.9	0	0
34:2 (18:1/16:1)	0	20.6	15.8	17.7	24.1	17.5	0	0
Ratios								
PE/PG	2.3	3.4	2.8	3.6	3.6	2.0	3.2	3.4
PE+98 Da/PE	5.8	9.7	13.3	7.0	7.8	5.1	8.7	7.2
Saturated fatty acids	36	41	46	38	37	38	38	36
Fatty acids <C17[a]	97	96	96	96	95	97	94	92

<DL, below detection limit.

[a] most probable composition as estimated by fatty acid residue analysis

[b] <C17, fatty acid residues with less than 17 carbon atoms.

Discussion

Growth experiments. The present growth experiments revealed, that the concentration-dependent extent of growth inhibition was different for toluene and ethylbenzene (Fig. 1 and Table 1). While an increase of the toluene concentration in the medium from 80 to 220 µM had no effect on any of the tested growth parameters (t_D, yield, nitrate consumption) a comparable increase in ethylbenzene concentration already resulted in deceleration of growth and nitrate consumption. At sublethal alkylbenzene concentrations, i.e. 740 µM toluene and 315 µM ethylbenzene, similar increases in doubling time and decreases in nitrate consumption rate, respectively, were observed. Notably, at 315 µM ethylbenzene, the maximal optical density was about 35 % lower as compared to cultures with 80 µM ethylbenzene. In contrast, highly similar optical densities were reached at all tested toluene concentrations. Differences in membrane solubility may explain this observation. At the sublethal concentrations of toluene and ethylbenzene (740 versus 315 µM in the medium) their theoretical concentrations in the membranes are 69 versus 83 mM, as calculated from their partition coefficients in the octanol/water and membrane/buffer systems (Sikkema et al. 1994).
In-situ concentrations of up to 10 mg/l (109 µM) toluene, 1.7 mg/l (16 µM) ethylbenzene or 33 mg/l total BTEX were measured (Aronson and Howard 1997) and *in-vitro* 132 mg/l BTEX was determined to be the highest possible equilibrium concentration reached in a mixture of gasoline with groundwater (10 % [v/v]; (Cline et al. 1991). Thus, the alkylbenzene concentrations applied in this study represent the upper range of *in situ* concentrations.

Proteomic studies. *Toluene and ethylbenzene degradation pathways.* The majority of all identified proteins from the anaerobic degradation pathways of toluene and ethylbenzene displayed constant abundances, irrespective of the applied alkylbenzene concentrations. Thus elevated abundance of the complete set of catabolic enzymes was apparently not used as a means to counteract the sublethal alkylbenzene concentrations. Exceptions to this trend were the catalytic subunit (BssA) and chaperone (BssE) of benzylsuccinate synthase, both of which are specifically up-regulated at sublethal toluene concentrations (740 µM). This may result in an increased transformation of toluene to (*R*)-benzylsuccinate, which could contribute to the observed higher tolerance of strain EbN1 towards toluene. However, a mutant of *Pseudomonas putida* strain DOT-T1 that was unable to use toluene aerobically did not differ in toluene

tolerance as compared to the wild-type. This suggested that the capacity to grow with toluene did not enhance the tolerance towards toluene (Mosqueda et al. 1999).

Polyhydroxyalkanoate (PHA) synthesis. Some bacteria accumulate PHAs as energy and carbon storage compound during conditions of nutrient excess or limitation (Anderson and Daves 1990). In *Ralstonia eutropha* 3 different enzymes are known to be involved in PHA formation. Initially, acetyl-CoA acetyltransferase (PhbA) forms acetoacetyl-CoA from 2 acetyl-CoA units. Then acetoacetyl-CoA reductase (PhbB) reduces acetoacetyl-CoA to (D)-3-hydroxyacyl-CoA, which is subsequently polymerized to polyhydroxybutyric acid by PHA synthase (PhaC). The product accumulates as granules which are coated with phospholipids and proteins, mostly phasins, which can amount to 5 % of the total cellular protein. Phasins are synthesized only in PHA accumulating cells, and only in amounts that can be bound to the granules (Wieczorek et al. 1995) and thus can be used as marker for PHA synthesis (York et al. 2001).

The genome of strain EbN1 contains genes required for PHA-synthesis (PhbA not unambiguously assigned) and several genes for phasin-like proteins. Based on the present proteomic analysis, formation of PhbB and PhaC and the possible phasins EbA1323, EbA6852 and EbA5033 was demonstrated. The abundance of these proteins increased most profoundly in the presence of 740 µM toluene. The phasins were also up-regulated in the presence of 315 µM ethylbenzene. EbA5033 showed also slightly increased abundances in cultures with 70 µM toluene and 210 µM ethylbenzene. The sequence similarities of the six predicted phasins of strain EbN1 (Rabus et al. 2005) to their orthologs in *R. eutropha*, a model organism for investigation of PHA synthesis, were all in the range of similarity of known or putative phasins (Table 4) (Potter et al. 2004). Phasins can amount to 3-5 % of total cellular protein in *R. eutropha* (Potter et al. 2004), while they represent only low abundant spots in strain EbN1. Considering the tight correlation between phasin and PHA amounts, it seems that storage compound formation of strain EbN1 in response to sublethal toluene and ethylbenzene concentrations is not as pronounced as in nutrient limited *R. eutropha*. However, one may speculated that PHA formation allows uncoupling of alkylbenzene degradation from a decelerated nitrate-reduction or provides hydrophobic surfaces for intracellular absorption of the toxic alkylbenzenes.

Table 4. Similarity[a] of the 6 paralogous phasins of strain EbN1 with their 4 orthologs from the model organism *R. eutropha*.

Strain EbN1	*R. eutropha*[b]			
	PhaP1	PhaP2	PhaP3	PhaP4
EbA1323	66	48	55	45
EbA2771	60	51	48	42
EbA6668	53	45	nd	45
EbA5598	47	42	49	43
EbA6852	51	45	40	40
EbA5033	42	49	44	50

[a] similar amino acids [%] as calculated by BLAST-p
[b] taken from Potter et al. 2004

Stress-related proteins. In the presence of sublethal concentrations of toluene or ethylbenzene strain EbN1 regulated proteins which are typically known from osmotic or oxidative stress response: the abundances of SodB, AcnA, AcnA2 and BetB increased, while those of two iron uptake related proteins decreased (Wilderman et al. 2004).

The SodB superoxide dismutases (Fe-containing) removes O_2-anions, which are formed as by-product during oxygen respiration. Even though dioxygen and superoxide are not present in anoxic media, formation of superoxide dismutase has been observed under anoxic conditions in facultative anaerobes, possibly to keep cells prepared for a return of oxic conditions (Karagalioglu and Imlay 1994), and even in obligate anaerobes (Brioukhanov and Netrusov 2004). However, there are also reports of increased Sod activity in *Porphyromonas gingivalis* (Amano et al. 1992) and *sodB* expression in *E. coli* as response to nitrate (Privalle and Fridovich 1991). As this was not observed in a nitrate reductase deficient strain of *E. coli*, a correlation to intermediates of denitrification such as radical-containing NO appeared possible. NO can be accidentally converted to the highly reactive nitroxyl-anion (NO^-) via several intracellular routes (Hughes 1999). NO^- is a known substrate of superoxide dismutase which re-oxidises it to NO (Reif et al. 2001).

The proteins EbA1861 and EbA4918, which are predicted to act as periplasmatic binding proteins for iron uptake, are down-regulated in the presence of high alkylbenzene concentrations. EbA4918 is similar (46 %) to the HitA protein (ANN70449) of *P. putida* KT2440, the abundance of which is decreased upon exposure to phenol (Santos et al. 2004). It is also highly similar (58 %) to a protein (ANN53820) of *Shewanella oneidense* MR-1, which was shown to belong to the fur-regulon (Wan et al. 2004). The main function of Fur (ferric uptake regulator) is to control iron homeostasis by repressing gene expression of iron uptake systems (Ernst et al. 1978; Hantke 1981). Interestingly, Fur is also known to indirectly induce *sodB* expression in *E. coli* (Wilderman et al. 2004).

The AcnB isoenzyme of aconitase contains a [4Fe-4S] cluster, which is in equilibrium with the intracellular iron pool. Under normal conditions it is more abundant than the AcnA isoenzyme. However, AcnB loses one of its iron atoms under oxic stress or iron starvation conditions and is then replaced by AcnA, which contains a more stable [4Fe-4S] cluster (Varghese et al. 2003). Thus one may speculate, that growth at sublethal concentrations of alkylbenzenes affects denitrification in that more NO is formed. This could lead to increased oxidative stress, translating into Fur-mediated changes in the expression of a variety of genes (*sodB, ebA1861, ebA4918* and *acnA*).

Betaine aldehyde dehydrogenase (BetB), catalyzes the last reaction in betaine biosynthesis. The increased abundance of BetB at sublethal concentrations of toluene and ethylbenzene indicated production of betaine. Synthesis of the osmotically active betaine is usually associated with adaptation to osmotic stress, but there are also reports about an involvement in solvent tolerance. In ethanol stressed *E. coli* expression of *betB* and a benefical effect of betaine supplementation was shown (Gonzalez et al. 2003). Osmotically stressed cells of *Oceanomonas baumannii* increased betaine synthesis upon phenol treatment (Brown et al. 2000). An alterated phase transition temperatures of artificial phospholipid membranes in presence of betaine was also observed (Rudolph et al. 1986).

The products of several genes (*wrbA*, *aldB* and *gabD*) which are under σ^S control in *E. coli* and indicative of reduced growth rates (Ihssen and Egli 2004), were found to be of increased abundance with sublethal alkylbenzene concentrations in strain EbN1. WrbA enhances repression of genes of the tryptophane operon by binding to the tryptophane repressing protein TrpR. It was 2.4 to 5.8 -fold up-regulated at sublethal alkylbenzene concentrations. In a *rpoS* (σ^S-encoding) mutant of *E. coli*, *wrbA* expression was 5-fold lower than in the wild-type (Lacour and Landini 2004). In glucose-limited *E. coli* cells, *wrbA* expression increased by 7.2-fold during transition from the exponential to the stationary growth phase (Schembri et al. 2003). *WrbA* expression seems to be gradually regulated, since it was 2.1-fold up-regulated in slowly growing, salicylate treated cells (Pomposiello et al. 2001) and 14-fold up-regulated in growth-arrested, antibiotic agent (DHCP) treated cells (Phadtare et al. 2002).

The putative 4-aminobutyraldehyde dehydrogenase (AldB) and the succinate semialdehyde dehydrogenase GabD belong to the putrescine degradation pathway and were up-regulated in the presence of 740 µM toluene. In *E. coli* expression of *aldB* and *gabD* is positively

regulated by σ^S (Xu and Johnson 1995; Metzner et al. 2004) and a 4-fold up-regulation of *adhB*-expression during transition from the exponential to the stationary growth phase was observed (Schembri et al. 2003). GabD also increased in abundance in phenol-stressed *P. putida* (Santos et al. 2004) and in paraquat or hydrogen peroxide treated *Bacillus subtilis* (Mostertz et al. 2004). Elevated putrescine concentrations are generally observed in cultures entering the stationary growth phase or being exposed to oxidative stress (Tkachenko and Nesterova 2003). Conversely up-regulation of enzymes involved in the putrescine degradation may indicate a return to stable conditions. Substrate, role or σ^S dependence of the co-regulated AdhB or its ortholog in *E. coli* YiaY are unknown.

Solvent efflux pumps like the AcrAB-TolC system belonging to the RND (resistance-nodulation-cell division family) are supposed to be the major factor rendering bacteria solvent tolerant and their constitutive or hydrocarbon-induced formation has often been observed (Ramos et al. 2002). TolC is the outer membrane protein of this complex and was previously detected by 2 DE in *E. coli*, *P. putida*, *Caulobacter crescentus* and *Synechocystis* sp. (Otto et al. 2001; Huang et al. 2002; Ireland et al. 2002; Santos et al. 2004; Yohannes et al. 2004). Even though the genome of strain EbN1 contains two copies of the AcrAB-TolC system, none of its components has been detected on 2DE in the present study.

Similarily, the often observed induction of heat shock or general stress response proteins upon treatment with diverse chemicals was not observed in this study with strain EbN1. However, detailed analysis of the literature revealed, that hydrocarbons were only once reported to induce these protein classes (Blom et al. 1992; Vercellone-Smith and Herson 1997; Bott et al. 2001; Hayashi et al. 2003).

Respiratory proteins. After the membrane-bound nitrate reductase NarGHI, the periplasmatic cytochrome cd_1 nitrite reductase NirS is the second enzyme of denitrification. In the presence of sublethal alkylbenzene concentrations NirS increased about 3-fold in abundance with a remarkable share of 6 % of all 2DE-separated proteins. It exhibits similarities of 74-84 % to its paralogs in *Pseudomonas stuzeri*, *Paracoccus denitrificans* and *Pseudomonas fluorescens* where the coding gene *nirS* was described to be induced sequencially i.e. later than *narGHI* (Baumann et al. 1996; Härtig et al. 1999; Philippot et al. 2001). In strain EbN1 grown on succinate under nitrate-reducing conditions a strong up-regulation of NirS was observed in the late exponential growth phase where the accumulated high nitrite concentrations were

reduced. Earlier up-regulation might be due to an increased energy demand or hampered nitrate reduction or due to regulatory interference.

Fatty acid biosynthesis. The reduced abundances of the biotin-dependent acetyl-CoA carboxylase (subunit AccD) and biotin synthase (BioB) may point to changes in fatty acid biosynthesis.

Phospholipid analysis. Chemical analysis of the intact phospholipids revealed that strain EbN1 altered the head group composition significantly in response to changes in alkylbenzene concentration, most remarkable is the appearance of PC not observed in benzoate or succinate grown cells (Table 3). However, the adaptation to sublethal concentrations differed between toluene and ethylbenzene for other PL. In case of ethylbenzene, the share of PE decreased in favour of PG, whereas in case of toluene both PE and PG decrease to the benefit of the methylcyclohexyl-modified PE (PE-MCH). Based solely on the empirical formula of this tentatively identified PL, one might also expect an increased head group area for this species. An increased incorporation of PL with larger head group area than PE is supposed to have a stabilizing effect on the phospholipid bilayer despite their lower phase transition temperatures compared to PE (Cullis et al. 1996). Using a mathematical model it has been shown that a 1:3 mixture of PG to PE results in tightest packing, optimal bond formation between PL molecules and thus in the most stable and impermeable membrane (Dowhan 1997; Murzyn et al. 2005). Observations like the reduced share of PE (78 to 63 %) in favour of PG and diphosphatidylglycerol (DPG or cardiolipin) in *P. putida* DOT-T1 after exposure to toluene (Ramos et al. 1997a) were frequently described (Weber and de Bont 1996; Isken and de Bont 1996; Ramos et al. 2002). Differences in the shares of PL with larger head groups than PE might be either directly due to the different molecular structures of toluene and ethylbenzene or due to the cellular localization of the activating enzymes as benzylsuccinate synthase is a cytoplasmic and ethylbenzene dehydrogenase a periplasmic enzyme. Furthermore, regarding the PC synthesis as common response of strain EbN1 to increasing alkylbenzene concentrations 80 µM ethylbenzene seem to excert a similar stress level as 240 µM toluene, an observation which is in agreement with the physiological data.

Next to changes of the head group composition also modification of the fatty acid residues can contribute to solvent tolerance. E.g. isomerization of unsaturated fatty acids from *cis* to

trans or saturation of the double bond increase the degree of order and thus the phase transition temperature which also increases with chain length (Weber and de Bont 1996; Isken and de Bont 1996; Ramos et al. 2002). Analysis of the composition of saponified phospholipids fatty acids showed a slightly increasing chain length with increasing ethylbenzene concentrations but a clear increase of saturation for toluene. Cells grown with benzoate or succinate resembled ethylbenzene or 240 µM toluene grown cells except for the increased share of 18:1. This was in general agreement with the pattern of the intact PLFA apart from a strong increase in 16:1/16:1 in expense of 16:1/16:0 of PC with increasing ethylbenzene concentration. In general, the distribution pattern of the PLFA with $C_{16:0}$ and $C_{16:1}$ as predominant fatty acids with a proportion of 95-97 % reflects a common pattern for bacterial organisms (Harwood and Russel 1984; Wilkinson 1988).

PHA analysis. Linearly growing cells of strain EbN1 contained up to 10 % (dry mass) poly-β-hydroxybutyrate (PHB) with increased concentrations of the anaerobic growth substrates toluene and ethylbenzene. In comparison the well-studied *R. eutropha* accumulated PHB up to 90 % (dry mass) under nutrient limited conditions or in the stationary growth phase (York et al. 2001). PHB aggregates in granules which are encapsulated by a membrane containing phasin proteins like PhaP. The PHB content correlates with the share of PhaP from the total cellular protein (up to 5 % PhaP) (Wieczorek et al. 1995). In case of strain EbN1, the combined share of the three identified phasin-like proteins from the total of DIGE-separable proteins was as follows: 0.28 % phasins versus 5 % PHB in cells grown with 740 µM toluene and 0.38 % phasins versus 10 % PHB in cells grown with 315 µM ethylbenzene. These ratios roughly fit to the values reported for *R. eutropha*.

Conclusions

This study revealed that strain EbN1 is able to anaerobically grow with toluene (2.2 to 740 µM) and ethylbenzene (10 to 315 µM) over a wide concentration range. In environments contaminated with gasoline maximal concentrations of toluene and ethylbenzene were 110 µM and 16 µM, respectively (Aronson and Howard 1997). Biological formation gave rise of toluene concentrations of 3 µM in the hypolimnion of pristine waters (Jüttner 1990) and 456 µM in sewage treatment plants (Mrowiec et al. 2005). Thus, strain EbN1 seems to be well adapted to different concentrations of hydrocarbon growth substrate as observed in its natural habitat of soils and limnic sediments. Studying adaptation to increasing alkylbenzene concentrations on the level of the membrane, the most significant change was the utilization of choline as phospholipid (PL) head group residue. Next to this, we observed an ethanolamine PL head group residue modified by an added methylcyclohexane, which was never reported before to our knowledge.

On the level of the proteome, little changes between low and medium alkylbenzene concentrations were observed, but numerous at sublethal concentrations. As inferred from the predicted functions of the regulated proteins, cells suffered of low energy status and of oxidative stress, possibly due to a disturbed membrane structure and the concomitant release of reactive NO species. On the other hand cells were active enough to use the surplus of reducing equivalents for the formation of storage compounds. This last proteomic finding was confirmed by the determination of up to 10 % PHB indicating the swift redirection of metabolism by strain EbN1 and the benefit from global proteome analysis.

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Solvent (alkylbenzene) tolerance of aromatic degrading denitrifying strain EbN1

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Abstract

Hydrocarbons with a *n*-octanol : water partition coefficient of log 1.5 to 4 accumulate in biological membranes, leading to increased fluidity and impaired function. The membrane solubility of hydrocarbons determines their toxic potential, posing another challenge to hydrocarbon degrading microorganisms, which already have to employ specific biochemical reactions for the degradation of these recalcitrant compounds. The denitrifying bacterium strain EbN1 anaerobically degrades toluene and ethylbenzene. To study the stress response of strain EbN1 to solvent pressure exerted by these two alkylbenzenes, anaerobically growing, succinate-adapted cultures were abruptly exposed to the alkylbenzenes. Growth was still observed at up to 0.98 mM toluene and 0.37 mM ethylbenzene, however growth rates were strongly reduced, and nitrate consumption and intermediate nitrite formation decelerated. The impact on the protein composition was quantitatively analyzed by 2D DIGE, with –fold changes in relative protein abundances ranging between –5.37 and 11.95. Overall exposition to toluene and ethylbenzene induced the differential regulation of 68 and 57 proteins, respectively, with 27 shared under both conditions. Known proteins of the anaerobic toluene and ethylbenzene pathways could not be detected, demonstrating that neither of the two alkylbenzenes could be actively degraded during the incubation time. Most prominently up-regulated proteins included heat shock proteins, nitrite reductase (NirS) and nitrous reductase (NosZ). Regulation of the latter agreed with the impaired denitrification.

Introduction

Alkylbenzenes such as toluene and ethylbenzene are major constituents of crude oil (up to 4 % [v/v]; Tissot and Welte 1984) and gasoline (about 20 % [v/v], Cline et al. 1991) and are produced at large scale as solvents and starting compounds for chemical synthesis. As part of industrialization hydrocarbons have increasingly been released into the natural environment due to accidental spillages during production, transport and storage (EPA 1986). Such contaminations are of particular concern in the vicinity of groundwater aquifers, since several hydrocarbons (e.g. BTEX) are both water-soluble and toxic. E.g. 1:20 mixtures of gasoline and water contain 70 mg/l toluene, 3.2 mg/l ethylbenzene and 130 mg/l BTEX (Cline et al. 1991). These aromatic hydrocarbons also have toxic and carcinogenic potential (Dean 1985). The mainly O_2-limited or even anoxic conditions in such environments emphasize the significance of anaerobic biodegradation. Besides ultimate bioremediation goals, biodegradation also contributes to improve the living conditions for the non-degraders within the autochtonous flora.

Anaerobic biodegradation has to overcome the low chemical reactivity of hydrocarbons in the absence of highly reactive oxygen species and was first shown in microcosm experiments with alkylbenzenes (Kuhn et al. 1988). Subsequently, numerous pure cultures with the respective degradation capacities have been isolated (for overview see: Widdel and Rabus 2001; Rabus 2005). Strain EbN1 and most other denitrifying alkylbenzene degraders are related to the *Azoarcus*/*Thauera*–cluster within *Betaproteobacteria* and will be described as new genus. Strain EbN1 is unique for its capacity to degrade toluene and ethylbenzene anaerobically (Rabus and Widdel 1995a), also directly from crude oil (Rabus and Widdel 1996). The anaerobic degradation pathways of both alkylbenzenes to their first common intermediate (benzoyl-CoA) differ completely. Toluene is initially degraded via a radical-addition to fumarate forming (*R*)-benzylsuccinate (Leuthner et al. 1998), a reaction that is widespread among anaerobic toluene degraders (Rabus 2005). In contrast, ethylbenzene is directly oxidized at the methylene-carbon via (*S*)-1-phenylethanol (Johnson et al. 2001; Kniemeyer and Heider 2001a). Recently, the substrate-dependent regulation of both pathways was demonstrated (Kühner et al. 2005) and the complete genome sequence of strain EbN1 determined (Rabus et al. 2005).

The toxicity of organic solvents corresponds to their partition coefficient log P_{OW} in a mixture of *n*-octanol and water. Organic solvents with a log P_{OW} between 1.5 and 4 e.g. 2.69 for toluene and 3.2 for ethylbenzene are extremely toxic for microorganisms. Due to their lipophilicity these compounds accumulate in and thereby increase the fluidity of biological membranes. As a consequence interactions of membrane proteins are influenced and the membranes become permeabilized. Leakage of ions, ATP and even DNA, RNA and proteins after toluene treatment of *E. coli* was demonstrated (Jackson and de Moss 1965).

Solvent tolerance of microorganisms was studied mainly with respect to biotechnological applications. In case of biotransformations, solvents may be used as primary substrate, to supply another poorly water-soluble substrate or to remove a hydrophobic product when applied as inert second phase (Isken and de Bont 1998). In the latter case the aqueous phase will be saturated with the organic solvent which could be of highly toxic potential. Isolated toluene tolerant-strains predominantly belong to the genus *Pseudomonas* and are usually capable of aerobic hydrocarbon degradation (Isken and de Bont 1998). Their most important mechanisms of toluene tolerance are enhancing membrane stability by altering the composition of membrane fatty acids and phospholipid head groups and by energy-dependent active efflux pumps belonging to the resistance-nodulation-cell division (RND) family also known to confer antibiotic resistance (Ramos et al. 2002).

Recently, growth behaviour and molecular response of strain EbN1 at sublethal (almost growth preventing) concentrations of toluene or ethylbenzene, respectively, were investigated (Kühner et al., in preparation). Briefly, growth was observed with up to 0.74 mM toluene and 0.315 mM ethylbenzene, respectively. Under these conditions doubling times decreased markedly and intermediate excretion of nitrite was strongly reduced. Also the composition of the phospholipid head groups was changed. Proteomeic analysis by two-dimensional gel electrophoresis revealed, that abundances of the thus far identified proteins of toluene and ethylbenzene degradation (Kühner et al. 2005) did not change significantly. In contrast, nitrite reductase and proteins for the synthesis of storage compounds were of increased abundance. The reduced accumulation of nitrite and together with the formation of PHB (polyhydroxybutyrate) of up to 10 % dry weight could point towards an impeded respiration.

In the present work strain EbN1 was not adapted to growth with toluene or ethylbenzene, but with succinate. Actively growing cells were abruptly exposed to various concentrations of

toluene and ethylbenzene, in order to study the physiological and proteomic response of strain EbN1 to solvent stress when the respective degradation pathways were not operative.

Materials and methods

Medium and cultivation. The denitrifying bacterium strain EbN1 was cultivated under nitrate-reducing conditions as previously described (Rabus and Widdel 1995a). Cultivation was carried out in 500 ml flat glass bottles, which contained 400 ml medium with 10 mM succinate and were anoxically sealed under an N_2/CO_2 (90/10 [v/v]) atmosphere. Growth was determined by measuring the optical density at 660 nm (UV-mini 1240; Shimadzu, Duisburg, Germany).

Physiological experiments. Cells adapted for more than 5 passages to anaerobic growth with succinate were used as inoculum. During early linear growth, toluene and ethylbenzene were added directly to the aqueous medium and the bottles were vigorously shaken to dissolve the alkylbenzenes. The controls without alkylbenzene addition were treated similarly. Experiments with various toluene and ethylbenzene concentrations demonstrated 1.2 mM toluene and 0.5 mM ethylbenzene as sublethal, i.e. highest concentrations that still allowed growth.
Samples (2.5 ml) for determination of alkylbenzene- and nitrate/nitrite- concentrations and optical density were taken from the aqueous phase of the cultures using N_2-flushed, sterile syringes. From these samples, two subsamples were processed as follows: For HPLC-analysis of alkylbenzenes one 1 ml subsample was diluted with 1 ml methanol (HPLC-grade), filtrated (Spartan 13/0.2 RC; Schleicher & Schuell, Dassel, Germany) and stored in Teflon-sealed, 1.5 ml vials at 4°C. The other 1 ml subsample was first used to determine the optical density and then processed for HPLC-analysis of nitrate and nitrite as follows: The subsample was filtrated (Spartan 13/0.2 RC), stored at 4°C and diluted 1 : 10 in water (HPLC-grade) prior to analysis. Three cultures were analyzed for each tested condition. Parallel cultures yielded highly similar time courses of growth, alkylbenzene and nitrate/nitrite consumption. Controls lacked either organic substrate, hydrocarbon or inoculum.

Mass cultivation. Mass cultivation was performed to supply sufficient cell material for proteomic analysis. Three bottles (400 ml) of toluene, ethylbenzene or untreated parallel cultures were harvested at 45, 250 and 600 min after alkylbenzene addition. Cells were pelleted (8900 g for 20 min at 4°C; J2-MC centrifuge, Beckman, Fullerton, USA) washed twice with 100 mM Tris-HCl pH 7.5 containing 5 mM $MgCl_2$, immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Chemical analysis. Alkylbenzene concentrations were determined with an HPLC system (Sykam, Fürstenfeldbruck, Germany) as previously described (Rabus and Widdel 1995a) separation was achived with a C18 column (Grom-Sil 100 ODS-2 Fe, 250 × 4.6, Grom, Rottenburg, Germany) temperature controlled at 25°C. The eluent was composed of 80 % (v/v) acetonitrile and 0.75 mM phosphoric acid, and the flow rate was 1 ml/min. Toluene and ethylbenzene were detected with a UV-detector at 215 nm and had retention times of 4.5 and 5.1 min, respectively.

Nitrate and nitrite were analyzed with an HPLC-system (Sykam) as previously described (Rabus and Widdel 1995a). Separation was performed with an anion exchange column (60 × 4, IBJ A3, Sykam) and temperature-controlled at 50°C. The eluent was composed of 45 % ethanol with 20 mM NaCl, the flow rate was 1 ml/min, and nitrate and nitrite were detected at 220 nm. Acetate concentrations were determined with an HPLC system (Sykam) equipped with an anion exchange column (Aminex HPX-87H, 300 × 7.8 mm, Biorad, Munich, Germany) that was temperature controlled to 60°C. With 5 mM H_2SO_4 in HPLC-grade water as eluent and a flow rate of 0.6 ml/min, acetate was detected at 210 nm with a retention time of 14.7 min.

2-Dimensional difference gel electrophoresis (2D DIGE). Cells were disrupted with the PlusOne Sample Grinding Kit (Amersham Biosciences, Freiburg, Germany) and preparation of protein extracts was carried out as recently reported (Gade et al. 2003). Protein concentration was determined according to the method described by Bradford (1976). Isoelectric focussing (IEF) was performed as described before (Gade et al. 2003; Kühner et al. 2005), using the IPGphor system and 24 cm IPG strips with a nonlinear pH gradient of 3-10 (Amersham Biosciences). The EttanDalt II system (Amersham Biosciences) was used for separation according to molecular weight in 12.5 % acrylamide gels strengthened with Rhinohide (Molecular Probes, Eugene, OR, USA). Low fluorescent glass plates (Amersham Biosciences) were used for 2D DIGE.

2D DIGE was essentially carried out as described before (Gade et al. 2003; Kühner et al. 2005). For labeling 200 pmol CyDye were used per 50 µg of protein sample. Pre-electrophoretic labeling with different fluorescent dyes allows co-separation of three samples in a single gel. An individual experiment in the present study contained per gel: reference state, test state and internal standard; 4 parallel gels were run per experiment. Protein extracts from alkylbenzene treated cultures harvested at one of the three time points represented the six test states and were each labeled with Cy3. Protein extracts from untreated cultures

harvested at the respective time points served as reference states and were each labeled with Cy5. To determine proteome changes due to the growth phase, extracts from untreated cells harvested at 45 and 600 min were labeled with Cy3 and compared to the Cy5 labeled reference state harvested at 250 min. All performed experiments contained the same preparation of internal standard, which was composed of equal amounts of all reference and test states and was labeled with Cy2.

2D DIGE gels were scanned immediately after electrophoresis with a Typhoon 9400 scanner (Amersham Biosciences). Analysis of cropped images was performed with the DeCyder software (version 5.0; Amersham Biosciences). Parameters for co-detection of spots were: (i) detection of 3000 spots and (ii) exclusion of signals with slope > 1, area < 200, peak height < 190 and volume < 60000. Statistical analysis was based on independent spot maps. Differentially regulated spots were manually controlled to fulfill the following criteria: average ratio of < -2.5 and > 2.5, ANOVA p-value of < 0.05, t-test value of < 10^{-4}, number of matched gels > 81/108. The master gel contained 1999 matched protein spots. For better perception the Cy2 channel was omitted in Figs. 2-4.

The scatter plots beside the gel images in Figs. 2, 3 and 4 give an overview of the number of regulated proteins plotting fold change in abundance against the share from total protein. The latter was determined as follows: The total sum of all spot volumes was determined from the Cy3 images and the share of each regulated spot was calculated from that sum. In case of identified proteins represented by more than one spot on the gels, the respective volumes were added and the –fold changes averaged.

Since 2D DIGE is a minimal labeling technique one lysine in 3 % of a protein species is labeled, i.e. the majority of the protein remains unlabeled. For identification, gels were therefore subsequently stained with colloidal Coomassie Brilliant Blue (cCBB) (Doherty et al. 1998). Selected regulated proteins were manually excised in triplicates and identified by mass spectrometry.

Protein identification by mass spectrometry. Tryptic digest of excised, pooled proteins was performed as described before (Jeno et al. 1995). Peptide masses were determined by MALDI–TOF–MS (Corthals et al. 2000). Protein identification and genome analysis were based on the published list of annotated genes from the genomic sequence of strain EbN1 (Rabus et al. 2005). Peptide mass fingerprints were mapped by using the MS-Digest program (Clauser et al. 1999).

Results

Growth experiments. Cultures of strain EbN1 actively growing with succinate were suddenly exposed to toluene and ethylbenzene with concentrations ranging from 0.7 – 5.6 and 0.3 – 1.5 mM, respectively. Undiluted alkylbenzenes were added directly to the cultures during the early linear growth phase. The adaptive response of strain EbN1 was initially analyzed *in vivo* by determining growth (Fig. 1AB). Concentrations of 1.2 mM toluene and 0.5 mM ethylbenzene proved to be sublethal (almost growth arresting). While lower concentrations only slightly reduced growth rates relative to the untreated controls, growth was completely arrested at higher concentrations. All further experiments were carried out with these two sublethal alkylbenzene concentrations, which were added to succinate utilizing cultures after about 8 h of incubation.

Toluene and ethylbenzene were monitored when sublethal concentrations were added. In both cases they remained considerably stable during the incubation time, even though the average concentrations were somewhat lower than originally calculated. Since this was also observed in the sterile controls, absorption by the butyl rubber stoppers appears likely. In subsequent experiments with sublethal concentrations of toluene and ethylbenzene, respectively, only the experimentally determined average concentrations are indicated instead of the calculated concentrations.

The time courses of nitrate and nitrite concentrations (Fig. 1D) differed markedly between the untreated control and the cultures exposed to sublethal concentrations of toluene and ethylbenzene, respectively. In the latter case nitrate consumption was slowed down and less nitrite was intermediately accumulated. Also nitrite was then consumed more slowly and not completely.

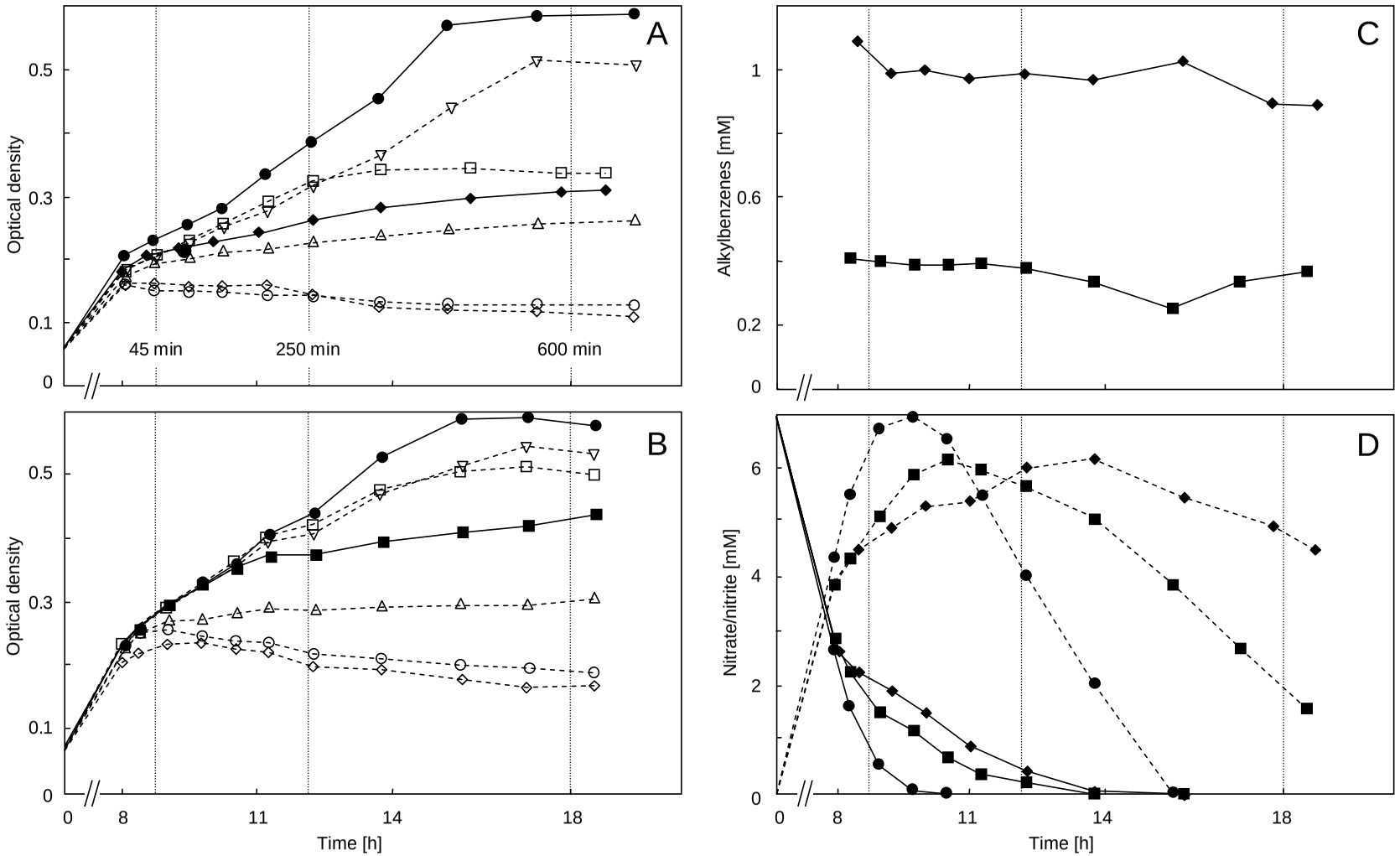

Fig. 1. Physiological response of strain EbN1 growing with succinate to sudden solvent exposure (after 8 h incubation). (A) Added concentrations of toluene [mM]: ●, 0; ▽, 0.7; □, 1; ◆, 1.2; △, 1.5; ○, 3; and ◇, 5.8. (B) Added concentrations of ethylbenzene [mM] ●, 0; ▽, 0.3; □, 0.4; ◆, 0.5; △, 0.6; ○, 0.8; and ◇, 1.67 mM. Sublethal (almost growth arresting) concentrations of toluene (1.2 mM) and ethylbenzene (0.5 mM) are indicated by full lines. (C) Time courses of toluene (◆; calculated addition; 1.0 determined average concentration) and ethylbenzene (■; 0.5 mM calculated addition; 0.4 mM determined average concentration). (D) Time courses of nitrate (full lines) and nitrite (dashed lines) concentrations in cultures without alkylbenzene (●), with 1.2 mM toluene (◆) and 0.5 mM ethylbenzene (■) addition. The dotted lines indicate the time points of sampling mass cultures for proteomic studies 45, 250 and 600 min after addition of toluene and ethylbenzene, respectively.

Proteomic analysis. The adaptive response of strain EbN1 to the sudden solvent pressure was subsequently analyzed on the protein level by means of 2D DIGE. For this purpose, treated (0.98 mM toluene and 0.37 mM ethylbenzene, respectively) and untreated (control) cultures were harvested 45, 250 and 600 min after alkylbenzene addition, giving rise to a total of 9 growth conditions. For each time point, the respective control served as reference state for treatment with toluene (Fig. 2) and ethylbenzene (Fig. 3). To consider proteins the abundance of which were merely regulated in response to transition into stationary phase, differential protein profiles of control cultures were analyzed independently (Fig. 4). Based on the require $\geq |2.5|$ –fold changes in abundance, a total of 81 regulated proteins were determined, with their intersections among the different growth conditions displayed in Fig. 5. E.g. 7 and 19 proteins, respectively, were specifically regulated in response to sublethal concentrations of toluene and ethylbenzene, respectively, while 25 were affected by both growth conditions. Two proteins were apparently only regulated during early linear growth in the untreated cultures; one of them could be identified as OmpC. For each investigated growth condition, the determined fold changes in protein abundance were also plotted against their calculated shares from the total protein (right panels in Figs. 2-4). The two most striking cases were observed 600 min after treatment with 0.37 mM ethylbenzene (Fig. 3C). EbA1323 had with 2.6 % the highest share from the total protein, even though the fold change in abundance was rather low with 2.91. In contrast, the most highly up-regulated (8-fold) protein NosZ had only a 0.21 % share from the total protein. Since 30 proteins changed their abundance also in a growth-dependent manner, only 51 proteins were affected by solvent pressure. This is a rather small fraction considering that the master gel contained almost 2000 matched protein spots. Remarkably, also the growth-phase depended changes in the protein profile of strain EbN1 were not very pronounced (Fig. 4).

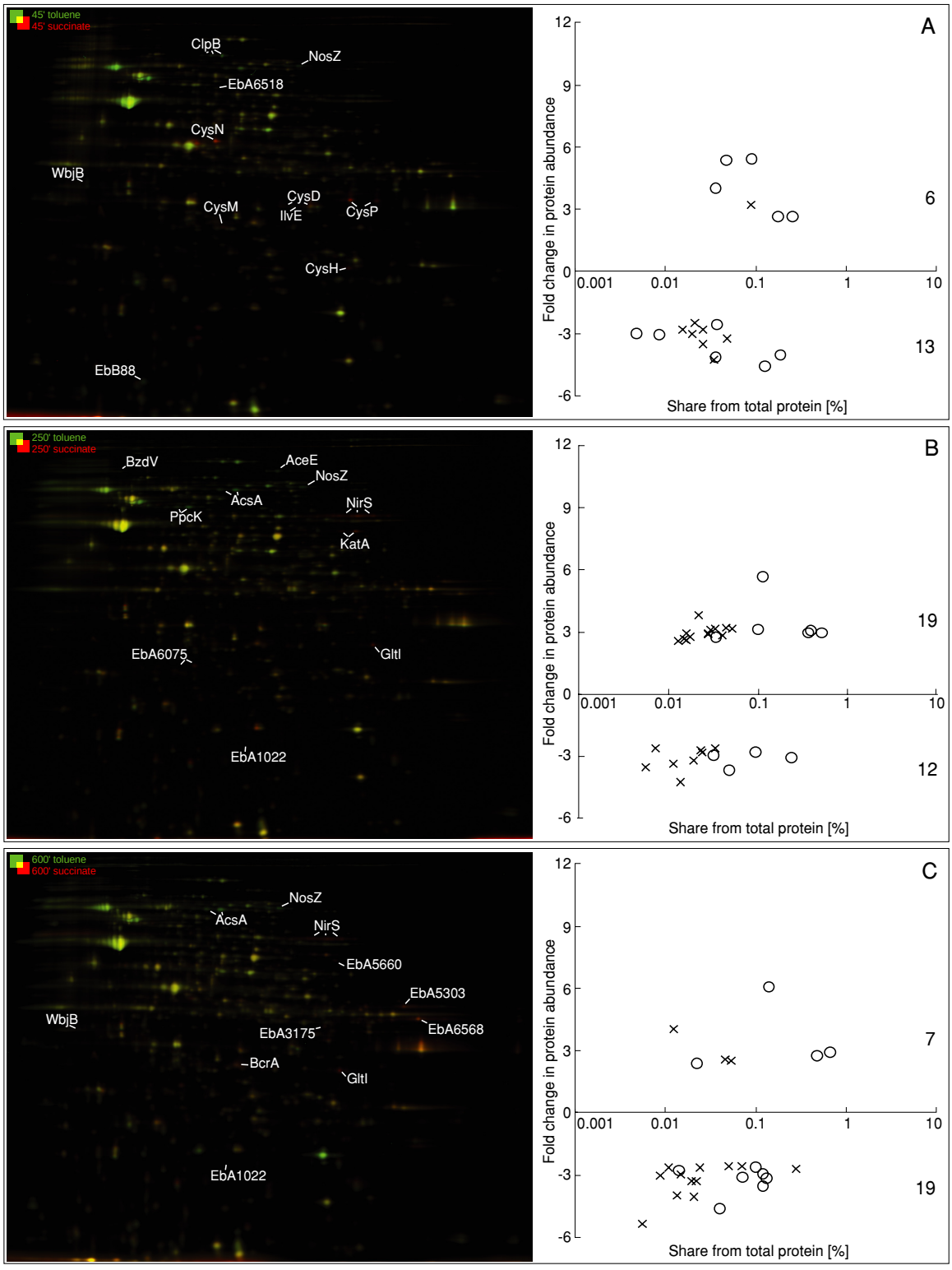

Fig. 2. Time courses of changes in protein profiles of strain EbN1 upon sudden exposure to 1.0 mM toluene, as determined by 2D DIGE. (A) 45 min, (B) 250 min and (C) 600 min after addition of toluene to cultures growing with succinate. In each case extracts from succinate-grown, untreated cells harvested at the respective time point served as reference state. The left panel displays false-colored 2DE images. Proteins with increased and decreased abundances, appear green- and red-colored, respectively, while proteins with unchanged abundance appear yellow. Proteins, that are regulated by more than |2.5|-fold and are mass-spectrometrically identified are annotated. The totalities of proteins regulated by more than |2.5|-fold are displayed in the right panel for each tested time point with respect to the share of each regulated protein from the sum of all proteins regulated by 2DE. Symbols: ○, identified proteins; ×, not identified proteins. Total numbers of up- and down-regulated proteins are indicated. Further information on the identified proteins is provided in Table 1.

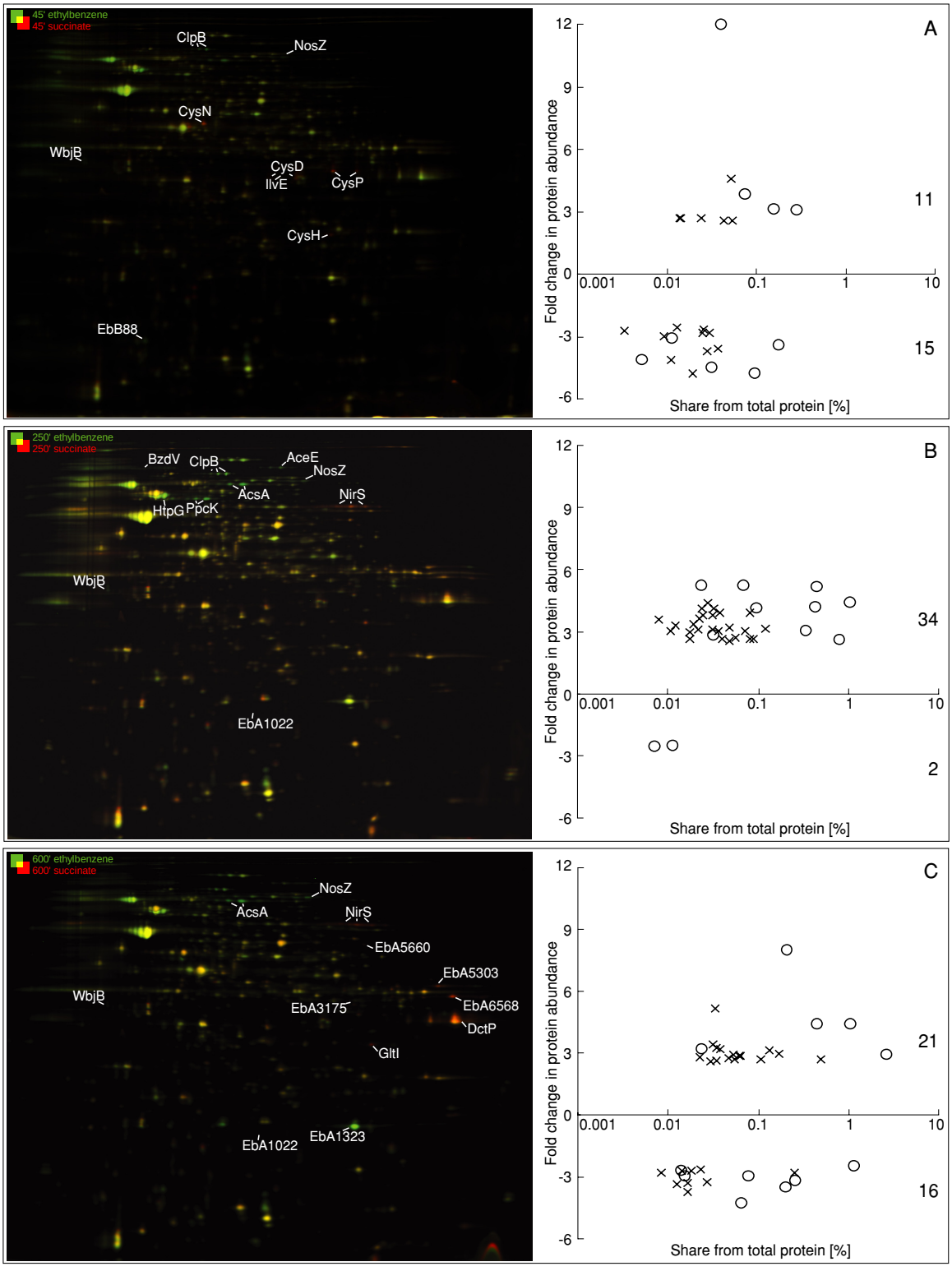

Fig. 3. Time courses of changes in protein profiles of strain EbN1 upon sudden exposure to 0.4 mM ethylbenzene, as determined by 2D DIGE. (A) 45 min, (B) 250 min and (C) 600 min after addition of ethylbenzene to cultures growing with succinate. Symbols: ○, identified proteins; ×, not identified proteins. Total numbers of up- and down-regulated proteins are indicated. For further information refer to the legend of Fig. 2 and Table 1.

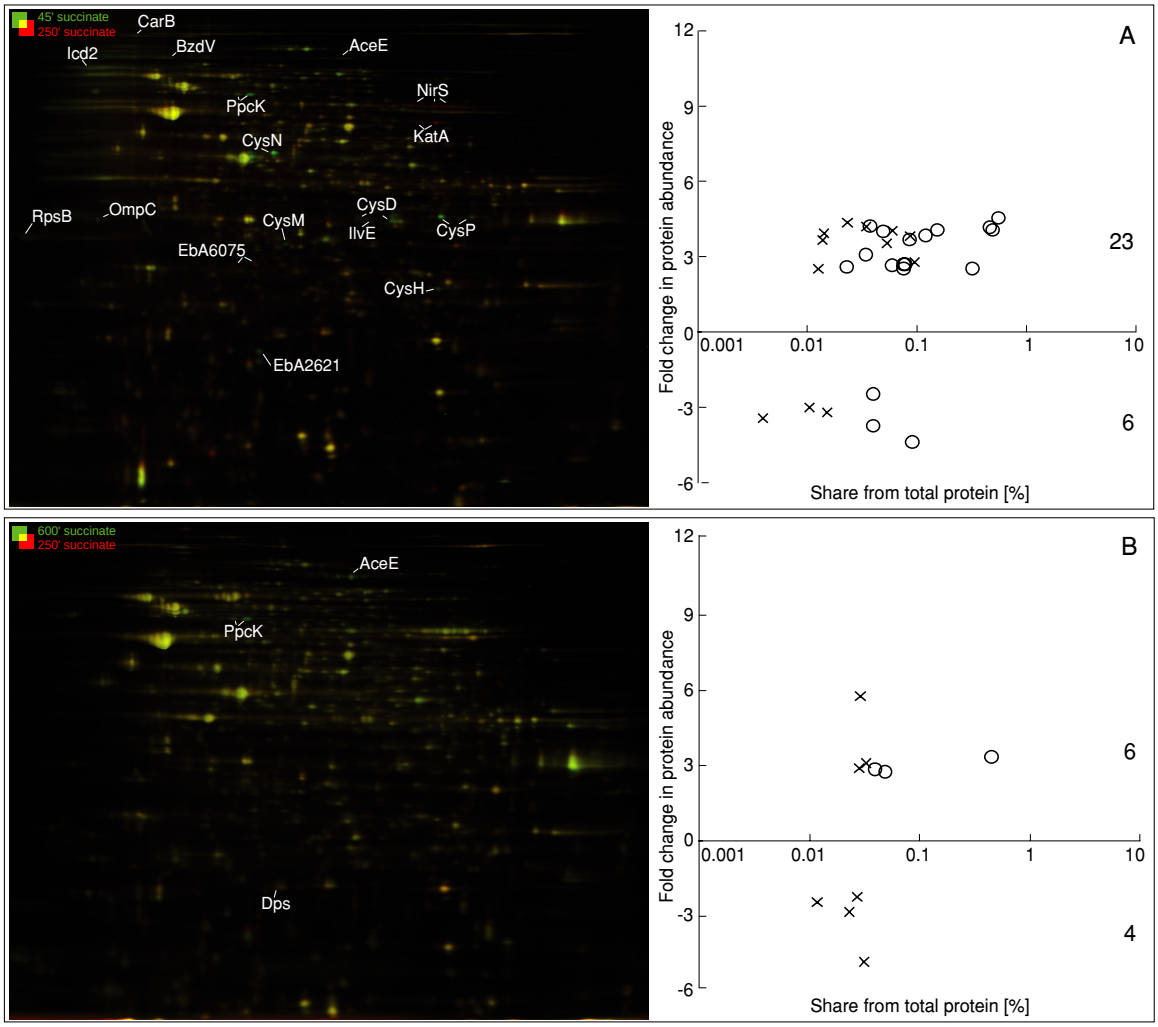

Fig. 4. Growth phase dependent changes in the protein profile of strain EbN1 grown with succinate in the absence of solvent pressure. (A) Cells harvested during early linear growth (corresponds to 45 min in Figs. 2 and 3). Cells harvested during late linear growth (corresponds to 250 min in Figs. 2 and 3) served as reference state. Symbols: ○, identified proteins; ×, not identified proteins. For further information refer to the legend of Fig. 2 and Table 1.

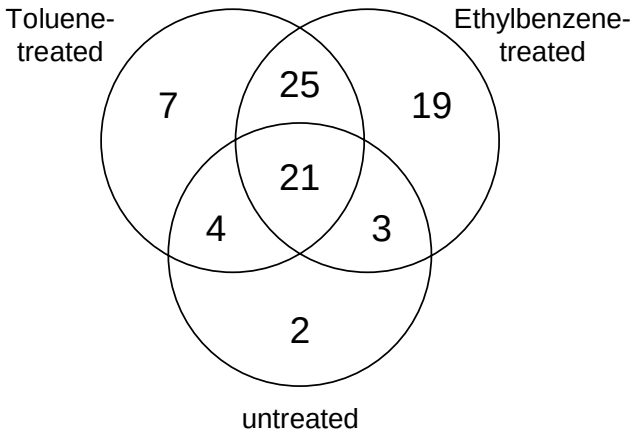

Fig. 5. Overview of regulatory specificity depending on growth conditions. Proteins were grouped according to (i) regulation exclusively under a single condition, (ii) co-regulation nder two or more different growth conditions. The latter gives rise to the different intersections. Different incubation times upon addition of sublethal alkylbenzene concentrations were not differentiated.

For protein identification, DIGE-gels were stained with cCBB immediately after scanning. Thirty-five reasonable abundant proteins were then excised from the gels and identified by mass-spectrometric analysis and PMF-mapping (MASCOT-score >50). Certainty of identification was assessed as follows. Three different protein spots were repeatedly excised from 8 different states and analyzed independently. IlvC and RpoA were identified 8 out of 8 times with average score of 130 and 195, respectively. CysN was identified 6 out of 8 times with an average score of 111. In the case of the two unsuccessful attempts mixtures of 2-3 proteins were present. Accordingly, all samples giving rise to multiple identifications were excluded. Protein identification was additionally confirmed by comparison to the evolving proteomic database of strain EbN1 (Wöhlbrand and Rabus, unpublished). Results of protein identification and difference in abundance are compiled in Table 1.

Stress-related proteins. Several heat-shock proteins (HtpG, EbB88 and ClpB) displayed temporarily increased abundances, with the highest fold changes (up to 3.03-fold) observed 45 min and lowest fold changes (down to 1.37-fold) 600 min after addition of alkylbenzenes. The *ebB88* gene is flanked by two genes coding for other putative heat shock proteins; the product identification of the downstream gene *ebA2733* was not of sufficient certainty (MASCOT-score 30, coverage 54 %). ClpB is a chaperone that resolubilizes proteins aggregated after heat shock.

The relative abundance of WbjB, a protein involved in *N*-acetyl-L-fucosamine biosynthesis needed for the O-antigen chain synthesis showed a similar course. Early (45 min) after addition of ethylbenzene and toluene the abundance of WbjB had a maximal increase by 11.95- and 3.15-fold, respectively. This increased abundance was lowered to 5.3- and 2.4-fold, respectively, after 600 min. Some proteins with changed abundance were also observed in the controls without addition of alkylbenzenes. OmpC, a porin of the outer membrane, had a decreased abundance in the stationary phase (600 min). In contrast, Dps, the DNA-binding ferritin protein protecting DNA against oxidative damage and catalase KatA showed increased abundance in the stationary phase.

Table 1. Identified proteins, the abundance of which changed in response to sudden exposure to toluene and ethylbenzene, respectively.

Protein name[a]	Predicted function[a]	Coverage[b]	Score[c]	Fold changes in protein abundance[d]							
				Toluene-			Ethylbenzene-			No- addition	
				45'[e]	250'[e]	600'[e]	45'[e]	250'[e]	600'[e]	45'[e]	600'[e]
	Stress Response										
HtpG	Heat shock protein	58%	142	2.18	2.11	1.74	2.37	2.58	1.81	1.09	1.29
EbB88	Putative heat shock protein (IbpA-COG)	79%	103	2.58	1.41	1.37	3.07	2.38	1.93	-1.64	1.14
ClpB	Chaperone	62%	148	2.6	1.71	1.56	3.01	3.03	2.4	-1.16	1.23
WbjB	Similar to Polysaccharide biosynthesis protein	47%	90	5.3	1.93	2.37	11.95	5.23	3.15	-1.48	-2.16
OmpC	Outer membrane protein (porin)	57%	66	1.02	1.25	1.35	1.52	1.61	1.26	2.7	1.73
Dps	DNA-binding ferritin-like protein	53%	65	2.34	1.34	1.13	2.12	1.4	1.09	1.42	2.87
KatA	Catalase	52%	150	1.81	-2.81	-2.1	1.75	-1.6	-1.48	-3.76	1
	Denitrification										
NirS	Cytochrome *cd* $_1$ nitrite reductase precursor	61%	321	2.14	-3.08	-3.54	2.06	-2.54	-3.53	-4.44	1.32
NosZ	Nitrous-oxide reductase precursor	61%	270	5.35	5.68	6.05	3.77	5.2	7.99	1.17	1.48
	Transporters										
EbA3175	Putative amino acid ABC transporter	62%	70	1.2	-2.07	-2.97	1.11	-1.51	-2.72	-1.82	1.09
EbA5660	Probable ABC transporter substrate binding protein	54%	227	-1.1	-1.54	-2.82	-1.17	-1.52	-2.99	-1.15	1.29
GltI	Putative ABC transporter, glutamate receptor	85%	157	-1.17	-3.66	-4.66	-1.15	-2.26	-4.3	-2.25	1.13
ebA5303	Putative ABC transporter subunit, benzoate uptake?	52%	74	-1.21	-1.8	-3.11	-1.13	-1.37	-3	-1.29	1.34
DctP	C4-dicarboxylate-binding periplasmic protein	26%	56	1.15	-1.13	-2.18	1.18	-1.13	-2.52	-1.02	1.52
	Metabolism										
AcsA	Acetyl-CoA synthetase	40%	56	1.77	2.96	2.88	2.24	5.17	4.38	1.78	1.35
EbA6518	Probable CoA ligase (AMP-forming)	36%	55	3.96	1.4	1.66	1.68	1.72	1.89	1.99	1.78
EbA1323	Conserved hypothetical protein, predicted phasin	38%	79	1.03	1.18	2.11	-1.06	1.53	2.91	-1.11	-1.61
BcrA	Benzoyl CoA reductase subunit	67%	62	-1.47	-1.89	-2.63	-1.48	-1.13	-1.33	1.56	-1
AceE	Pyruvate dehydrogenase multienzyme complex, E1 component	41%	266	-1.01	3.14	1.29	1.09	4.14	1.53	4	2.72
Icd2	Isocitrate dehydrogenase isozyme 2, monomeric type, NADP-specific	43%	50	1.39	2.15	2.19	2.2	2.1	1.62	2.51	1.26
BzdV	Subunit of oxidoreductase of unknown function, conserved in Azoarcus-type benzoate degradation gene clusters	54%	131	-1.03	2.77	1.36	-1.04	2.83	1.62	3.65	1.68
IlvE	Branched-chain amino acid aminotransferase	63%	165	-3.12	-1.06		-4.15	-1.1	-1.08	4.21	-1.02
CarB	Carbamoyl-phosphate synthase large chain	42%	151	-1.02	2.15	1.87	1.07	2.08	1.85	2.52	-1.52
RpsB	30S ribosomal protein S2	73%	71	1.29	2.09	1.85	2.22	2.01	1.49	2.55	1
PpcK	Phosphoenolpyruvate carboxykinase	49%	346	1.21	3.11	1.01	1.35	4.18	1.43	4.17	3.33

Protein name[a]	Predicted function[a]	Coverage[b]	Score[c]	Fold changes in protein abundance[d]							
				Toluene-			Ethylbenzene-			No- addition	
				45'[e]	250'[e]	600'[e]	45'[e]	250'[e]	600'[e]	45'[e]	600'[e]
	Sulfur assimilation										
CysP	Periplasmic thiosulfate-binding protein	38%	62	-4.65	-1.56	-2.33	-4.8	-1.38	-2.34	4.05	1.11
CysD	Putative ATP sulfurylase small subunit	55%	120	-3.08	-1.11	-1.23	-3.13	-1.01	1.14	3.05	-1.08
CysN	ATP sulfurylase large subunit	54%	126	-4.07	1.01	-1.25	-3.46	1.14	-1.16	4.51	1.15
CysH	APS-reductase	86%	179	-4.18	-1.19	-1.24	-4.54	-1.21	-1.37	3.81	-1.07
CysM	cysteine synthase B (anaerob)	73%	216	-2.61	-1.01	-1.15	-1.19	-1.03	-1.31	2.7	1.05
	Proteins with unknown function										
EbA1022	Conserved hypothetical protein	51%	63	1.77	2.96	2.72	2.24	5.17	4.38	1.78	1.35
EbA2621	Conserved hypothetical protein	66%	54	-1.3	1.96	1.87	-1.58	1.6	1.19	4.06	2.18
EbA3357	Hypothetical protein	64%	54	-3.13	-3	1.14	-1.77	-1.98	-3.56	1.05	3.51
EbA6075	Predicted short chain dehydrogenase	63%	75	-1.06	-2.76	-2.47	-1.05	-1.74	-1.74	-2.52	-1.05
EbA6568	Hypothetical protein	50%	56	-1.18	-1.52	-3.2	-1.3	-1.42	-3.2	-1.1	1.5

[a] Protein name and predicted function as published, www.micro-genomes.mpg.de/ebn1 (Rabus et al. 2005).

[b] Coverage [%] of the mass-spectrometrically determined peptides relative to the total amino acid sequence of the identified proteins.

[c] Score of MS-based identification as calculated using mascot.

[d] Cultures of strain EbN1 actively growing with succinate were treated with 0.98 mM toluene, 0.37 mM ethylbenzene or no alkylbenzenes (control). Fold changes in protein abundance were determined by 2D DIGE. Samples for 2D DIGE analyses were obtained 45, 250 and 600 min after addition of alkylbenzenes.

[e] Time after shock with alkylbenzenes.

Denitrification. Two enzymes of denitrification, NirS [nitrite (NO_2^-) reductase] and NosZ [nitrous (N_2O) reductase] showed changes in abundance (Fig. 6). In the absence of alkylbenzenes, the abundance of NirS increased by 4.4- and 5.7-fold in late linear (250 min) and stationary (600 min) growth phase, respectively, relative to the early linear growth phase (45 min). The up-regulation of NirS coincided with the depletion of nitrate. In the presence of alkylbenzenes however, NirS was already about 2-fold up-regulated at the early time point (45 min) and decreased only slightly towards the end of incubation. NosZ behaved differently, since it appeared un-regulated in the untreated controls and displayed increasing abundance (5.35- to 6.05-fold and 3.77- to 7.99-fold) with time in the presence of toluene and ethylbenzene, respectively.

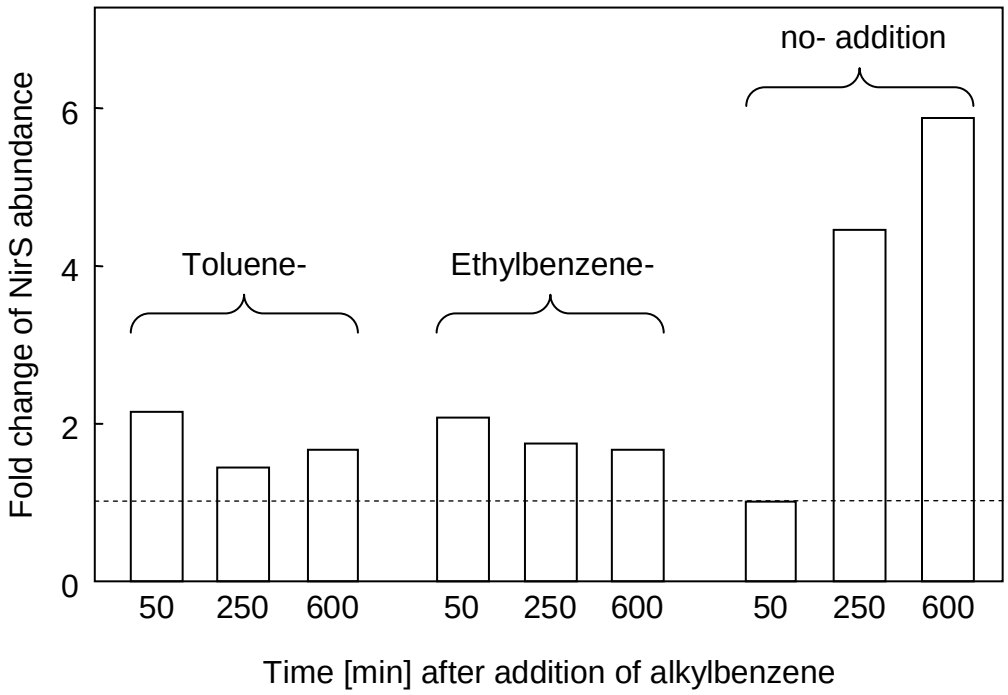

Fig. 6. Changes in abundance of nitrite reductase (NirS). Due to the dynamic behaviour of the reference states (untreated cells after 45, 250 and 600 min), a single reference state (untreated cells after 45 min) was used for all growth conditions.

Transporters. They are all organized in operon-like structures containing the genes for the other subunits of the respective transport systems. EbA3175 belongs to an ABC-transporter (Liv) for the uptake of leucine, isoleucine and valine. EbA5660 could possibly be specific for peptide-reception. GltI is part of a glutamate transporter. EbA5303 probably belongs to an ABC-transporter, the genes of which are closely localized to the catabolic genes of anaerobic benzoate degradation. DctP belongs to a TRAP transporter for the H^+-driven uptake of dicarboxylates.

Metabolism. Several metabolic proteins were specifically changed in abundance after alkylbenzene treatment. The acetyl-CoA synthetase AcsA (1.77 to 5.17-fold) and EbA6518 a probable CoA-ligase (3.96-fold) were of increased abundance. Similarly, EbA1323 a hypothetical protein predicted to be a phasin, which are part of the coating membrane of PHA (polyhydroxyalkanoate) granules, was of slightly increased abundance 600 min after treatment. The coding *ebA1323* gene is in 28 bp distance of a gene possibly coding for an enoyl-CoA hydratase/carnitine racemase. BcrA, a benzoyl-CoA reductase subunit, showed a 2.6-fold decrease 600 min after toluene treatment.

During the transition from the linear (45 min) to the stationary (600 min) growth phase of untreated cells the abundance of several proteins decreased markedly: AceE, a subunit of the pyruvate dehydrogenase complex; Icd2, an isocitrate dehydrogenase; BzdV, an oxidoreductase subunit of unknown function linked to benzoate degradation; IlvE, an amino acid amino-transferase involved in valine or alanine biosynthesis; CarB, carbamoylphosphate synthase, which is involved in nucleotide synthesis; and RpsB, a subunit of the 30S ribosome. Interestingly, the abundances of CarB and RpsB remained at a high level in the presence of alkylbenzenes. Phosphoenolpyruvate carboxykinase (PpcK) of gluconeogenesis showed an exceptional regulatory pattern. While its abundance was decreased by about 3.5-fold in mid-linear growth phase (250 min) of untreated cells, it was increased by 3- to 4-fold in the presence of alkylbenzenes at the same time point.

Sulfur assimilation. Proteins involved in uptake (CysP) and activation (CysDN) of sulfate, reduction of sulfate to sulfite (CysH) and addition of sulfide to acetylserine (CysM) were found to decrease in abundance from 4- to 1-fold during transition into stationary phase. Alkylbenzene treatment lead to abundances similar to stationary phase levels of untreated cells.

Conserved / unique proteins of unknown function. The five regulated proteins of this category are encoded by genes distributed across the entire chromosome of strain EbN1. The abundance of EbA1022 increased 3- to 5-fold 250 min after addition of alkylbenzenes. EbA2621 had a maximum in relative abundance during mid-linear growth (250 min) in untreated cells. The coding gene overlaps 4 bp with the one of CysI, a sulfite reductase subunit suggesting coregulation and thus indirectly indicating differential regulation of the whole sulfur assimilation pathway. In contrast, the abundances of EbA3357 and EbA6075 increased at 250 min in untreated cells. Interestingly, in *Pseudomonas stutzeri* the coding gene of the Orf247 (59 % similarity to EbA6075) is located downstream of *nnrS* and both are transcribed when nitrite and nitric oxide reduction occurs (Honisch and Zumft 2003).

Finally, the relative abundance of EbA6568 decreased steadily after alkylbenzene treatment.

Toluene and ethylbenzene degradation. In a previous study, 12 and 11 proteins of the anaerobic degradation pathways for toluene and ethylbenzene, respectively, could be identified on 2D DIGE-gels (Kühner et al. 2005). In the present study, non of these proteins could be detected in the succinate-utilizing culture 45, 250 or 600 min after addition of toluene or ethylbenzene, respectively. Thus the two catabolic pathways were not operative during the incubation time.

Discussion

Growth experiments. The initial growth experiments (Fig. 1) revealed a progressive decrease in growth rate and yield in response to increasing concentrations of toluene and ethylbenzene added to cultures of strain EbN1 actively growing with succinate under nitrate-reducing conditions. Application of average (across incubation time; Fig. 1C) concentrations of 0.98 mM toluene or 0.37 mM ethylbenzene established sublethal conditions, which were characterized by markedly reduced growth rate and yield, decreased nitrate reduction and incomplete nitrite consumption. This is reminiscent of the behaviour of strain EbN1 during growth with ethylbenzene, when supplied as sole source of organic carbon at the sublethal concentration of 0.32 mM (Kühner et al., in preparation). Interestingly, growth at sublethal concentrations of toluene (0.74 mM) did not affect the yield (Kühner et al., in preparation). In *Paracoccus denitrificans* treatment with pesticides resulted also in reduced cell yields and changes in the denitrification pattern: Untreated cells reduced 23 mM nitrate with intermediate excretion of 7 mM nitrite; dimethoate treated cells formed 16 mM nitrite and were apparently incapable of further reduction; in contrast, methylparathion, atrazine or simazine treated cells grew without any nitrite accumulation (Saéz et al. 2003).

Relevance of applied alkylbenzene concentration. The average sublethal concentrations of toluene (0.97 mM) and ethylbenzene (0.37 mM) present in the medium during the entire incubation time translate into theoretical membrane concentrations of 91 mM (toluene) and 97 mM (ethylbenzene), respectively. These values were calculated from the partition coefficients in the *n*-octanol/water and membrane/buffer systems (Sikkema et al. 1994). When strain EbN1 is grown with toluene and ethylbenzene as sole source of organic carbon at sublethal concentrations (0.74 mM and 0.32 mM, respectively) the respective theoretical membrane concentrations are 66.9 mM and 82.8 mM, respectively. Thus, strain EbN1 tolerates apparently 27 % higher membrane concentrations of alkylbenzene during growth with succinate rather than with the alkylbenzenes themselves. The highest reported *in situ* concentration of BTEX was 30 mg/l (Aronson and Howard 1997); for comparison, 100 mg/l toluene or ethylbenzene correspond to 1.09 mM and 0.94 mM, respectively. During anaerobic digestion of municipal sludge intermediate formation of up to 40 mg/l toluene was observed in large laboratory-scale reactors (Mrowiec et al. 2005). Laboratory partitioning experiments with 1:20 (v/v) mixtures of gasoline (average BTEX weight content of 21 %) and water yielded average BTEX concentration in the water phase of 130 mg/l (Cline et al. 1991). The weight content of BTEX

is several-fold higher in gasolines than in crude oil. Previous experiments demonstrated that strain EbN1 can readily grow with a 1:40 (v/v) mixture of crude oil and mineral medium under nitrate reducing conditions (Rabus and Widdel 1996). Thus one might speculate that strain EbN1 could tolerate natural partition systems at sites containing crude oil, diesel or jet fuel or even gasoline.

Proteomic studies. Sudden exposure of strain EbN1 to sublethal concentrations of toluene or ethylbenzene resulted in maximal fold-changes of 11.95 and -5.37 in relative abundance. Among all regulated proteins the maximal share of a single protein from the total cellular protein as separated by 2DE amounted to 2.6 % (Figs. 2-4). These values are however not extraordinarily high, considering that previously fold changes of up to 281 (Kühner et al. 2005) and maximal shares of 6 % from total protein were observed (Kühner et al., in preparation). In the present study, a total of 81 regulated protein spots were detected, which are distributed across the various intersections of untreated cells and those treated with toluene or ethylbenzene (Fig. 5). Fifty-one protein spots were specifically regulated upon alkylbenzene treatment with 7 and 19 of them specific for toluene and ethylbenzene, respectively. The 30 other proteins were also regulated in untreated cells (2 of them exclusively), most probably in response to changing growth phases. Protein profiles of untreated cells of strain EbN1 revealed that most differential regulation occurred between the 45 min and the 250 min time points, where growth rates were rather similar. In contrast, the transition to stationary phase (600 min) did not translate into profound changes of the protein profiles. This observation was unexpected, since adaptation of *E. coli* to stationary phase correlate to changes in growth rates (Ihssen and Egli 2004). Nevertheless, proteomic analyses of Gram-positive *Listeria monocytogenes* also demonstrated an early adaptation to stationary phase. During mid-exponential growth already more than half of the protein changes attributed to transition to stationary phase were observed (Weeks et al. 2004). Earlier studies with *E. coli* revealed the formation of 23 new 2DE-separated protein spots in cultures growing in glucose-containing mineral medium and exposed to benzene (Blom et al. 1992). Two of these proteins could be correlated to carbon starvation response. Similarly, *Pseudomonas putida* mt-2 induced the formation of 22 carbon starvation proteins during aerobic growth with toluene (Vercellone-Smith and Herson 1997).

To learn more about the underlying molecular mechanisms and to differentiate between adaptation to solvent stress and growth phases, more than 30 of the regulated proteins were excised from the 2DE gels and identified by mapping of PMFs.

Stress related proteins. Proteins directly related to stress response are the HtpG- and IbpA- heat shock proteins and the heat shock induced chaperone ClpB, maintaining proteins in the biological active form. This is an unexpected finding since *E. coli* K12 or *P. putida* mt-2 did not form heat shock proteins upon treatment with benzene or toluene (Blom et al. 1992; Vercellone-Smith and Herson 1997). However, the solvent sensitive *E. coli* strain K12-JA300 induced genes for 4 heat shock proteins (*hslJ, htpX, ibpAB*) after 30 min exposure to *n*-hexane which was not observed with the tolerant mutant strain OST3410 (Hayashi et al. 2003). In strain EbN1 the abundance of the induced heat shock proteins decreased with prolonged incubation below threshold. When strain EbN1 was growing with sublethal concentrations of either toluene or ethylbenzene heat shock proteins were not identified among the up-regulated proteins (Kühner, in preparation).

WbjB was the most highly up-regulated (~12-fold) protein in the present study. Its orthologs are involved in *N*-acetyl-D-fucosamine synthesis, a constituent of surface polysaccharide structures (O-antigens of LPS and capsule, respectively) of *Pseudomonas aeruginosa* (WbjBCD) and *Staphylococcus aureus* (Cap5EFG). Gene knockout of *wbjB* and *cap5E* resulted in rough LPS of *P. aeruginosa* and abolished capsule polysaccharide synthesis of *S. aureus* (Kneidinger et al. 2003). While solvent tolerant *P. putida* strain Idaho showed rough modified LPS upon exposure to toluene, the toluene sensitive *P. putida* strain MW1200 had smooth LPS (Pinkart et al. 1996). This and similar observations in other organisms lead to the assumption that changes in the LPS composition/architecture might confer a certain degree of tolerance against solvents (Weber and de Bont 1996). Noteworthy, however, a *wbpL* knock-out mutant of *P. putida* strain DOT-T1E, which is impaired in O-antigen synthesis, displayed similar toluene tolerance as the wild type (Junker et al. 2001).

Other stress related proteins of strain EbN1 that were up-regulated in response to alkylbenzene treatment or stationary phase were Dps, KatA, OmpC. Dps is a non-specific DNA-binding protein that was originally found to protect DNA (possibly against oxidative damage) in stationary phase *E. coli* (Almiron et al. 1992). The expression of *dps* is independently controlled by the transcriptional regulator OxyR together with σ^{70} during exponential growth and by σ^{S} together with IHF in the stationary growth phase (Altuvia et al.

1994). Considering that Dps was most highly up-regulated in untreated, stationary cells and that the genome apparently lacks an *oxyR* gene, *dps*-regulation is likely to be under σ^S-control in strain EbN1. The predicted bifunctional catalase/peroxidase KatA was most highly up-regulated in the mid-linear growth phase (250 min) of untreated strain EbN1 cultures. A growth phase dependent regulation of catalase has been reported for several organisms. During transition of *Agrobacterium tumefaciens* into stationary growth phase the expression of *katA* (encoding a bifunctional catalase) was repressed and that of *catE* (encoding a monofunctional catalase) was induced (Prapagdee et al. 2004). In *E. coli* the *katE* gene is part of the RpoS (σ^S) regulon (Huisman et al. 1996). The genome of strain EbN1 contains only a single catalase encoding gene (*katA*). Similarly, *Caulobacter crescentus* possesses only a catalase/peroxidase, the activity of which is 9-fold increased in early stationary growth phase (Schnell and Steinman 1995). OmpC belongs to the outer membrane porins (omps) which are regulated in response to e.g. cell density (Liu and Ferenci 2001) or metal treatment (Egler et al. 2005). In addition, OmpC acts as inducer of σ^E-activity, which controls the expression of genes for periplasmic enzymes and proteins related to the cell envelope (Alba and Gross 2004). A σ^E encoding gene is predicted in the genome of strain EbN1.

Denitrification. While NirS (periplasmic nitrite reductase) showed a similar regulatory pattern (~2-fold up-regulated) at all timepoints after alkylbenzene addition, up-regulation of NosZ (nitrous oxide reductase) increased steadily with incubation time. Expression studies with *Paracoccus denitrificans* (Baumann et al. 1996), *Pseudomonas stuzeri* (Härtig et al. 1999) and *Pseudomonas fluorescens* (Philippot et al. 2001) indicated that the genes for the individual reduction steps of denitrification were sequentially regulated; i.e. *nirS* was transcribed later than *narGHI* and *nosZ*. This agrees with the unlinked organization (also observed with strain EbN1) of the *nar* (NO_3^-–reductase encoding), *nir* (NO_2^-–reductase encoding), *nor* (NO–reductase encoding) and *nos* (N_2O–reductase encoding) genes. These regulatory patterns are paralleled by varying levels of the denitrification intermediates (Baumann et al. 1996). It should also be considered that nitrate reduction is energetically preferred over nitrite reduction (Nicholls 1992). Thus the early up-regulation of NirS in the presence of about 2 mM nitrate (45 min after addition of alkylbenzenes to strain EbN1 cultures) was unexpected. When strain EbN1 was growing with sublethal concentrations of toluene (0.7 mM) or ethylbenzene (0.32 mM), a 3-fold increase of NirS abundance was correlated with a relative low maximum of 2 – 3 mM intermediately excreted nitrite (Kühner et al., in preparation). The reduced nitrite consumption rate observed under both experimental conditions could indicate a decreased

efficiency of denitrification. Noteworthy, the regulatory pattern of NosZ was somewhat different. NosZ displayed an about 5-fold increased abundance in response to alkylbenzene treatment but –unlike NirS– did not change in abundance in untreated cells transitioning to stationary growth phase. NosZ was not differentially regulated when strain EbN1 was grown with sublethal concentrations of toluene and ethylbenzene.

Transporters. The periplasmatic binding proteins of 4 ABC- and 1 TRAP transport systems, presumably functioning in amino acid, benzoate and succinate uptake, decreased in abundance upon alkylbenzene treatment. Similarly, a further periplasmic binding protein of an ABC-transport system was down-regulated during growth of strain EbN1 with sublethal concentrations (0.7 mM) of toluene (Kühner et al., in preparation). In contrast, ABC-transporters for amino acids display a different regulatory behaviour in other bacteria. E.g. five such transporters are up-regulated in *P. putida* in response to phenol shock (Santos et al. 2004) and three in *E. coli* in response to carbon starvation (Wick et al. 2001).

Metabolism. Succinate metabolism probably proceeded via succinate dehydrogenase, fumarase, malic enzyme and pyruvate dehydrogenase to acetyl-CoA, which is channeled to TCA cycle for oxidation to CO_2. Correspondingly, protein components of this pathway (AceE, a subunit of pyruvate dehydrogenase and Icd2, isocitrate dehydrogenase) were resolved on the 2DE gels. However, the strong up-regulation (about 5-fold) of acetyl-CoA synthetase (AcsA) in alkylbenzene-treated cells (this study) or cultures growing with sublethal concentrations of toluene (Kühner et al., in preparation) was unexpected, as the medium did not contain acetate in either of the two cases. Alkylbenzene-treated cultures excreted low amounts of acetate (around the detection limit of 0.5 mM), indicating hydrolysis of acetyl-CoA. Such an activity could lead to substrate-level ATP-generation (Brown et al. 1977). Correspondingly, *E. coli* excreted acetate during growth with succinate under O_2-limiting conditions (Edwards et al. 2001). During growth with 0.32 mM ethylbenzene (sublethal), enzymes involved in PHA biosynthesis (PhbB, PhaC) and phasins (EbA1323, EbA6852 and EbA5033) were specifically up-regulated and PHB accumulated up to 10 % of dry weight (Kühner et al., in preparation). This finding suggested a re-direction of acetyl-CoA from TCA to PHB synthesis, probably due to impaired respiration. The possibility of PHB-granules functioning as sink for toxic solvents is tempting, however, in the present study where alkylbenzenes were not catabolized only one probable phasin (EbA1323) was slightly up-regulated (2.9-fold).

Toluene and ethylbenzene degradation pathways. The absence of any known proteins of the anaerobic degradation pathways of toluene or ethylbenzene under any of the investigated growth conditions demonstrated, that neither of the two alkylbenzenes could be metabolized during the incubation time. Conversely, the experimental set up allowed to study stress response to solvents only. However, *P. putida* DOT-T1 and a mutant incapable of aerobic toluene degradation differed not in toluene tolerance leading to the suggestion, that toluene metabolism is not involved in toluene tolerance (Mosqueda et al. 1999).

Solvent efflux pumps like the AcrAB-TolC system belonging to the RND (resistance-nodulation-cell division family) are supposed to be the major factors rendering bacteria solvent tolerant and their constitutive or hydrocarbon-induced formation has often been observed (Ramos et al. 2002). TolC is the outer membrane protein of this complex and was previously detected by 2 DE in *E. coli, P. putida, C. crescentus* and *Synechocystis sp.* (Otto et al. 2001; Huang et al. 2002; Ireland et al. 2002; Santos et al. 2004; Yohannes et al. 2004). Even though the genome of strain EbN1 contains at least two copies of the AcrAB-TolC system or gives rise to 7 hits when searching for COG 1538 of TolC, none has been detected with 2DE in the present study. Thus, strain EbN1 might have an unusual high tolerance to solvents as compared to other bacteria.

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C Weitere Publikationen und Ergebnisse

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Formation of *n*-alkane- and cycloalkane-derived organic acids during anaerobic growth of a denitrifying bacterium with crude oil

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Abstract

The formation of metabolites during anaerobic biodegradation of saturated hydrocarbons directly from crude oil in the absence of oxygen was investigated using a denitrifying bacterium, the *Azoarcus*-like strain HxN1, which can utilise C6–C8 *n*-alkanes anaerobically as growth substrates. Various alkylsuccinates (apparently diastereomers) with alkyl chains (probably linked at carbon-2) ranging from C4 to C8 were detected by gas chromatography-mass spectrometry. These metabolites apparently result from the activation reaction of C4–C8 alkanes with cellular fumarate, analogous to the recently established reaction of pure *n*-hexane with fumarate in strain HxN1 to yield (1-methyl-pentyl)succinate. Other succinates carried substituents derived from cyclopentane and possibly methylcyclopentane and hence indicated an activation of such cycloalkanes. Since *n*-butane, *n*-pentane or cycloalkanes as single compounds did not support growth of strain HxN1, their apparent products point at co-metabolic reactions during utilisation of the C6–C8 *n*-alkanes. Furthermore, methyl-branched and cyclopentyl-substituted fatty acids were detected. This finding is explained by a further metabolism of the substituted succinates via carbon skeleton rearrangement and decarboxylation. All metabolites detected in the oil-grown cultures were also identified in cultures grown with defined mixtures of saturated hydrocarbons. Results are of potential value for an understanding of metabolite formation in hydrocarbon-rich anoxic environments from the viewpoint of bacterial physiology.

Introduction

n-Alkanes represent the most abundant type of petroleum hydrocarbons. The disappearance of *n*-alkanes, like that of particular aromatic hydrocarbons, in oil reservoirs is generally regarded as an indication of in situ biodegradation (e.g. Peters and Moldowan 1993). Since for many decades hydrocarbon-degrading microbial cultures could only be established in the presence of oxygen, the selective depletion of *n*-alkanes and other hydrocarbons in oil reservoirs has for a long time been attributed to aerobic bacteria (Palmer 1993).

During the past decade, however, numerous novel isolates of anaerobic bacteria were shown to degrade not only aromatic, but also the chemically less reactive saturated hydrocarbons under strict exclusion of O_2 (for overview see Heider et al. 1999; Spormann and Widdel 2000; Widdel and Rabus 2001). Utilisation of *n*-alkanes was demonstrated with novel species of sulphate-reducing (Aeckersberg et al. 1991, 1998; Rueter et al. 1994; So and Young 1999a) or nitrate-reducing (Ehrenreich et al. 2000) bacteria that oxidised *n*-alkanes. Anaerobic degradation of *n*-alkanes was in addition detected in enriched bacterial communities that reduced nitrate (Bregnard et al. 1997; Rabus et al. 1999) or sulphate (Caldwell et al. 1998), or formed methane and CO_2 (Zengler et al. 1999; Anderson and Lovley 2000). Even the branched alkane, pristane, was degraded anaerobically (Bregnard et al. 1997). The proven ability of anaerobic bacteria to degrade alkanes offers an explanation for the observed selective loss of these compounds from petroleum in reservoirs that are usually oxygen-depleted (Connan et al. 1996).

Reactive oxygen species derived from O_2, which are used in aerobic organisms to activate alkanes (Groh and Nelson 1990; White and Coon 1980), cannot be generated in an anaerobic metabolism. Therefore, anaerobic alkane activation must mechanistically differ completely from aerobic alkane activation. Indeed, recent investigations into the anaerobic *n*-alkane metabolism point at an unprecedented reaction of hydrocarbons. Enriched sulphate-reducing bacteria utilising *n*-dodecane (Kropp et al. 2000) and a denitrifying isolate (strain HxN1) growing with *n*-hexane (Rabus et al. 2001) formed alkyl-substituted succinates. In case of strain HxN1, an organic radical was specifically detected in cells grown with *n*-hexane. These findings pointed at a radicalic addition of the *n*-alkane to fumarate, a common cell metabolite. Hence, there was an analogy to the anaerobic activation of toluene, the methyl group of which is added to fumarate by a radical mechanism yielding benzylsuccinate (for overview see Heider et al. 1999; Spormann and Widdel 2000). However,

the *n*-alkanes were not activated at their methyl groups (Kropp et al. 2000; Rabus et al. 2001). With *n*-hexane, activation at carbon-2 was evident as the principal reaction that apparently yielded diastereomers of (1-methylpentyl)succinate. Further studies suggested that (1-methylpentyl)succinate is subsequently metabolised via activation to the coenzyme A (CoA) thioester and subsequent carbon skeleton rearrangement yielding (2-methyl-hexyl)malonyl-CoA (Wilkes et al. 2002). The latter compound allows α-decarboxylation to 4-methyloctanoyl-CoA, an activated branched fatty acid that can undergo conventional β-oxidation and thus finally lead to CO_2 as end product (Wilkes et al. 2002). The simultaneously derived reducing equivalents ("electrons") are used to reduce nitrate to dinitrogen, which yields energy for growth. The specific formation of alkylsuccinates (Kropp et al. 2000; Rabus et al. 2001; Gieg and Suflita 2002) or methyl-branched fatty acids (Wilkes et al. 2002; So and Young 1999b) during utilisation of different *n*-alkanes by various types of enriched or isolated anaerobic bacteria suggests that the pathway for *n*-hexane degradation can be generalised for the utilisation of *n*-alkanes in various anaerobic microorganisms as depicted in Fig. 1.

Succinates with hydrocarbon-derived substituents have been regarded as valuable indicators (biomarkers) of an anaerobic biodegradation of aromatic and aliphatic hydrocarbons in subsurface environments contaminated with petroleum or other complex hydrocarbon mixtures (Beller 2000; Elshahed et al. 2001; Gieg and Suflita 2002; Reusser et al. 2002). Bacterial populations in or from such environments formed metabolites that indicate the transformation of a wider range of alkylbenzenes (Rabus et al. 1999; Wilkes et al. 2000; Elshahed et al. 2001) or saturated hydrocarbons (Gieg and Suflita 2002) than utilised by cultures established with single compounds. This suggests the occurrence of so far uncultured anaerobic bacteria with degradative capacities that have not been observed in the established cultures. On the other hand, certain hydrocarbons may also undergo by-reactions or co-metabolic reactions. It has been shown that, for instance, toluene-degrading anaerobic bacteria formed aromatic organic acids also from xylene isomers that did not serve as growth substrates (Evans et al. 1992; Biegert and Fuchs 1995; Rabus and Widdel 1995a; Beller and Spormann 1999). The latter probably reacted due to promiscuous activity of the toluene-activating enzyme (Beller and Spormann 1999).

Here, we investigated by chemical analysis of metabolites whether also anaerobic *n*-alkane-degrading bacteria can, in principle, activate a wider range of saturated hydrocarbons than used as direct growth substrates. We used crude oil as a natural hydrocarbon mixture and

a denitrifying *Azoarcus*-like bacterium, strain HxN1, which is most easily cultivated among the presently available anaerobic *n*-alkane-degrading isolates (Ehrenreich et al. 2000). Anaerobic bacterial growth with crude oil and simultaneous depletion of *n*-alkanes has been demonstrated before (Rueter et al. 1994; Rabus et al. 1999), but formation of substituted succinates in such cultures has not been investigated. The present analyses were also intended as a further confirmation of the postulated generalised anaerobic degradation pathway for *n*-alkanes (Fig. 1).

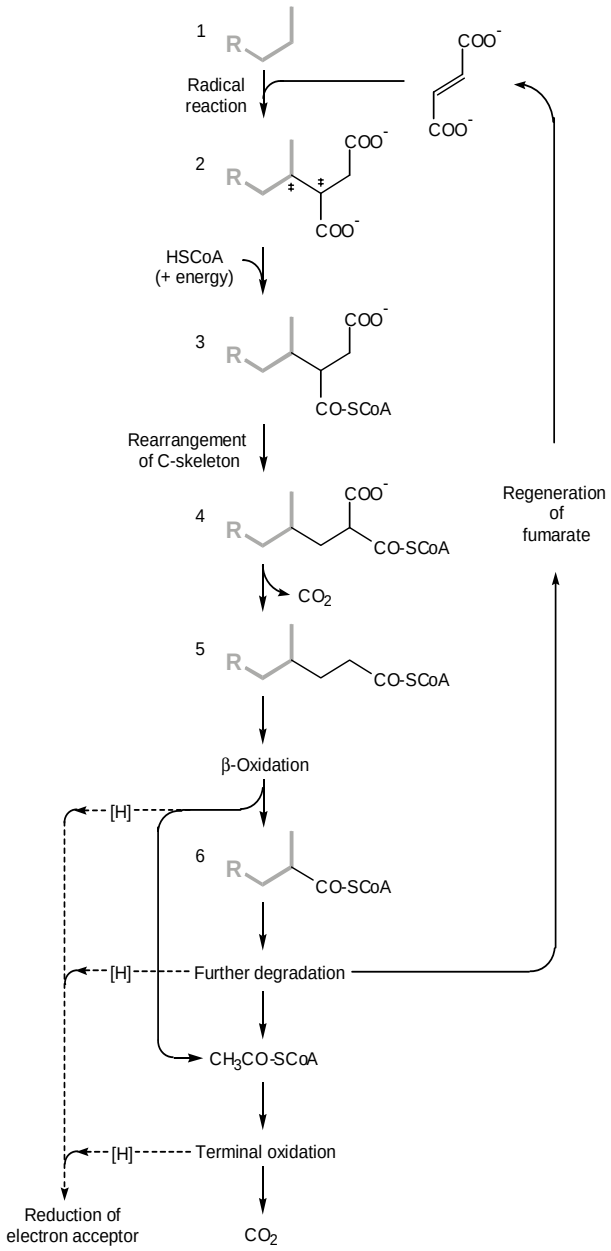

Fig. 1. Proposed generalised pathway for the anaerobic degradation of *n*-alkanes. The *n*-alkane (1) reacts at carbon-2 in a radical mechanism (not shown) with fumarate yielding a (1-methylalkyl)succinate (2) which is then activated with HSCoA (coenzyme A, CoA) to (1-methylalkyl)succinyl-CoA (3). The latter undergoes carbon skeleton rearrangement (hydrogen-carboxyl-CoA exchange) yielding (2-methylalkyl)malonyl-CoA (4) that allows α-decarboxylation to 4-methylalkanoyl-CoA (5). This is further oxidised via conventional β-oxidation, yielding intermediates such as 2-methylalkanoyl-CoA (6), propionyl-CoA (not shown) and acetyl-CoA. Propionyl-CoA could be recycled to fumarate (details not shown; for further information see Wilkes et al. 2002), and acetyl-CoA is oxidised to CO_2 (details not depicted). Compounds 2, 5, and 6 have been identified in the present study (upon hydrolyses of the assumed thioesters and esterification with diazomethane during sample preparation). Formation of diastereomers of (1-methylalkyl)succinate (2) has been suggested to be due to reduced stereoselectivity at the *n*-alkane chain (Rabus et al. 2001); the two chiral carbon atoms of this compund are marked by double dagger. In the case of cyclopentane or methylcyclopentane as reacting hydrocarbons, the methyl esters of intermediates analogous to compounds 2 and 5 were observed (carbon chain marked in grey to be replaced by cyclic moieties); further processing is uncertain.

Materials and Methods

Cultivation. A pure culture of the denitrifying *Azoarcus*-like strain HxN1 has been maintained in our laboratory since the previous isolation of this organism with *n*-hexane and nitrate (Ehrenreich et al. 2000). The culture was grown at 28°C in stoppered 500 ml bottles containing 400 ml of defined anoxic mineral medium with 9 mM $NaNO_3$ and 20 ml of sterile deaerated crude oil (from the North Sea) as the only source of organic carbon under an anoxic headspace (9:1 mixture of N_2 and CO_2, v/v). Before addition, the crude oil was deaerated and sterilised as described elsewhere (Rabus and Widdel 1995b, 1996). Furthermore, strain HxN1 was grown with various defined mixtures of hydrocarbons. To avoid inhibitory effects of such hydrocarbons, they were diluted in 2,2,4,4,6,8,8-heptamethylnonane as inert carrier phase (5 ml per 400 ml medium) so as to achieve the indicated concentrations (percentages as v/v). The mixed hydro-carbons always included *n*-hexane (4%) and as additional hydrocaron either methane, ethane, propane, *n*-butane (each 101 kPa in head space), *n*-pentane (1%), *n*-heptane (2%), *n*-octane (3%), *n*-nonane, *n*-decane (each 6%), cyclopentane, methylcyclopentane, or cyclohexane (each 1%). The inoculum size was 10% (v/v). The bottles were incubated nearly horizontally with orifices below the medium surface level so as to avoid contact between the hydrocarbon phase and the stopper (Rabus and Widdel 1995b, 1996). Cultures were processed for chemical analyses towards the end of growth (with oil after 6 days, with defined alkanes after 24 days).

Sample preparation and gas chromatography-mass spectrometry. Extraction of acidified cultures with diethyl ether and derivatisation of extracts was carried out as described previously (Wilkes et al. 2000; Rabus et al. 2001). Methylated culture extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) with a type 5890 gas chromatograph (Hewlett Packard, Waldbronn, Germany) connected to a type 95SQ mass spectrometer (Finnigan MAT/ThermoFinnigan, Egelsbach, Germany) as described by Wilkes et al. (2000) and Rabus et al. (2001). A standard of (1-methylpentyl)succinic acid dimethyl ester was synthesised as reported elsewhere (Rabus et al. 2001). Cyclopentylsuccinic acid dimethyl ester was prepared in analogy to cyclohexylsuccinic acid dimethyl ester (Giese and Meister 1977; Giese and Kretzschmar 1982, 1984) using dimethyl fumarate, cyclopentyl mercuric acetate, and sodium borohydride. ^{1}H- and ^{13}C-NMR data were measured on a Varian Inova 400 (Varian, Darmstadt, Germany): ^{1}H-NMR (400 MHz,$CDCl_3$) δ = 1.14-1.34 (m, 2H), 1.50-1.82 (m, 6H), 1.92-2.02 (m, 1H), 2.50 (dd, J = 3.0, 15.7 Hz, 1H), 2.65-2.79 (m, 2H), 3.66 (s, 3H), 3.70 (s, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ = 24.85, 24.95, 30.41, 30.47,

35.37, 42.36, 46.16, 51.56, 51.68, 172.40, 175.13. Further standard compounds (used as methyl esters after derivatisation) were cyclohexylsuccinic, cyclopentylpropionic, 2-methylpentanoic, 2-methylhexanoic, 4-methylheptanoic, and 4-methyl-octanoic acids (Sigma-Aldrich, Deisenhofen, Germany). Coinjection experiments were performed as described previously (Rabus et al. 2001).

Results and Discussion

Strain HxN1 was able to grow in mineral medium with nitrate (being reduced to dinitrogen) and crude oil as the only source of organic compounds under strict exclusion of air. No growth was observed in the absence of either crude oil or nitrate. Growth with crude oil was approximately four times slower (shortest doubling time, 40–50 h) than the previously documented growth with *n*-hexane as single substrate (Ehrenreich et al. 2000). If limiting amounts of crude oil were added, consumption of *n*-hexane, *n*-heptane and *n*-octane from crude oil was evident (Behrends and Widdel, unpublished data). To favor an accumulation of metabolites for their chemical identification, a high (excess) amount of crude oil was added in the present study such that *n*-alkanes were not significantly depleted during growth. With 9 mM nitrate added, optical cell densities (measured at 660 nm) of 0.15–0.2 were reached in these cultures.

GC-MS analysis of extracts from the grown culture revealed a complex suite of organic acids that were not detectable in controls lacking either nitrate or oil. Since the metabolism of *n*-hexane in strain HxN1 leads to (1-methylpentyl)succinate and branched fatty acids that also carry the *n*-hexane-derived 1-methylpentyl moiety (Rabus et al. 2001; Wilkes et al. 2002; see also Introduction and Fig. 1), chemical analysis of the organic acids was focused on analogous compounds.

To unequivocally correlate metabolites formed with crude oil to the utilisation of particular hydrocarbons, strain HxN1 was grown in addition in separate cultures with *n*-hexane in combination with other hydrocarbons that were added individually. Since the metabolites of *n*-hexane are known (Wilkes et al. 2002), additional metabolites can be assigned to the utilisation of the second hydrocarbon in each experiment. The second hydrocarbon was methane, ethane, propane, *n*-butane, *n*-pentane, *n*-heptane, *n*-octane, *n*-nonane, *n*-decane, cyclopentane, methylcyclopentane or cyclohexane.

Alkyl- and cycloalkyl-substituted succinates. Since fragment ions m/z 114 and m/z 146 from the *n*-hexane-derived (1-methylpentyl)succinic acid dimethyl ester (Rabus et al. 2001) do not include the alkyl moiety, they should be also detectable if anaerobic bacteria form alkylsuccinates from saturated hydrocarbons other than *n*-hexane. Indeed, the summed (partial) ion chromatogram for these two fragments revealed a series of seven GC peaks (Fig. 2, Table 1) that occurred singly or as double peaks. The Kováts-indices provide evidence that these compounds represent a homologous series (Table 1). Characteristic mass spectral data of all of these GC peaks are compiled in Table 1.

Table 1 Selected characteristic mass spectral data of identified (1-methylalkyl)succinic acid dimethyl esters in the methylated extract from the culture of strain HxN1 after anaerobic growth with crude oil. A base peak at *m/z* 114 was observed for all listed compounds.

Peak no. [a]	Identified compound (as dimethyl ester)	Parent hydrocarbon	Formula	M_r	Relative intensity (%) of key ions			Kováts-Index
					m/z 146	$[M-31]^+$	$[M-73]^+$	
1a	(1-Methylpropyl)-succinic acid	*n*-Butane	$C_{10}H_{18}O_4$	202	35	19	30	1306
1b					38	16	28	1309
2a	(1-Methylbutyl)-succinic acid	*n*-Pentane	$C_{11}H_{20}O_4$	216	34	12	24	1387
2b					38	14	21	1389
3a	(1-Methylpentyl)-succinic acid [b]	*n*-Hexane	$C_{12}H_{22}O_4$	230	42	12	28	1475
3b					42	11	20	1478
4	Cyclopentylsuccinic acid [b]	Cyclopentane	$C_{11}H_{18}O_4$	214	48	17	42	1490
5	(Methylcyclopentyl)-succinic acid	Methyl-cyclopentane	$C_{12}H_{20}O_4$	228	66	15	42	1533
6a	(1-Methylhexyl)-succinic acid	*n*-Heptane	$C_{13}H_{24}O_4$	244	49	11	32	1569
6b					48	11	24	1571
7a	(1-Methylheptyl)-succinic acid	*n*-Octane	$C_{14}H_{26}O_4$	258	62	12	41	1664
7b					60	11	30	1666

[a] Peak numbers correspond to those in Fig. 2. Double peaks of identified compounds were interpreted as diastereomers (see text). Selected mass spectra are shown in Fig. 3.
[b] Identification was based on comparison of retention times and mass spectra with those of an authentic standard. Identification of all other peaks was based on relative retention times and similarity of fragmentation patterns.

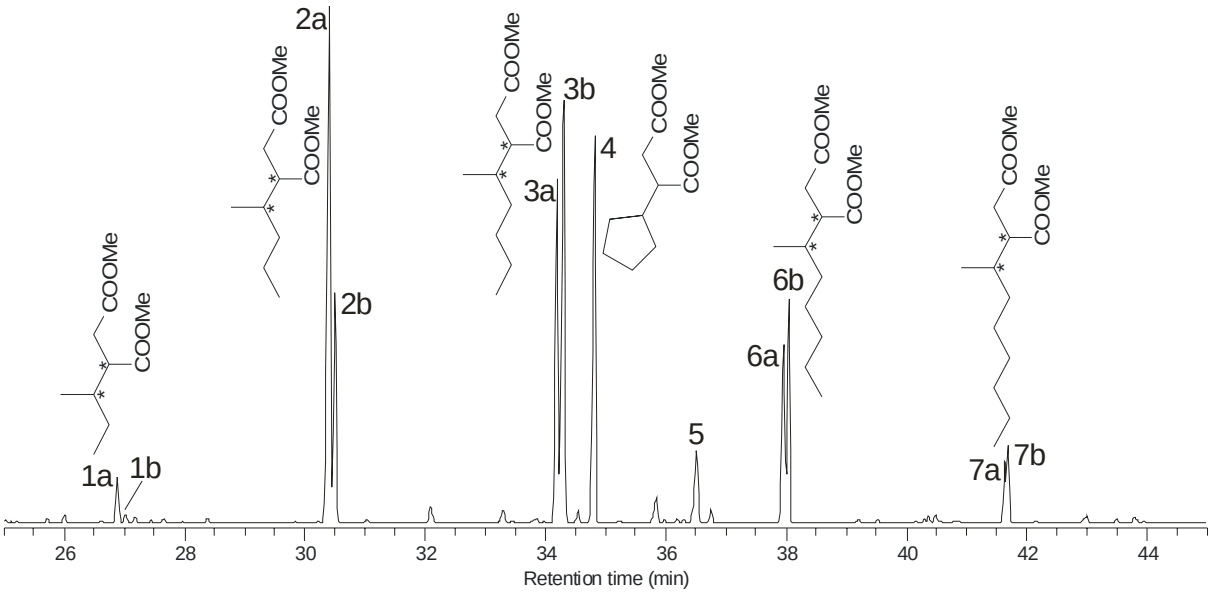

Fig. 2. Partial ion chromatogram (*m/z* 114 + 146) representing the alkylsuccinic acid dimethyl esters in the methylated extract from the culture of denitrifying strain HxN1 upon anaerobic growth with crude oil. Peak numbers correspond to those used in Table 1.

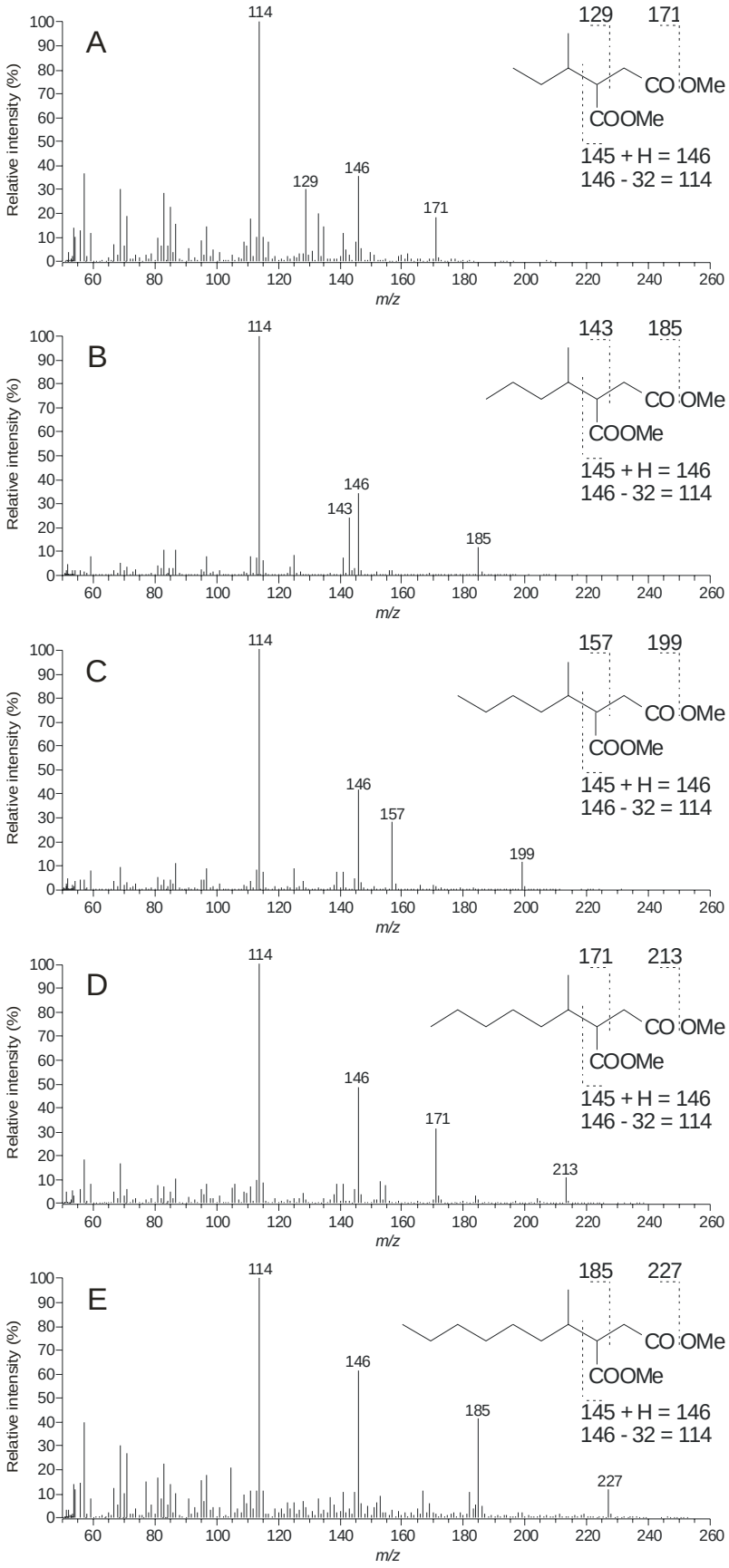

Fig. 3. Mass spectra of GC-peaks (all from Fig. 2) representing dimethyl esters of (A) (1-methylpropyl)succinic acid (peak 1a), (B) (1-methylbutyl)succinic acid (peak 2a), (C) (1-methylpentyl)succinic acid (peak 3a), (D) (1-methylhexyl)succinic acid (peak 4a), and (E) (1-methylheptyl)succinic acid (peak 5a).

The two GC peaks from each double peak, i.e. from those numbered 1, 2, 3, 6 and 7 (Fig. 2), exhibited essentially the same fragmentation patterns (Fig. 3). By comparison with the mass spectrum of an authentic standard, GC peaks 3a and 3b were identified as the (postulated) diastereomers of (1-methylpentyl)succinic acid dimethyl esters (see also Rabus et al. 2001). Besides the common base peak at m/z 114 and the second most abundant fragment ion at m/z 146, the mass spectra of the GC double peaks exhibited specific fragment ions at m/z 129, 143, 157, 171, 185 and m/z 171, 185, 199, 213, 227 and thus revealed two series of homologs differing by 14 amu. This value is characteristic of CH_2-groups, and therefore the most probable interpretation of the double peaks 1a/1b, 2a/2b, 4a/4b and 6a/6b (Fig. 2; Table 1) is that they represent the diastereomers of (1-methylpropyl)succinic, (1-methylbutyl)succinic, (1-methylhexyl)-succinic and (1-methylheptyl)succinic acid dimethyl esters, respectively. We therefore conclude that *n*-butane, *n*-pentane, *n*-heptane and *n*-octane from crude oil were activated in addition to *n*-hexane by the postulated radical-catalyzed addition to fumarate (Rabus et al. 2001). It has been recently discussed that the formation of diastereomers of (1-methylpentyl)succinate may be due to reduced stereoselectivity at reacting carbon-2 of *n*-hexane, whereas the fumarate carbon may react with high stereoselectivity (Rabus et al. 2001). The chromatogram of the various (1-methylalkyl)succinic acid dimethyl esters (Fig. 2) shows that in case of the C_4 and C_5 alkyl chains the earlier eluting diastereomers clearly dominate; with longer alkyl chains, the diastereomers are formed at almost equal amounts. This indicates that in the case of short-chain n-alkanes the reaction at carbon-2 is more stereoselective.

GC-Peaks 4 and 5 (Fig. 2) yielded specific fragment ions at m/z 141, 183 and m/z 155, 197, respectively, in addition to the common ones at m/z 114 and 146 (Fig. 4 A, B). The specific ones are by 2 amu lower than those of the dimethyl esters of (1-methylbutyl)-succinate and (1-methylpentyl)succinate, respectively (Fig. 3 B, C). This observation suggests that the substituents at the succinate moiety of GC-peaks 4 and 5 also had five and six carbon atoms, respectively, but were either monocyclic or monounsaturated and hence derived from mono-cyclic or mono-unsaturated hydrocarbons. Since the latter are not common constituents of crude oil, these metabolites are interpreted as activation products of cycloalkanes. Indeed, the use of an authentic standard allowed a clear identification of GC-peak 4 as cyclopentylsuccinic acid dimethyl ester. The fragmentation pattern (Fig. 4 B) of GC-peak 5 leaves open the possibility of a cyclohexane- or a methylcyclopentane-derived substituent. However, the retention time of an authentic standard of cyclohexylsuccinic acid dimethyl ester was different from that of GC-peak 5, indicating that the substituent must have

been derived from methylcyclo-pentane. Methylcyclopentane can theoretically be attacked at any of the four chemically distinguished carbon atoms leading to four structurally different succinate derivatives. However, it may be speculated that the reaction preferentially takes place at carbon atoms 2 or 3. Carbon atom 1 is sterically hindered by the methyl group, and an activation at the methyl group appears unlikely in view of the activation principle of *n*-alkanes (see Introduction).

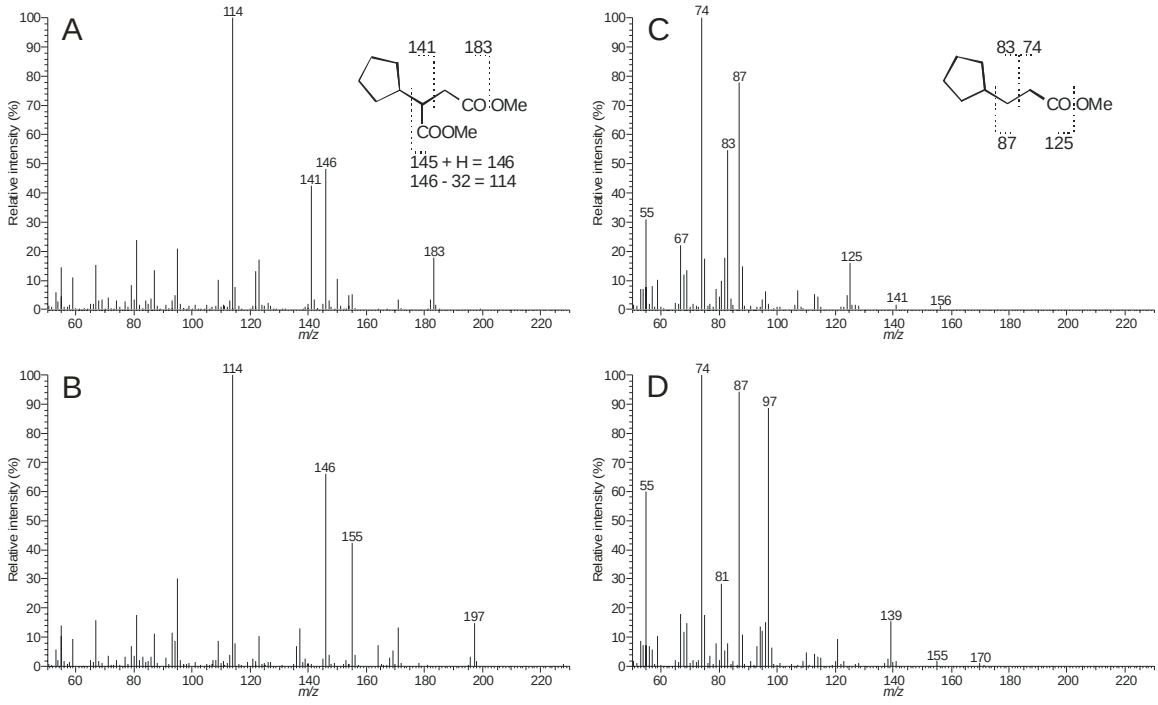

Fig. 4. Mass spectra of GC-peaks representing dimethyl esters of (A) cyclopentylsuccinic acid (peak 4 from Fig. 2), (B) an assumed methylcyclopentylsuccinic acid (peak 5 from Fig. 2) and methyl esters of (C) 3-cyclo-pentylpropionic acid (peak 6 from Fig. 6), and an assumed 3-(methylcyclopentyl)propionic acid (peak 8 from Fig. 6). Identity of (A) and (C) was confirmed by analysis of authentic standards.

In the growth experiments with defined binary mixtures of hydrocarbons, substituted succinates other than (1-methylpentyl)succinate from the commonly added *n*-hexane were identified at significant quantities if *n*-butane, *n*-pentane, *n*-heptane, *n*-octane, cyclopentane, or methylcyclopentane had been present in addition. The additional succinates formed with these hydrocarbons had the same retention times and fragmentation patterns as GC-peaks 1a/1b, 2a/2b, 4, 5, 6a/b or 7a/7b (Fig. 2), respectively, from the growth experiment with crude oil. Hence, this result confirmed that in addition to the C_6–C_8 n-alkanes, which are the only ones that definitely allow growth, also *n*-butane, *n*-pentane, cyclopentane and methylcyclo-pentane were activated. Cyclopentylsuccinate showed significant accumulation in comparison

to the *n*-hexane derived (1-methylpentyl)succinate (Fig. 5). In contrast, the experiment with *n*-hexane and cyclohexane yielded only traces of cyclohexylsuccinate, a compound which was not detectable at all in the experiment with crude oil.

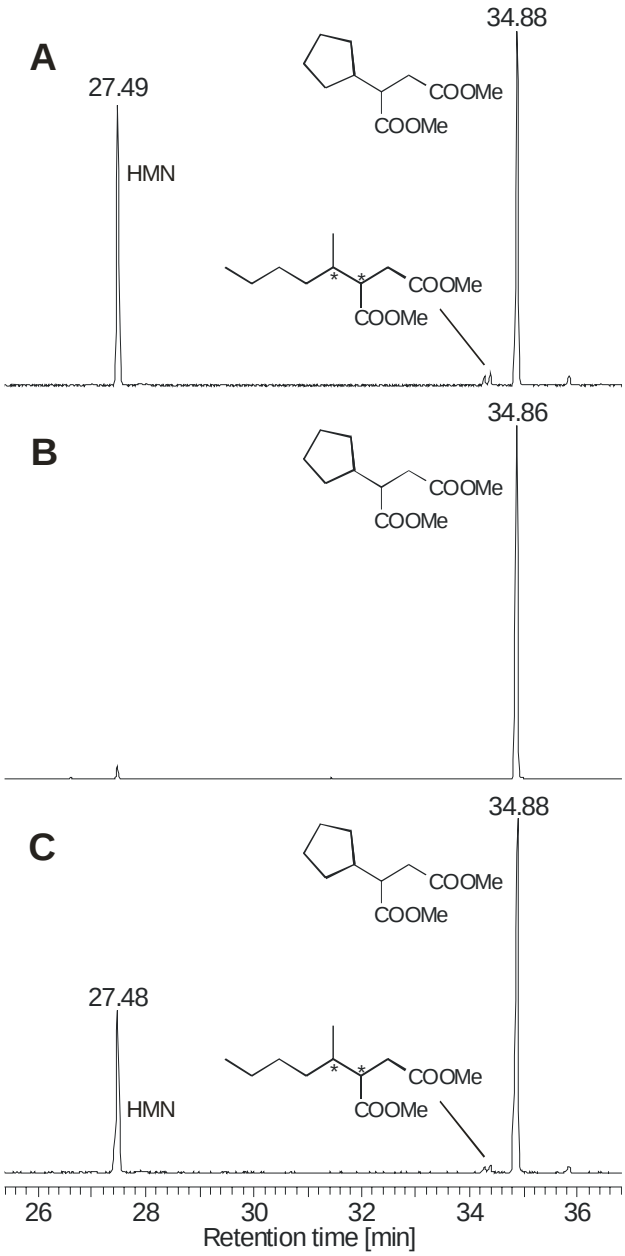

Fig. 5. Partial gas chromatograms of (A) methylated extract from a culture of strain HxN1 grown with a mixture of *n*-hexane and cyclopentane, (B) a synthetic standard of cyclopentylsuccinic acid dimethyl ester, and (C) a co-injection experiment of the samples shown under (A) and (B). HMN designates 2,2,4,4,6,8,8-heptamethylnonane that was used as an inert carrier phase and reservoir for the volatile hydrocarbons (see Experimental).

Branched and cyclopentyl-substituted fatty acids. The further metabolism of *n*-hexane-derived (1-methylpentyl)succinate in strain HxN1 has been suggested to proceed through 4-methyloctanoate and 2-methylhexanoate (as thioesters; Wilkes et al. 2002; see also Intro-

duction and Fig. 1). Summed ion chromatograms for selected mass fragments of methyl esters of these fatty acids and postulated analogs in the oil-grown culture revealed a series of GC peaks; eight of these could be identified as methyl esters of different 2-methyl- and 4-methyl-branched fatty acids (Fig. 6, Table 2). These branched fatty acids are fully in agreement with an activation and further metabolism of different *n*-alkanes analogous to that of *n*-hexane.

Table 2 Mass spectral data of methyl-branched and cyclic fatty acid methyl esters in methylated extract from the culture of strain HxN1 after anaerobic growth with crude oil.

Peak no. [a]	Identified compound (as methyl ester)	Parent hydrocarbon	Formula	M_r	Key ions: *m/z* (% relative intensity)	Kováts - Index
1	2-Methyl-pentanoic acid [b]	*n*-Pentane	$C_7H_{14}O_2$	130	M^+ absent, 115 (3), 101 (16), 99 (18), 88 (100), 71 (33), 59 (10)	n.d.
2	2-Methyl-hexanoic acid [b]	*n*-Hexane	$C_8H_{16}O_2$	144	144 (M^+, <1), 113 (7), 101 (24), 88 (100), 87 (9) 85 (15), 59 (10)	969
3	2-Methyl-heptanoic acid [b]	*n*-Heptane	$C_9H_{18}O_2$	158	158 (M^+, <1), 127 (5), 115 (8), 101 (26), 99 (4), 88 (100), 71 (16), 59 (8)	1067
4	4-Methyl-heptanoic acid	*n*-Pentane	$C_9H_{18}O_2$	158	158 (M^+, <1), 129 (6), 127 (19), 115 (23), 87 (100), 85 (58), 74 (97), 59 (21)	1092
5	2-Methyl-octanoic acid	*n*-Octane	$C_{10}H_{20}O_2$	172	M^+ absent, 141 (3), 115 (3), 113 (7), 101 (100), 88 (69), 85 (11), 71 (18), 59 (22)	1158
6	Cyclopentyl-propionic acid [b]	Cyclopentane	$C_9H_{16}O_2$	156	156 (M^+, <1), 141 (2), 125 (16), 87 (78), 83 (54), 74 (100), 67 (22), 55 (31)	1180
7	4-Methyl-octanoic acid [b]	*n*-Hexane	$C_{10}H_{20}O_2$	172	172 (M^+, <1), 143 (5), 141 (14), 115 (25), 99 (24), 87 (100), 74 (78), 59 (13)	1187
8	(Methylcyclo-pentyl) propionic acid [b]	Methyl-cyclopentane	$C_{10}H_{18}O_2$	170	170 (M^+, <1), 155 (1), 139 (15), 97 (89), 87 (94), 81 (28), 74 (100), 55 (60)	1231
9	4-Methyl-nonanoic acid [b]	*n*-Heptane	$C_{11}H_{22}O_2$	186	186 (M^+, <1), 157 (4), 155 (10), 129 (11), 115 (16), 113 (19), 87 (100), 74 (73), 71 (29), 59 (14)	1283
10	4-Methyl-decanoic acid	*n*-Octane	$C_{12}H_{24}O_2$	200	200 (M^+, <1), 171 (4), 169 (7), 143 (12), 127 (16), 115 (14), 87 (100), 85 (16), 74 (69), 59 (10)	1381

n.d., not determined

[a] Peak numbers correspond to those used in Fig. 6.

[b] Identification was based on comparison of retention times and mass spectra with those of an authentic standard. Identification of all other peaks was based on relative retention times, similarity of fragmentation patterns and comparison with published mass spectra (McLafferty and Stauffer 1989).

Two minor metabolites, in the summed ion chromatogram visible as GC peaks 6 and 8 (Fig. 6), revealed mass spectra with molecular ions at m/z 156 and m/z 170, respectively, specific fragment ions at m/z 83, 125 and m/z 97, 139, respectively, and common fragment ions at m/z 74, 87 (Fig. 4 C, D). The ions from GC peak 6 suggest the structure of cyclo-

pentylpropionic acid methyl ester, which was confirmed by GC-MS of an authentic standard of this compound. The molecular ion and specific fragment ions of GC-peak 8 were by 14 amu higher than those of GC-peak 6 and therefore indicate the formation of a fatty acid with a substituent derived from methylcyclopentane. There was no evidence for the formation of cyclohexylpropionate.

The experiments with the defined binary mixtures of hydrocarbons confirmed the formation of methyl-branched fatty acids also from *n*-alkanes other than *n*-hexane, and the formation of fatty acids with the cyclic substituents from cyclopentane and methylcyclopentane.

Conclusions

The present study not only confirmed the postulated generalised pathway for the anaerobic degradation of *n*-alkanes (Fig. 1), but also revealed the activation of short-chain and cyclic saturated hydrocarbons that do not serve as growth substrates for strain HxN1. It is unlikely that the organism possesses additional activating enzymes (alkylsuccinate synthases) for saturated hydrocarbons that are not used for growth. Rather, the alkylsuccinate synthase for the activation of the C_6–C_8 *n*-alkanes in strain HxN1 is assumed to exhibit promiscuous activity (relaxed substrate specificity) towards various saturated hydrocarbons. However, aromatic metabolites were not detectable in the presently oil-grown cultures of strain HxN1, indicating that the relaxed substrate specificity does not extend to an activation of alkylbenzenes. It is also obvious from the detected fatty acids that the alkyl-substituted succinates are processed further also in case of the alkanes that do not serve as growth substrates.

It is presently unknown whether the biochemical processing of the hydrocarbons that alone do not support growth is an incomplete co-metabolism that ceases at some stage in an organic dead-end product, or whether this is a complete co-metabolism (leading to CO_2) that can contribute to certain extent to the energy gain and growth of the bacteria. Either possibility may exist, depending on the type of hydrocarbon and bacterium. Even if such processes would be very slow, they may contribute to the compositional alteration of crude oil in petroleum reservoirs on a geological time scale. A more detailed investigation of the range and limits of co-metabolic reactions in anaerobic hydrocarbon-degrading bacteria would be a valuable contribution from the side of cultivation-based microbiology to a causal understanding and interpretation of hydrocarbon-derived biomarkers observed in situ.

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2

Fumarat-abhängige Aktivierung von Kohlenwasserstoffen bei anaerobem Wachstum von Aromaten- oder Alkan-verwertenden Bakterien mit Rohöl

-Diese Arbeit wurde bislang nicht in Form eines Manuskripts verfaßt-

Wie bereits in A.2.5 vorgestellt, werden zunehmend Fumarat-Addukte von Kohlenwasserstoffen in Umweltproben, Anreicherungen und Reinkulturen nachgewiesen. Dabei konnte in Reinkulturen die Entstehung folgender Produkte gezeigt werden:

a) Benzylsuccinat (BS) und dessen Derivate, die durch Addition von Fumarat an die Methylgruppe von Toluol bzw. *o*-, *m*-, *p*-Xylol entstehen (Evans et al. 1992; Rabus und Widdel 1995b; Beller et al. 1996; Biegert et al. 1996; Krieger et al. 1999)
b) (1-Methylpentyl)succinat (MPS) und Derivate, die in Folge einer subterminalen Addition von Fumarat an *n*-Hexan bzw. andere *n*-Alkane und Cycloalkane gebildet werden (Rabus et al. 2001; Wilkes et al. 2003; Cravo-Laureau et al. 2005).
c) (1-Phenylethyl)succinat (PES), das nach Aktivierung von Ethylbenzol durch Addition an die Aromaten-ständige Methylengruppe auftritt (Kniemeyer et al. 2003a).

Bei Untersuchungen des Reaktionsmechanismus wurde festgestellt, daß Aromaten-verwertende Organismen z.B. deuteriertes d_8-Toluol unter Erhalt aller Markierungen in deuteriertes Benzylsuccinat umsetzen. Analog verläuft die Aktivierung von markiertem *n*-Hexan in Stamm HxN1, d.H. das H-Atom des reagierenden C-Atoms des Kohlenwasserstoffs bleibt bei der Reaktion erhalten. Bei Zusatz von 2,3-d_2-Fumarat bildete Stamm HxN1 überaschenderweise nur einfach deuteriertes 3-*d*-(1-Methylpentyl)-succinat (Rabus et al. 2001). Da ein entsprechender Versuch beim anaeroben Alkylbenzolabbau noch nicht durchgeführt wurde, ist bislang unklar, ob dieser Markierungseffekt spezifisch für den *n*-Alkanabbau ist. Dieser Effekt wurde als Anpassung an die erhöhte Dissoziationsenergie der subterminalen C-H Bindung (410 kJ/mol) gegenüber der in der Methylgruppe von Toluol (360 kJ/mol, siehe auch Tab. 1) diskutiert (Rabus et al. 2001). Insgesamt 11 Alkylbenzol- und 5 Alkan-verwertende denitrifizierende oder Sulfat-reduzierende Stämme wurden auf Metabolit-Ebene hinsichtlich der folgenden Fragestellungen untersucht:

– Wie weit verbreitet ist die Fumarat-abhängige Aktivierung von Kohlenwasserstoffen zu den entsprechenden Aryl- bzw. Alkyl-Succinaten?
– Kann *n*-Hexan ko-metabolisch von Alkylbenzol-Verwertern aktiviert werden?
– Kann Toluol ko-metabolisch von Alkan-Verwertern aktiviert werden?

– Wie groß ist das Spektrum Fumarat-abhängig aktivierter Kohlenwasserstoffe bei anaerobem Wachstum mit Rohöl?
– Ist der Verlust eines Deuteriums aus 2,3-d_2-Fumarat im Aktivierungsprodukt bei allen Alkan-Verwertern zu beobachten? Wie verhält es sich bei den Alkylbenzol-Verwertern?

Die Kultivierung (durchgeführt von D. Lange) erfolgte entsprechend der Angaben in A2.1 Tab. 2 bzw. der dort ausgewiesenen Referenzen. Im Fall des thermophilen Stamms TD3 erfolgte die Inkubation bei 60°C. Die eingesetzten Substrate waren von p.a.-Qualität, Nordsee-Rohöl wurde verwendet wie bereits beschrieben (Rabus und Widdel 1996), 2,3-d_2-Fumarat wurden von Campro-Scientific bezogen. Metabolite wurden mit Diethylether extrahiert und analysiert wie beschrieben (Rabus et al. 2001). Bei Abschluß der Arbeit lagen noch nicht alle Ergebnisse der gaschromatographischen Metabolitanalyse vollständig vor (durchgeführt von H. Wilkes, Potsdam).

2.1 Fumarat-abhängige Verwertung von Kohlenwasserstoffen

In Tabelle 1 werden die bei anaeroben Wachstum auf Kohlenwasserstoffen gebildeten Succinat-Addukte gezeigt. In allen mit Toluol wachsenden Stämmen wurde Benzylsuccinat detektiert. Bei Wachstum mit *o*- bzw. *m*-Xylol wurde entsprechend 2- bzw. 3-Methylbenzyl-succinat gebildet. Stamm pCyN2 aktivierte *p*-Cymol zu (4-Isopropylbenzyl)succinat. Im Gegensatz dazu konnte bei Stamm pCyN1 kein Succinat-Addukt von *p*-Cymol oder *p*-Ethyltoluol nachgewiesen werden. D.H. Stamm pCyN1 könnte über zwei verschiedene Eingangsreaktionen verfügen, ähnlich wie es bei Stamm EbN1 der Fall ist.

Bei den Nitrat-reduzierenden Stämmen HxN1 und OcN1 wurden die entsprechenden Alkylsuccinate von *n*-Hexan, *n*-Heptan und *n*-Oktan bzw. *n*-Octan bis *n*-Undekan detektiert. Der Sulfat-reduzierende Stamm TD3 bildete bei Wachstum mit *n*-Heptan bis *n*-Tridekan ebenfalls die der subterminalen Aktivierung entsprechenden Metabolite von (1-Methylhexyl)-succinat bis (1-Methyldodecyl)succinat. Weder beim Nitrat-reduzierenden Stamm HdN1 noch beim Sulfat-reduzierenden Stamm Hxd3 konnte bei Wachstum mit *n*-Hexadekan ein Succinat-Addukt identifiziert werden. Im Fall von Stamm Hxd3 stimmt diese Beobachtung mit der postulierten Carboxylierung als Eingangsreaktion überein (So et al. 2003).

Insgesamt zeigen diese Ergebnisse bereits, daß die Fumarat-abhängige Eingangsreaktion die dominante Strategie beim anaeroben Kohlenwasserstoffabbau darstellt.

Tabelle 1. Anaerobe Bildung von Aryl- und Alkyl-Succinaten bei Wachstum mit Alkylbenzolen und *n*-Alkanen durch verschiedene Nitrat- und Sulfat-reduzierende Bakterien.

Stamm	Elektronen akzeptor	Kohlenwasserstoff-Substrat[k]	Gebildete Aryl- /Alkylsuccinate	Referenz
Thauera aromatica K172[a]	NO_3^-	Toluol (2%)	Benzylsuccinat	Seyfried et al., 1994
T[b]	NO_3^-	Toluol (2%)	Benzylsuccinat	Seyfried et al., 1994
		m-Xylol (2 %)	(3-Methylbenzyl)succinat	Krieger et al., 1999
EbN1[c]	NO_3^-	Toluol (2%)	Benzylsuccinat	Rabus and Heider, 1998
		Ethylbenzol (5 %)	nd[l]	
ToN1[c]	NO_3^-	Toluol (2%)	Benzylsuccinat	Hier beschrieben
mXyN1[c]	NO_3^-	Toluol (2%)	Benzylsuccinat	Hier beschrieben
		m-Xylol (2%)	(3-Methylbenzyl)succinat	Hier beschrieben
pCyN1[d]	NO_3^-	Toluol (2%)	Benzylsuccinat	Hier beschrieben
		p-Ethyltoluol (2 %)	nd[l]	Hier beschrieben
		p-Cymol (2 %)	nd[l]	Hier beschrieben
pCyN2 [d]	NO_3^-	*p*-Cymol (2 %)	(4-Isopropylbenzyl)succinat	Hier beschrieben
Desulfobacula toluolica Tol2[e]	SO_4^{2-}	Toluol (2%)	Benzylsuccinat	Rabus and Heider, 1998
mXyS1[f]	SO_4^{2-}	*m*-Xylol (2 %)	(3-Methylbenzyl)succinat	Wilkes et al., 2000
		Toluol (2 %)	Benzylsuccinat	
oXyS1[f]	SO_4^{2-}	*o*-Xylol (2 %)	(2-Methylbenzyl)succinat	Hier beschrieben
HxN1[h]	NO_3^-	*n*-Hexan (5 %)	1-Methylpentylsuccinat	Rabus et al., 2001
		n-Heptan (5 %)	1-Methylhexylsuccinat	Rabus et al., 2001
		n-Oktan (5 %)	1-Methylheptylsuccinat	Rabus et al., 2001
OcN1[h]	NO_3^-	*n*-Oktan (5 %)	1-Methylheptylsuccinat	Hier beschrieben
		n-Nonan (5 %)	1-Methyloktylsuccinat	Hier beschrieben
		n-Dekan (5 %)	1-Methylnonylsuccinat	Hier beschrieben
		n-Undekan (10 %)	1-Methyldekylsuccinat	Hier beschrieben
HdN1[h]	NO_3^-	*n*-Hexadekan (100 %)	nd[l]	Hier beschrieben
TD3[i]	SO_4^{2-}	*n*-Heptan (5 %)	1-Methylhexylsuccinat	Hier beschrieben
		n-Oktan (5 %)	1-Methylheptylsuccinat	Hier beschrieben
		n-Nonan (5 %)	1-Methyloktylsuccinat	Hier beschrieben
		n-Dekan (5 %)	1-Methylnonylsuccinat	Hier beschrieben
		n-Undekan (10 %)	1-Methyldekylsuccinat	Hier beschrieben
		n-Dodekan (10 %)	1-Methylundekylsuccinat	Hier beschrieben
		n-Tridekan (10 %)	1-Methyldodekylsuccinat	Hier beschrieben
Hxd3[j]	SO_4^{2-}	*n*-Hexadekan (100 %)	nd[l]	So et al. 2003

[a] Anders et al.,1993 (DSM 6984.
[b] Dolfing et al., 1990 (DSM 9506).
[c] Rabus and Widdel, 1995.
[d] Harms et al., 1999a (DSM 12567).
[e] Rabus et al., 1993 (DSM 7467).
[f] Harms et al., 1999b (DSM 13228).
[g] Kniemeyer et al., 2003
[h] Ehrenreich et al., 2000.
[i] Rueter et al., 1994.
[j] Aeckersberg et al., 1991 (DSM 6200).
[k] Konzentrationen der eingesetzten Kohlenwasserstoffe [% (v/v)] als Verdünnung in HMN.
[l] nd, Aryl- oder Alkylsuccinate unter den angegebenen Bedingungen nicht detektierbar.

2.2 Ko-metabolische Aktivierung

Die Fumarat-abhängige, ko-metabolische Aktivierung von Kohlenwasserstoffen wurde zunächst in Wachstumsansätzen mit Rohöl untersucht. Wegen der großen Isomeren-Vielfalt sowie dem Fehlen authentischer Standards erlaubte die massenspektrometrische Analyse dieser Proben lediglich Vermutungen über die chemische Struktur der aktivierten Kohlenwasserstoffe. Um dennoch eine eindeutige Identifizierung zu ermöglichen, wurden „biologische" Standards gebildet, indem die vermuteten Edukte ko-metabolisch mit bekanntermaßen verwertbaren Kohlenwasserstoffen (z.B. Toluol oder *n*-Hexan) umgesetzt wurden. Die Ergebnisse der Versuche mit Rohöl und den definierten Gemischen von Reinsubstraten sind für Aromaten-Verwerter in Tabelle 2 und für Alkan-Verwerter in Tabelle 3 zusammengefaßt.

Tabelle 2. Ko-metabolisch zu den entsprechenden Aryl- und Alkylsuccinaten aktivierte Kohlenwasserstoffe (% [v/v] in HMN) bei aneroben Wachstum Alkylbenzol-verwertender Bakterien.

Stamm	**Toluol**	***n*-Hexan**	**Xylol**			**Ethyl-**	**Ethyltoluol**			***p*-Cymol**	**Trimethylbenzol**	
			***o*-**	***m*-**	***p*-**	**benzol**	***o*-**	***m*-**	***p*-**		**1,3,5-**	**1,2,4-**
T. aromatica K172	**1**[a]	40?	0.1?	0.1?	0.1?	-	-	-	-	-	-	-
T	**1**[a]	40?	0.5[b]	**0.5**[a]	0.5[c]	-	-	-	-	-	-	-
EbN1	**1**[a]	60?	0.5?	0.5?	0.5?	**(5)**[a]	0.5?	0.5?	0.5	-	-	-
ToN1	**1**[a]	40?	0.1?	0.1?	0.1?	-	-	-	-	-	-	-
mXyN1	**1**[a]	40?	0.5?	**2**[a]	0.5?	-	-	-	-	-	-	-
pCyN1	**1**[a]	10?	0.1?	0.1?	0.1?	-	-	-	**(2)**[a]	**(2)**[a]	-	-
pCyN2	0.5	10?	0.5?	0.5?	0.5?	-	-	-	-	**2**[a]	-	-
D. toluolica Tol2	**1**[a]	10?	0.5?	0.5?	0.5?	-	-	-	-	-	-	-
oXyS1	1	40?	**1**[a]	0.5?	0.5?	-	0.5?	-	-	-	-	1?
mXyS1	0.5[d]	40?	0.5[d]	**1**[ad]	**0.5**[d]	-	-	0.5?	-	-	1?	1?

[a] Konnte als Kohlenstoff- und Energiequelle zum Wachstum verwendet werden.
[b] (Beller and Spormann 1997)
[c] (Beller and Spormann 1999)
[d] (Wilkes et al. 2000)
( ), bei angegebener Konzentration (% [v/v] in HMN) kein Aryl- oder Alkyl-Succinat entdeckt
-, nicht untersucht
?, Ergebnisse stehen noch aus

Die Stämme EbN1 und pCyN1 können in Gegenwart von Toluol *p*-Ethyltoluol und *p*-Cymol nicht aktivieren. Aus der Literatur sind andere anaerob Toluol-abbauende Stämme bekannt, die Xylole ko-metabolisch aktivieren können (Evans et al. 1992; Beller et al. 1996; Beller und Spormann 1997a; Beller und Spormann 1999; Verfürth et al. 2004). So verminderte der Stamm mXyS1 bei Wachstum mit Rohöl die Konzentration von Toluol,

m-, *p*-Xylol, *m*-Ethyltoluol und 1,2,4- bzw. 1,3,5-Trimethylbenzol. Entsprechend wurden neben Benzyl-succinat und den (Methylbenzyl)succinat-Isomeren der Xylole drei Peaks mit Massenspektren entdeckt, die auf Ethyltoluol bzw. Trimethylbenzol als Vorläufer schließen lassen (Wilkes et al. 2000).

Bei Wachstum mit Rohöl umfasst das Spektrum Fumarat-abhängig aktivierter *n*-Alkane bei Stamm HxN1 *n*-Butan bis *n*-Nonan, bei Stamm OcN1 *n*-Propan bis *n*-Tridekan und beim thermophilen Stamm TD3 Ethan bis *n*-Tridekan. Das aus Ethan gebildete Ethylsuccinat ist dabei ein Sonderfall, weil es terminal aktiviert wurde.

Darüber hinaus wurden bei den Stämmen OcN1 und TD3 weitere Produkte mit den Massenspuren der (1-Methylalkyl)succinate gefunden, die aber im Vergleich zu den entsprechenden *n*-Alkanvorläufern kürzere Retentionszeiten aufwiesen. Möglicherweise handelt es sich um verzweigte Alkane oder die Aktivierung könnte am z.B. C3-Kohlenstoff eines *n*-Alkans erfolgt sein. Zur Klärung dieser Frage wurden Wachstumsversuche mit unterschiedlich Methyl-verzweigten Alkanen durchgeführt, deren massenspektrometrische Ergebnisse noch ausstehen.

Tabelle 3. Ko-metabolische Aktivierung von Kohlenwassertoffen zu den entsprechenden Aryl- und Alkyl-Succinate bei anaeroben Wachstum Alkan-verwertender Bakterien mit Reinstoffen oder Erdöl.

Aktiviertes Substrat (% [v/v])[a]	**Art des Produkts**[b]	**Peak Anzahl**[c]	**Produkt beobachtet**[d]					
			HxN1		OcN1		TD3	
			Rein-stoff	Roh-öl	Rein-stoff	Roh-öl	Rein-stoff	Roh-öl
<u>*n*-Alkane</u>								
Methan, 202 KPa		1	-	-	?	-	?	-
Ethan, 202 KPa		1	-	-	?	-	?	+
n-Propan, 202 KPa	MPS	1	-	-	?	+	?	+
n-Butan, 202 KPa	MPS	2	+	+	?	+	?	+
n-Pentan, 1 %	MPS	2	+	+	?	+	?	+
n-Hexan, 5/1/5 %	MPS	2	+[e]	+[e]	?	+	?	+
n-Heptan, 5/1/5 %	MPS	2	+[e]	+[e]	?	+	?	+[e]
n-Oktan, 3/5/5 %	MPS	2	+[e]	+[e]	+[e]	+[e]	?	+[e]
n-Nonan, 10/5/5 %	MPS	2	-	+	?	+[e]	?	+[e]
n-Dekan, 6/5/5 %	MPS	2	-	-	?	+[e]	+[e]	+[e]
n-Undekan, 10 %	MPS	2		-	?	+[e]	?	+[e]
n-Dodekan, 10 %	MPS	2		-	?	+	?	+[e]
n-Tridekan, 10 %	MPS	2		-	?	+	?	+[e]
n-Hexadekan, 96/100 %	MPS	2		-	?	-	?	-
<u>Verzweigte Alkane</u>								
$C_6H_{14} - C_{12}H_{26}$[f]	MPS	26				+		
$C_6H_{14} - C_{11}H_{24}$[f]	MPS	12						+
<u>Cyclische Alkane</u>								
Cyclopentan, 1 %	MPS	1	+	+	?	+	?	+
Methylcyclopentan, 1 %	MPS	1	+	+	?	+	?	+
$C_7H_{14} - C_{10}H_{20}$[g]	MPS	6				+		
<u>Alkylbenzole</u>								
Toluol, 0.5 %	BS	1	+	+	+	+	+	+
Ethylbenzol, 0.5 %	PES	2	?	+	?	+	?	+
n-Propylbenzol, 1 %	PES	2	?	+	?	+	?	+
n-Butylbenzol, 1 %	PES	2	?	+	?	+	?	+
n-Pentylbenzol, 2 %	PES	2	?	-	?	+	?	+
n-Hexylbenzol, 5 %	PES	2	?	-	?	+	?	+
C_8H_{10}[h]	BS	1		+				
$C_8H_{10} - C_{11}H_{16}$[h]	BS	4				+		
$C_8H_{10} - C_{12}H_{18}$[h]	BS	8						+

[a] Unten aufgeführte Substanzen wurden in angegebener Konzentration als Mischung mit einem Wachstumssubstrat (4 % *n*-Hexan (HxN1), *n*-Oktan (OcN1) bzw. *n*-Dekan (TD3), [v/v] HMN) eingesetzt.

[b] Aktivierung und Art des Produkts wie beschrieben für *n*-Hexan ((1-Methylpentyl)succinat, MPS), Toluol (Benzylsuccinat, BS) und Ethylbenzol ((1-Phenylethyl)succinate, PES).

[c] Anzahl der beobachteten Peaks.

[d] + / -, entsprechendes Succinat-Addukt gezeigt bzw. nicht gezeigt.

[e] Verwertbar als alleinige Kohlenstoff- und Energiequelle.

[f] Als Reinsubstanz untersuchte Substrate: 2- bzw. 3-Methylpentan, (1 %); 2,2- bzw. 2,3-Dimethylbutan, (4 %).

[g] Als Reinsubstanz untersuchte Substrate: Ethylcyclopentan, Cyclohexan, (1 %).

[h] Als Reinsubstanz untersuchte Substrate: *o*-, *m*-, *p*-Xylol, *o*-, *m*-, *p*-Ethyltoluol, *o*-, *m*-, *p*-Cymol (0.5 %); 1,2,3-, 1,2,4-, 1,3,5-Trimethylbenzol, (1 %); 1,2,3,5-, 1,2,4,5-Tetramethylbenzol (5 %); Pentamethylbenzol, Hexamethylbenzol, (10 %); *n*-Heptylbenzol, (5 %); *n*-Octylbenzol, (10 %); *n*-Nonylbenzol, *n*-Decylbenzol, (20 %).

Außerdem können alle *n*-Alkan-verwertenden Stämme Cyclopentan und Methylcyclopentan aktivieren. Stamm OcN1 bildete darüber hinaus noch sechs weitere Produkte mit Massenspektren, die auf höhermolekulare Cycloalkan-Succinataddukte (bis zu 10 C-Atome) hinweisen. In Extrakten eines mit Ethylcyclopentan angereicherten Mikrokosmos wurden bis zu fünf Peaks mit identischen, für Ethylcyclopentylsuccinat erwarteten Massenspektren detektiert (Rios-Hernandez et al. 2003). Als Ursache werden unterschiedliche Diastereomere angenommen. Im Fall von Stamm OcN1 haben die sechs beobachteten Produkte insgesamt vier unterschiedliche Massenspektren.

Überraschend groß war die Anzahl ko-metabolisch aktivierter Alkylbenzole. Neben Benzylsuccinat und (1-Phenylethyl)succinat wurden Metabolite mit Massenspektren der *n*-Alkylbenzole bis Hexylbenzol, der Xylole und anderer, höher substituierter Toluol-Derivate entdeckt. Zur genaueren Identifizierung dieser Arylsuccinate wurden Reinsubstanzgemische der Xylol-, Ethyltoluol- und Cymol-Isomere, sowie Tri-, Tetra-, Penta- und Hexamethylbenzole verwendet. Die Identifizierung von Benzylsuccinat und (1-Phenylethyl)succinat als Ko-Metabolite bedeutet, daß die beiden, von unterschiedlichen Alkylbenzol-verwertenden Organismen angewandten Aktivierungs-Positionen gleichzeitig vorkommen. Die ebenfalls mögliche und bei Alkan-verwertenden Organismen naheliegendere subterminale Aktivierung von z.B. *n*-Hexylbenzol zu [Methyl(pentylphenyl)]-succinat wurde nicht beobachtet. Die Ursache könnte in der geringeren zur homolytischen Dissoziation der C-H Bindung benötigten Energie liegen. Diese beträgt für die in juxta-Position zum π-Elektronen-system stehende Methylengruppe des Ethylbenzols 358 gegenüber 398 kJ/mol für die subterminale Methylengruppe der *n*-Alkane (McMillen und Golden 1982).

2.3 Markierungsexperimente mit d_2-Fumarat

Den anaerob mit Kohlenwasserstoffen wachsenden Stämmen wurde zu Beginn der linearen Wachstumsphase 10 mM Deuterium-markiertes 2,3-d_2-Fumarat zugegegeben. Die Markierungsmuster der gebildeten Aryl- bzw. Alkylsuccinate wurden massenspektrometrisch bestimmt. Die entsprechenden Ergebnisse sind in Tabelle 7 zusammengefaßt. Die meisten Alkylbenzol-verwertenden Organismen bildeten Arylsuccinate, in denen beide Deuteriumatome erhalten blieben. Das in den Stämme mXyS1 und TD3 gefundene Benzylsuccinat bzw. (Methylnonyl-)succinat enthielt keine Deuterium-Markierung. Beide Stämme können Fumarat nicht als Wachstumssubstrat verwenden und es ist davon auszugehen, daß Aufnahmesysteme

für Fumarat fehlen. Entsprechend bilden diese beiden Stämme zu 100 % unmarkierte Succinat-Addukte.

Die Alkan-verwertenden Stämme HxN1 und OcN1 bilden übereinstimmend mit früheren Beobachtungen (Rabus et al. 2001) und im Gegensatz zu den Aromaten-verwertenden Stämmen keine bideuterierten Produkte, sondern ausschließlich 3-d_1-(Methylalkyl-)succinate oder unmarkierte Produkte. Das Markierungsmuster der ko-metabolisch aktivierten Benzylsuccinate ist zur Zeit noch nicht bekannt. Die Stämme HdN1 und Hxd3, die *n*-Hexadekan nicht durch Addition an Fumarat aktivieren, bildeten auch mit Toluol keine Succinat-Addukte.

Die bisherigen Ergebnisse unterstützen die Annahme, daß der Deuteriumverlust bei der Bildung von Alkylsuccinaten ursächlich mit dem spezifischen Mechanismus der *n*-Alkan-aktivierung zusammenhängt. An dieser Stelle gibt es demnach mechanistische Unterschiede in den Aryl- bzw. Alkylsuccinat-Synthasen.

Tabelle 7. Durch Zusatz von d_2-Fumarat hervorgerufenes Markierungsmuster von Aryl- und Alkylsuccinaten bei aneroben Wachstum mit Alkylbenzolen und *n*-Alkanen.

Stamm	**Kohlenwasserstoff-Substrat**[a]	**Aryl-Succinat [%]**[b]			**Alkyl-Succinat [%]**[c]		
		***d*2**	***d*1**	***d*0**	***d*2**	***d*1**	***d*0**
T. aromatica K172	Toluol + *n*-Hexan	15	20	65	nd	nd	nd
T. aromatica K172 (Zellextrakt)	Toluol	76	21	3	nd	nd	nd
T	Toluol + *n*-Hexan	11	5	84	nd	nd	nd
EbN1	Toluol + *n*-Hexan	12	16	72	nd	nd	nd
ToN1	Toluol + *n*-Hexan	35 / 57	9 / 14	56 / 29	nd	nd	nd
mXyN1	Toluol + *n*-Hexan	17	7	76	nd	nd	nd
mXyN1	*m*-Xylol+ *n*-Hexan	25	14	62	nd	nd	nd
pCyN1	Toluol + *n*-Hexan	23	17	60	nd	nd	nd
pCyN2	*p*-Cymol+ *n*-Hexan	14	11	75	nd	nd	nd
D. toluolica Tol2	Toluol + *n*-Hexan	10	4	86	nd	nd	nd
oXyS1	*o*-Xylol+ *n*-Hexan	?	?	?	?	?	?
mXyS1	*m*-Xylol+ *n*-Hexan	nd	nd	100	nd	nd	nd
EbS7	Ethylbenzol + *n*-Hexan	?	?	?	?	?	?
HxN1	*n*-Hexan + Toluol	?	?	?	nd	?	?
OcN1	*n*-Octan + Toluol	?	?	?	nd	?	?
TD3	*n*-Dekan + Toluol	nd	nd	100	nd	nd	100

[a] Eingesetzte Kohlenwasserstoff-Verdünnung wie in Tabelle 1 und 2 beschrieben.
[b] Arylsuccinate der Ausgangssubstanzen Toluol, *o*-Xylol, *m*-Xylol, *p*-Cymol sind Benzylsuccinat, (2-Methylbenzyl)succinat, (3-Methylbenzyl)succinat, (4-Isopropylbenzyl)succinat
[c] 1-Methylalkylsuccinate der Ausgangssubstanzen *n*-Hexan, *n*-Oktan oder *n*-Dekan sind (1-Methylpentyl)succinat, (1-Methylheptyl)succinat oderr (1-Methylnonly)succinat (siehe auch Tabelle 4).

Insgesamt konnte gezeigt werden, daß die überwiegende Mehrheit der untersuchten Stämme das jeweilige Kohlenwasserstoff-Substrat durch Fumarat-Addition aktiviert, zum Teil aber auch andere, bisher unbekannte Mechanismen verwendet. Außerdem konnte der Wasserstoffaustausch am Succinat-Rest auf die von Alkan-verwertenden Stämme gebildeten Alkylsuccinate beschränkt werden. Des weiteren konnte das Spektrum durch Fumarat-Addition aktivierbarer Kohlenwasserstoffe über die bisher in Grundwasserproben nachgewiesenen Produkte von

a) Toluol, *o*-, *m*-, *p*-Xylol und 2-Methylnaphthalin (Beller et al. 1995; Gieg et al. 1999; Elshahed et al. 2001; Gieg und Suflita 2002; Martus und Püttmann 2003; Griebler et al. 2004)

b) *n*-Alkanen von Propan bis Hexadekan und Ethylcyclopentan (Gieg und Suflita 2002; Rios-Hernandez et al. 2003)

c) Ethylbenzol (Gieg und Suflita 2002)

hinaus erweitert werden.

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