Die Gene der (1-Methylalkyl)succinat-Synthase im anaeroben *n*-Alkanabbau des Betaproteobakteriums Stamm HxN1

Dissertation

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Zusammenfassung

Das Betaproteobakterium Stamm HxN1 oxidiert *n*-Alkane einer Kettenlänge von C_5 bis C_8 unter denitrifizierenden Bedingungen vollständig zu CO_2. Die Aktivierung der *n*-Alkane durch Addition an Fumarat wird vermutlich von dem Glycylradikalenzym (1-Methylalkyl)succinat-Synthase katalysiert. Die (1-Methylalkyl)succinat-Synthase wird von den *mas* Genen kodiert, die in Stamm HxN1 ein Operon aus sieben offenen Leserahmen bilden.

Die in dieser Arbeit unternommenen Versuche die aus Stamm HxN1 gereinigte (1-Methylalkyl)succinat-Synthase zu kristallisieren, blieben erfolglos. Es wurden Anfangsstadien eines möglichen Proteinkristalls generiert, die jedoch für eine Röntgenstrukturanalyse ungeeignet sind.

In dieser Arbeit wurde ein genetisches System für Stamm HxN1 entwickelt, mit dem markierte Deletionsmutanten des Stammes hergestellt wurden. Durch die Deletion des *masD* Gens, das die postulierte katalytische Untereinheit der (1-Methylalkyl)succinat-Synthase kodiert, wurde ein zweites identisches *mas* Operon in Stamm HxN1 identifiziert. Die physiologische Charakterisierung der Mutante nach Deletion von *masD* und *masD´* bestätigte erstmals *in vivo* die Aktivierung von *n*-Alkanen unter anaeroben Bedingungen durch die (1-Methylalkyl)succinat-Synthase. Die Deletion der *masD* Gene verursachte polare Effekte auf die Transkription der benachbarten *mas* Gene, die die kleinen Untereinheiten und die Aktivase der (1-Methylalkyl)succinat-Synthase kodieren. Der Phänotyp wurde deshalb durch Komplementation mit dem gesamten *mas* Operon wiederhergestellt.

Erste Hinweise auf die Regulation des anaeroben *n*-Alkanabbaus in Stamm HxN1 lieferten in dieser Arbeit durchgeführte Induktionsstudien auf verschiedenen Wachstumssubstraten und Kohlenwasserstoffen. Es zeigte sich, dass die Induktion des *mas* Operons durch *n*-Hexan auch in Gegenwart einer Carbonsäure oder eines Zuckers als weitere verwertbare Kohlenstoffquelle stattfindet. Die Regulation erfolgt daher nicht über Katabolitrepression. Des Weiteren wurde gezeigt, dass nicht nur die *n*-Alkane der Kettenlänge von C_5 bis C_8, sondern auch länger- und kürzerkettige *n*-Alkane, Cycloalkane, Aromaten und Alkohole als Induktoren der Expression des *mas* Operons wirken. Die Induktion durch Kohlenwasserstoffe, die von Stamm HxN1 nicht vollständig oder überhaupt nicht oxidiert werden, deutet auf die Regulation durch einen unspezifischen Sensor hin.

Summary

The betaproteobacterial strain HxN1 oxidizes *n*-alkanes with a chain length of C_5 to C_8 under denitrifying conditions completely to CO_2. The *n*-alkanes are activated by addition to fumarate. This reaction is presumably catalyzed by the glycyl radical enzyme (1-methylalkyl)succinate synthase, whose encoding *mas* genes are organized in an operon of seven open reading frames in strain HxN1.

In this study it was attempted to crystallize the (1-methylalkyl)succinate synthase, which had been purified from strain HxN1. However, the obtained crystalline structures were insufficient for X-ray analysis.

A genetic system for strain HxN1 that allowed the generation of deletion mutants of strain HxN1 was developed in this thesis. The deletion of *masD*, which encodes the postulated catalytic subunit of the (1-methylalkyl)succinate synthase, revealed the presence of a second identical *mas* operon in strain HxN1. Following deletion of the second *masD* gene, *masD´*, the physiological characterization of the Δ*masD*, Δ*masD´* mutant confirmed *in vivo* the activation of *n*-alkanes under anaerobic conditions by the (1-methylalkyl)succinate synthase. The deletion of the *masD* genes caused polar effects onto the expression of the adjacent genes of the *mas* operon. The genes upstream and downstream of *masD* encode the other subunits and the activating enzyme of the (1-methylalkyl)succinate synthase. Therefore, the phenotype was restored by complementation with the entire *mas* operon.

First hints regarding the regulation of the anaerobic *n*-alkane degradation in strain HxN1 were obtained in this thesis by investigating the induction of the *mas* operon by a set of growth substrates and hydrocarbons. The induction of the *mas* operon by *n*-hexane was not inhibited in the presence of a carboxylic acid or a sugar as second carbon source. Thus, the *mas* operon is not regulated by catabolite repression. In addition, the range of hydrocarbons, which induce the expression of the *mas* operon was analyzed. It was shown that not only the growth substrates of strain HxN1, the *n*-alkanes from C_5 to C_8, but also *n*-alkanes with a shorter or longer chain length, cyclic alkanes, aromatic hydrocarbons and even alcohols induced expression. The induction of the *mas* operon by several hydrocarbons, which are not a growth substrate for strain HxN1, points to the regulation by an unspecific sensor.

Inhaltsverzeichnis

A Einleitung

1. Gesättigte Kohlenwasserstoffe

Kohlenwasserstoffe sind organische Verbindungen, die ausschließlich aus Kohlenstoff und Wasserstoff bestehen. Sie finden als Energiequelle, Lösungsmittel und Rohstoff der chemischen Industrie Verwendung. Alle anderen organischen Verbindungen leiten sich von ihnen ab, indem einzelne Wasserstoffatome durch funktionelle Gruppen ersetzt oder interne Kohlenstoffmehrfachbindungen aufgebaut werden.

1.1 Systematik der Kohlenwasserstoffe

Anhand der Bindung zwischen zwei Kohlenstoffatomen werden Kohlenwasserstoffe eingeteilt in gesättigte Kohlenwasserstoffe, die ausschließlich C–C Einfachbindungen enthalten, ungesättigte Kohlenwasserstoffe, die mindestens eine Doppel- oder Dreifachbindung enthalten und aromatische Kohlenwasserstoffe, die konjugierte Doppelbindungen haben (Abb. 1).

Gesättigte Verbindungen sind Alkane, die als lineare *n*-Alkane oder ringförmige Cycloalkane vorkommen und mit Alkylseitenketten substituiert sein können. *n*-Alkane mit Alkylseitenketten werden als verzweigte Isoalkane bezeichnet. Die allgemeine Summenformel für *n*-Alkane und Isoalkane lautet C_nH_{2n+2}, wobei für die Isoalkane $n > 3$ gilt. Das einfachste Alkan ist Methan (CH_4). Eine Verlängerung um jeweils eine Methylengrupppe (CH_2) bildet die homologe Reihe der *n*-Alkane. Ab dem Butan (C_4H_{10}) existiert für eine Summenformel mehr als eine Strukturformel, im Falle des Butans sind dies die Konstitutionsisomere *n*-Butan und das verzweigte Isobutan (2-Methylpropan). Cycloalkane haben die Summenformel C_nH_{2n} und bestehen aus mindestens drei Kohlenwasserstoffen ($n > 2$). Auch sie bilden eine homologe Reihe, in dem das Molekül um jeweils eine Methylengruppe verlängert wird.

Zu den ungesättigten Kohlenwasserstoffen gehören Alkene, als Verbindungen mit Doppelbindungen, und Alkine, die Dreifachbindungen besitzen. Alkane, Alkene und Alkine werden auch als aliphatische Kohlenwasserstoffe bezeichnet. Aromatische Verbindungen enthalten mindestens einen aromatischen Ring mit konjugierten Doppelbindungen. Dieser Benzolring ist bei Alkylbenzolen mit ein oder mehreren Alkylseitenketten substituiert. Polyzyklische aromatische Kohlenwasserstoffe (PAK) bestehen aus mehr als einem aromatischen Ring.

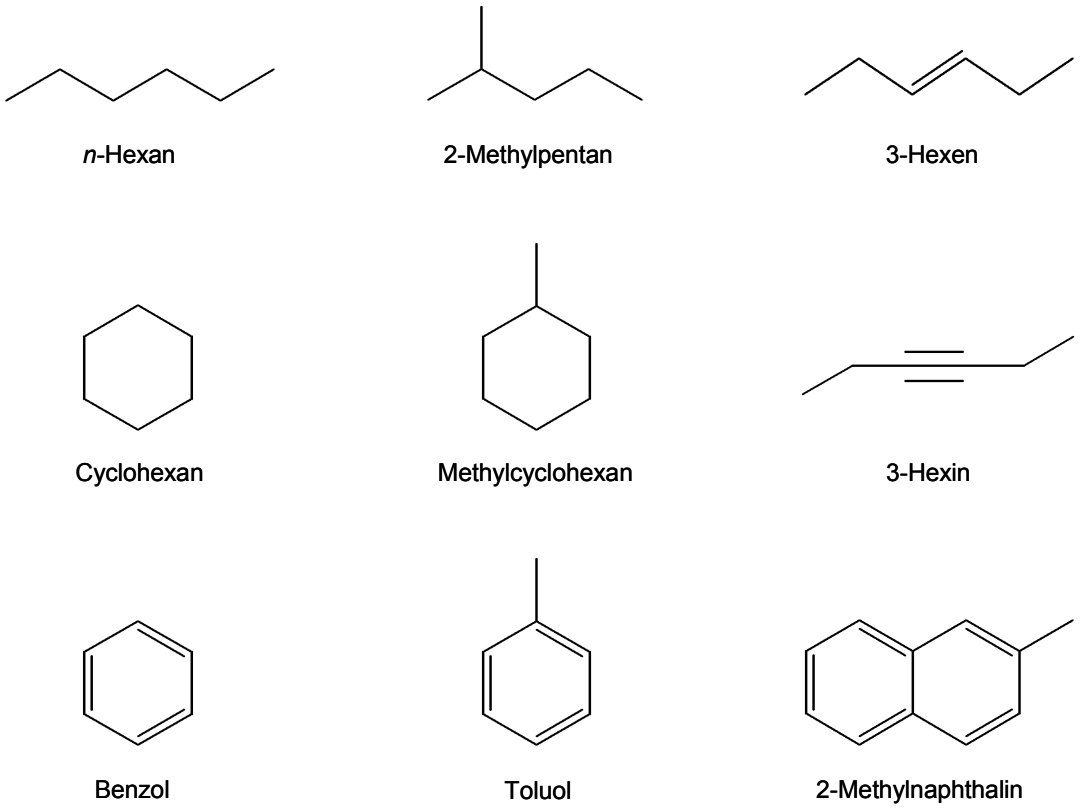

Abb. 1 Strukturformeln von Kohlenwasserstoffen aus der Gruppe der *n*-Alkane (*n*-Hexan), Isoalkane (2-Methylpentan), Cycloalkane (Cyclohexan, Methylcyclohexan), Alkene (3-Hexen), Alkine (3-Hexin), Mono- (Benzol, Toluol) und Polyzyklischen Aromaten (2-Methylnaphthalin).

1.2 Physikalisch-chemische Eigenschaften und Reaktionen der Alkane

Die physikalisch-chemischen Eigenschaften von Alkanen sind abhängig von der Molekülgröße. Die Dichte nimmt mit steigender molarer Masse zu (Abb. 2). Auch der Aggregatzustand von *n*-Alkanen wird bestimmt durch die Molekülgröße. Kurzkettige *n*-Alkane mit einer Kettenlänge von C_1 bis C_4 sind unter Normalbedingungen gasförmig. Die *n*-Alkane von C_5 bis C_{16} sind bei Raumtemperatur flüssig, während *n*-Alkane > C_{16} fest sind, da mit zunehmender Kettenlänge Schmelz- und Siedepunkte ansteigen (Abb. 2). Je größer die Oberfläche des Moleküls ist, desto höher sind Schmelz- und Siedepunkte, weil die van-der-Waals-Kräfte zwischen den einzelnen Molekülen stärker sind (Vollhardt, 1990). Am Beispiel der Isomere des Hexans zeigt sich, dass die verzweigten Isomere einen niedrigeren Siedepunkt als das unverzweigte *n*-Hexan haben, weil sie eine geringere Oberfläche haben und die Moleküle sich nicht so dicht zusammenlagern können wie die linearen *n*-Alkane (Abb. 2). Cyclohexan hingegen hat einen höheren Siedepunkt als *n*-Hexan, weil in dem starren, symmetrischen zyklischen System stärkere van-der-Waals-Kräfte wirken (Vollhardt, 1990). Die Löslichkeit von

n-Alkanen in Wasser nimmt mit zunehmender Kettenlänge ab, da die Moleküle aufgrund ihres zunehmenden hydrophoben Charakters unfähig sind, Wasserstoff-Brückenbindungen auszubilden (Abb. 2).

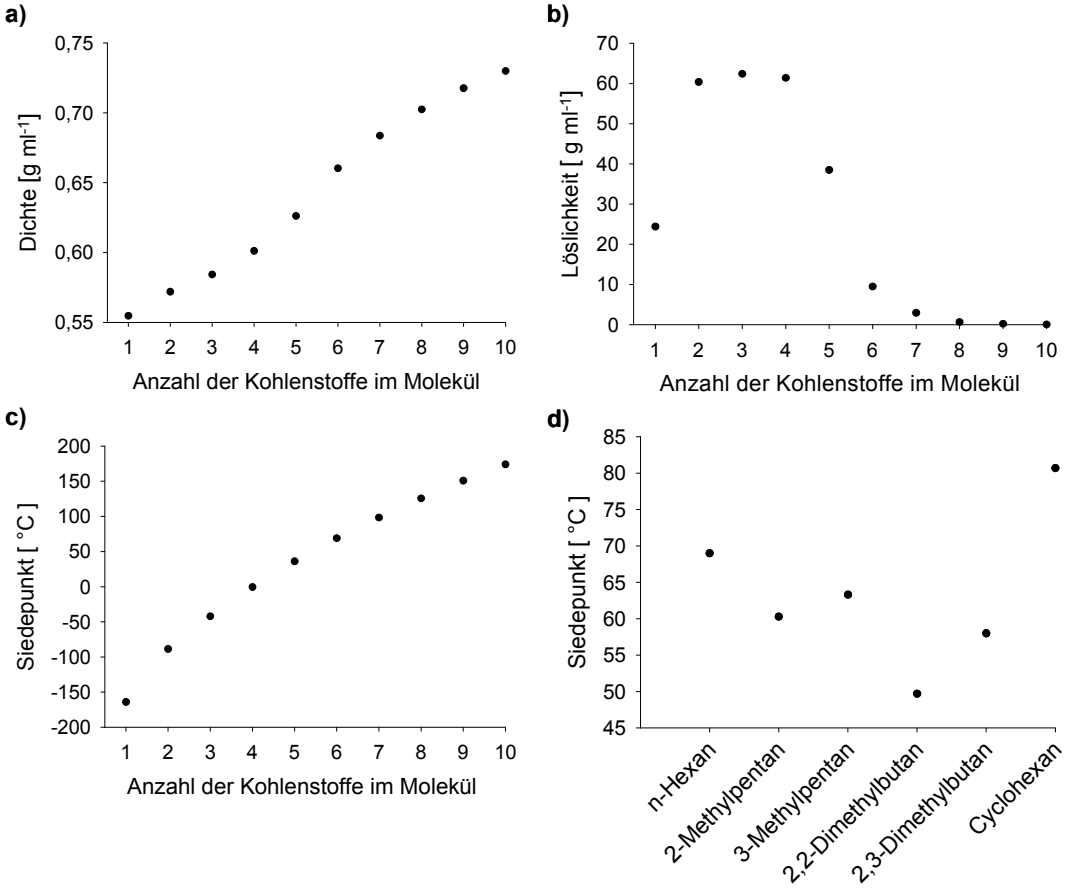

Abb. 2 Physikalisch-chemische Eigenschaften von Alkanen. **a)** Dichte von *n*-Alkanen bei 20 °C, Ausnahmen: Methan und Butan bei 0 °C, Ethan bei –100 °C, Propan bei –45 °C. **b)** Löslichkeit von *n*-Alkanen in Wasser bei 25 °C. **c)** Siedepunkte von *n*-Alkanen. **d)** Siedepunkte der Isomere des Hexans. Nach: Bell (1973); Weast (1990).

Alkane sind aufgrund der geringen Differenz der Elektronegativität (EN) zwischen einem Kohlenstoffatom (EN = 2,55) und einem Wasserstoffatom (EN = 2,2) nahezu unpolar (Vollhardt, 1990). Da keines der beiden Atome bei einer Dissoziation das bindende Elektronenpaar komplett zu sich herüberziehen kann, werden C–H Bindungen in Alkanen nicht heterolytisch in Ionen, sondern nur homolytisch gespalten. Bei der homolytischen Spaltung wird das bindende Elektronenpaar gleichmäßig auf die beteiligten Atome aufgeteilt und Radikale entstehen. Zur homolytischen Spaltung einer chemischen Bindung wird Energie, die Bindungsdissoziationsenergie (ΔH^0), benötigt, die je nach Art der Bindung und der miteinander verbundenen Atome einen

charakteristischen Wert besitzt und von der Stabilität der gebildeten Radikale abhängig ist (Vollhardt, 1990; Wilkes & Schwarzbauer, 2010). So beträgt die Bindungsdissoziationsenergie einer C–H Bindung im Methan 440 kJ mol^{-1}, im Ethan 411 kJ mol^{-1} und an den primären C-Atomen des Propans 410 kJ mol^{-1} (Wilkes & Schwarzbauer, 2010). Generell nimmt die Bindungsdissoziationsenergie einer C–H Bindung vom primären bis zum tertiären C-Atom ab (Tab. 1). Primär sind alle terminalen C-Atome in *n*- und Isoalkanen, sekundär alle dazwischenliegenden C-Atome, von denen keine Alkylseitenketten abzweigen, sowie die C-Atome in Cycloalkanen und tertiär die C-Atome in Iso- oder Cycloalkanen, an denen eine Alkylseitenkette substituiert ist (Abb. 3). Auch die Bindungsdissoziationsenergie einer C–C Einfachbindung ist abhängig von der Energie der bei der Homolyse entstehenden Radikale. Für die Homolyse der C–C Bindung des Ethans ist sie mit 377 kJ mol^{-1} am größten, da die entstehenden primären Alkylradikale eine größere Energie besitzen als sekundäre und tertiäre Alkylradikale (Vollhardt, 1990). Die Bindungen in Alkanen werden durch Pyrolyse oder Verbrennung aufgebrochen. Bei der Pyrolyse werden Alkane thermisch in kleinere Fragmente zerlegt. Die Verbrennung von Alkanen erfolgt vollständig zu CO_2 und Wasser. Desweiteren sind Alkylradikale in der Lage an Doppelbindungen zu addieren (Vollhardt, 1990).

Tab. 1 Bindungsdissoziationsenergien von C–H Bindungen am primären, sekundären und tertiären Kohlenstoffatom. Nach: Vollhardt (1990).

Kohlenstoffatom	kJ mol^{-1}
primär	410
sekundär	395,7
tertiär	389

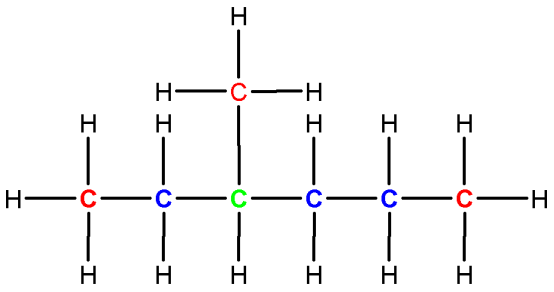

Abb. 3 Primäre (rot), sekundäre (blau) und tertiäre (grün) Kohlenstoffatome im 3-Methylhexan.

1.3 Entstehung und Vorkommen von Alkanen

1.3.1 Biologische Bildung

Alkane werden von Mikroorganismen, Pflanzen und Tieren gebildet. In Mikroorganismen sind sie entweder ein Stoffwechselprodukt der Atmung oder ihre Funktion ist noch nicht bekannt. Bei der Methanogenese durch Archaeen ist Methan das Produkt einer energieliefernden Reaktion (Thauer, 1998). Schätzungen zufolge macht die mikrobielle Methanproduktion bis zu 70% der Gesamtmenge an jährlich global produziertem Methan

(500 bis 600 Teragramm) aus (McInerney *et al.*, 2010). Neuere Untersuchungen postulieren, basierend auf der Kohlenstoff-Isotopen-Zusammensetzung, die biologische Bildung von Ethan und Propan aus Acetat in Tiefseesedimenten (Hinrichs *et al.*, 2006). Über die Mikroorganismen, welche diese Ethano- bzw. Propanogenese zur Energiegewinnung betreiben, ist bisher jedoch nichts bekannt. Längerkettige *n*-Alkane werden von einer Vielzahl an Bakterien, darunter u.a. Cyanobakterien, anaerobe phototrophe Bakterien und Clostridien, sowie von Hefen und anderen Pilzen synthetisiert (Ladygina *et al.*, 2006). Möglicherweise halten intrazelluläre Kohlenwasserstoffe die physikochemischen Eigenschaften der Plasmamembran aufrecht oder unterstützen die Akkumulation hydrophober Substanzen in der Zelle, während extrazelluläre *n*-Alkane in *Pseudomonas fluorescens* die Zelladhäsion und Zellaggregation regulieren (Ladygina *et al.*, 2006).

In Pflanzen und Tieren dienen Kohlenwasserstoffe meistens dem Schutz oder der Interaktion mit anderen Organismen (Wackett, 2010). Vor kurzem wurde berichtet, dass Pflanzen unter oxischen Bedingungen aus bisher unbekanntem Grund größere Mengen an Methan emittieren (Keppler *et al.*, 2006). Mit Ausnahme der Freisetzung des Treibhausgases Methan durch methanogene Bakterien und Pflanzen ist die Menge an biologisch produzierten und freigesetzten Alkanen gering.

1.3.2 Geologische Bildung

Die geologische Bildung von Kohlenwasserstoffen ist ein über große Zeitspannen (5 bis 100 Millionen Jahre) stattfindender Prozess (Tissot & Welte, 1984). Im Wasser absinkendes totes organisches Material (Plankton, Pflanzen) lagert sich als Sediment auf dem Meeresboden ab. Die Biopolymere werden durch mikrobiologische Aktivität in kleinere Fragmente zersetzt. Der mikrobielle Abbau findet nur in der obersten Sedimentschicht, vorwiegend durchgeführt von anaeroben Bakterien, statt. Methanogene Bakterien setzen hierbei Methan frei. Diese erste Phase der Zersetzung der Biomasse wird Diagenese genannt (Tissot & Welte, 1984). Durch Sedimentation weiterer Biomasse wird das schon abgelagerte Sediment bedeckt und mit zunehmender Tiefe einem Druck- und Temperaturanstieg ausgesetzt, der in der Phase der Diagenese zur Kondensation und Polymerisation der von den Mikroorganismen nicht genutzten Komponenten zunächst zu Fulvo- und Huminsäuren führt. Durch weitere Kondensation und den Verlust von funktionellen Gruppen werden hochkomplexe unlösliche Polymere, Kerogen genannt, gebildet. Der nächste Zersetzungsschritt wird Katagenese genannt (Tissot & Welte, 1984). Bedingt durch größere Tiefe und einen damit verbundenen weiteren Druck- und Temperaturanstieg wird das Kerogen thermisch durch Spaltung von

C–C Bindungen abgebaut, wobei Erdöl und Erdgas gebildet werden. Erdöl besteht aus *n*-Alkanen, Iso- und Cycloalkanen und Aromaten, deren Zusammensetzung in Erdölen verschiedener Fundorte variabel ist (Tissot & Welte, 1984). Erdgas besteht hauptsächlich aus Methan, in geringeren Mengen kommen auch Ethan, Propan, Butan und Isobutan vor (Tissot & Welte, 1984). Erdgas und Erdöl entweichen natürlicherweise aus ihren Lagerstätten und werden ins Meer freigesetzt. Dies geschieht insbesondere im Kontinentalschelf und in Gebieten, in denen Kontinentalplatten auseinanderdriften. Im Golf von Mexiko kommen mehrere Hundert dieser natürlichen Austrittsstellen vor. In noch größerer Tiefe werden durch einen weiteren Temperatur- und Druckanstieg Methan, CO_2 und fester Kohlenstoff gebildet. Dieser Prozess wird Metagenese genannt (Tissot & Welte, 1984).

1.3.3 Anthropogene Freisetzung

Erdöl und Erdgas sind wichtige fossile Energieträger, die in großen Mengen gefördert werden, um den steigenden Energiebedarf auf der Erde zu decken. Erdöl wird durch das Anbohren natürlicher Erdöllagerstätten in den Meeren an die Oberfläche gefördert oder aus dem Boden durch den Abbau von Ölsanden, wie beispielsweise in der kanadischen Provinz Alberta, gewonnen. Durch die Förderung und den Transport von Öl, sowie durch Unfälle wird Öl in die Umwelt freigesetzt. Wasser, das beim Abbau von Ölsanden kontaminiert wird, verschmutzt Flüsse oder versickert im Grundwasser (Schindler, 2010). Bei der Explosion der Ölplattform *Deep Water Horizon* im Golf von Mexiko im März 2010 traten ca. 780 Mio. Liter Öl aus (Atlas & Hazen, 2011). Die Havarie der *Exxon Valdez* 1989 vor der Küste Alaskas führte zur Freisetzung von 40 Mio. Liter Öl (Atlas & Hazen, 2011). Jüngstes Beispiel ist die *Rena*, die im Oktober 2011 vor der Küste Neuseelands auf ein Riff aufgelaufen ist und Leck geschlagen hat.

Eine Möglichkeit zur Beseitigung der Ölkontamination ist die biologische Sanierung, bei der die Fähigkeit von Bakterien zum Abbau von Kohlenwasserstoffen genutzt wird. Die Unglücke der *Exxon Valdez* und der *Deep Water Horizon* führten zu einer deutlichen Vermehrung der natürlicherweise vorkommenden kohlenwasserstoffabbauenden Bakterien, da diese nun nicht mehr substratlimitiert waren (Prince, 1993; Hazen *et al.*, 2010). Diese Bakterien tragen zum Abbau der Kohlenwasserstoffe bei, solange ihnen ein nutzbarer Elektronenakzeptor, wie z.B. Sauerstoff oder Sulfat, und ausreichend Nährstoffe, insbesondere Stickstoff und Phosphor, zur Verfügung stehen (Prince, 2010b). Um die biologische Sanierung als alternative Maßnahme zur chemischen Sanierung zukünftig besser nutzen zu können, ist es wichtig kohlenwasserstoff-abbauende Bakterien zu erforschen.

2. Mikrobieller Abbau von *n*-Alkanen

Alkane eignen sich aufgrund ihres hohen Energie- und Kohlenstoffgehaltes gut als Energie- und Kohlenstoffquelle für Mikroorganismen. Zur Aktivierung dieser reaktionsträgen Moleküle bedarf es aber spezieller Mechanismen. Mittlerweile ist eine Vielzahl von Organismen beschrieben, die in der Lage sind *n*-Alkane zur Energiegewinnung zu aktivieren und vollständig zu CO_2 abzubauen.

2.1 Verfügbarkeit und Aufnahme von *n*-Alkanen

Aufgrund ihrer guten Löslichkeit in Wasser sind die gasförmigen *n*-Alkane (C_1 bis C_4) für Bakterien leicht verfügbar (Abb. 2b). Sie gelangen vermutlich genauso wie H_2, N_2 und O_2 durch freie Diffusion in die Zelle. Die Löslichkeit flüssiger und fester *n*-Alkane nimmt mit zunehmender Kettenlänge immer weiter ab (Abb. 2b), so dass Bakterien Mechanismen entwickeln mussten, um sich die *n*-Alkane verfügbar zu machen. Zu diesen Mechanismen gehören die Adhäsion an die kohlenwasserstoffhaltige Phase mit hydrophoben Zelloberflächenstrukturen oder die Sekretion von Emulgatoren oder Tensiden, die die Verfügbarkeit des Substrates erhöhen (van Hamme *et al.*, 2003; Perfumo *et al.*, 2010; Satpute *et al.*, 2010). Durch Chemotaxis gelangen Bakterien in räumliche Nähe ihrer Kohlenstoff- und Energiequelle. Für einige Stämme, die *n*-Alkane oder Aromaten abbauen, wurde eine chemotaktische Antwort auf einen Kohlenwasserstoff gezeigt und in einigen Fällen wurde auch der Chemorezeptor identifiziert (Parales & Ditty, 2010). Aufgrund ihres hydrophoben Charakters diffundieren *n*-Alkane frei durch die Cytoplasmamembran. Die äußere Membran von Gram-negativen Zellen ist hingegen eine Barriere, die die Anwesenheit von Kanälen für den Substrattransport durch sie hindurch erforderlich macht. In Aromatenabbauern wurden Transportproteine für den aromatischen Kohlenwasserstoff Toluol identifiziert (Wang *et al.*, 1995).

2.2 Aerober Abbau von *n*-Alkanen

Neben Bakterien sind auch Hefen, Pilze und Algen in der Lage, *n*-Alkane unter aeroben Bedingungen abzubauen (van Beilen *et al.*, 2003). Der Sauerstoff dient nicht nur der Aktivierung des inerten Kohlenstoffmoleküls sondern auch als terminaler Elektronenakzeptor. Die *n*-Alkane werden durch die Hydroxylierung eines terminalen oder subterminalen Kohlenstoffs unter Bildung eines primären oder sekundären Alkohols aktiviert (Abb. 4) (Rojo, 2010a). Das zweite Sauerstoffatom wird zu H_2O reduziert, wofür ein Reduktionsmittel, z. B. NAD(P)H+H^+, benötigt wird. Ein primärer Alkohol wird weiter zu einem Aldehyd und anschließend zu einer Fettsäure oxidiert, die dann durch

β-Oxidation abgebaut wird. Sekundäre Alkohole werden über ein Keton und einen Ester zu einer Fettsäure und einem primären Alkohol abgebaut (Rojo, 2010a).

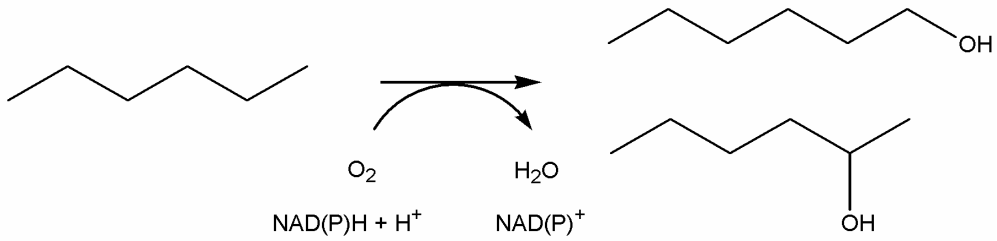

Abb. 4 Aktivierung von *n*-Hexan durch eine Oxygenase zum primären oder sekundären Alkohol.

Abhängig von der Kettenlänge des zu aktivierenden *n*-Alkans sind in Bakterien verschiedene Enzymklassen für die Hydroxylierung der *n*-Alkane zuständig. Methan wird von löslichen (sMMO) oder partikulären (pMMO) Methan-Monooxygenasen aktiviert (Hanson & Hanson, 1996). Andere kurzkettige *n*-Alkane werden von Methan-Monooxygenase ähnlichen Enzymen aktiviert (van Beilen & Funhoff, 2007). Längerkettige *n*-Alkane werden von Cytochrom P450 Alkanhydroxylasen oder in der Membran lokalisierten Alkanhydroxylasen aktiviert (van Beilen & Funhoff, 2005). Viele Organismen besitzen mehrere Hydroxylasen mit überlappenden Substratspektren, um ein großes Spektrum an *n*-Alkanen abbauen zu können (van Beilen & Funhoff, 2007).

Der aerobe *n*-Alkanabau ist u.a. in *Pseudomonas putida* Gpo1 eingehend charakterisiert worden (van Beilen *et al.*, 1994). Die benötigten Gene sind auf einem Plasmid kodiert. Gen *alkb* kodiert eine in der Membran lokalisierte Monooxygenase (van Beilen *et al.*, 1994). Desweiteren werden für die Hydroxylierung der *n*-Alkane zwei Elektronen-transferproteine, Rubredoxin und Rubredoxin-Reduktase, benötigt, die von *alkG* und *alkT* kodiert werden (Rojo, 2010b). Die Rubredoxin-Reduktase transferiert Elektronen über seinen Cofaktor FAD von NADH zum Rubredoxin, welches die Elektronen dann zur Monooxygenase AlkB transferiert (van Beilen & Funhoff, 2007; Rojo, 2010a).

2.3 Anaerober Abbau von *n*-Alkanen

Lange Zeit wurde davon ausgegangen, dass eine Aktivierung von *n*-Alkanen aufgrund ihrer geringen Reaktivität nur mithilfe von Sauerstoff als starkem Oxidationsmittel möglich ist, wodurch eine funktionelle Gruppe ins Molekül eingefügt wird. Diese ist notwendig für den Abbau von organischen Molekülen. Energetisch ist eine anaerobe *n*-Alkanaktivierung möglich. Die Energie, die bei der Oxidation zu CO_2 gewonnen wird, ist abhängig vom Redoxpotential des jeweiligen Elektronenakzeptors. Mit Nitrat wird

mehr Energie gewonnen als mit Sulfat. So wird bei der vollständigen Oxidation von *n*-Hexan mit Nitrat als Elektronenakzeptor eine Energie von 492,8 kJ mol^{-1} Nitrat frei, während es für die Oxidation von *n*-Hexan mit Sulfat nur 44,2 kJ mol^{-1} Sulfat sind (Spormann & Widdel, 2000).

Tatsächlich wurde auch unter anaeroben Bedingungen der Abbau von Kohlenwasserstoffen beobachtet. Zu Beginn der 1990er Jahre wurde das erste anaerob Alkan-verwertende Bakterium isoliert (Aeckersberg *et al.*, 1991). Mittlerweile sind einige Isolate beschrieben, die *n*-Alkane mit Nitrat oder Sulfat als terminalem Elektronenakzeptor vollständig zu CO_2 abbauen (Tab. 2). Kürzlich wurde berichtet, dass *Pseudomonas chloritidismutans* *n*-Decan mit Chlorat als Elektronenakzeptor abbaut (Mehboob *et al.*, 2009). Die Isolate entstammen unterschiedlichen Habitaten. Sulfatreduzierer wurden aus marinen Sedimenten, in denen die *n*-Alkane durch geologische Bildung natürlicherweise vorkommen, wie z.B. im Golf von Mexiko oder im Guaymas Basin im Golf von Kalifornien (Rueter *et al.*, 1994; Kniemeyer *et al.*, 2007), oder aus marinen Sedimenten und Schlämmen, die anthropogen mit *n*-Alkanen kontaminiert sind, isoliert (Aeckersberg *et al.*, 1998; So & Young, 1999; Cravo-Laureau *et al.*, 2004). Neben diesen Salzwasserisolaten wurden Bakterien auch aus Brackwasser (Grossi *et al.*, 2007) und aus Süßwassergrabenschlämmen (Ehrenreich *et al.*, 2000) isoliert. Desweiteren wurden Isolate aus Abwässern, die bei der Erdölförderung anfallen (Davidova & Suflita, 2005), oder aus Erdölförderanlagen gewonnen (Aeckersberg *et al.*, 1991). Stamm HdN1 wurde aus Schlamm einer Kläranlage isoliert (Ehrenreich *et al.*, 2000). Mit Ausnahme des thermophilen Stammes TD3, der aus dem Guaymas Basin stammt (Rueter *et al.*, 1994), sind alle anderen Isolate mesophil. Das Substratspektrum der Isolate ist auf einen bestimmten Kettenlängenbereich beschränkt (Tab. 2). Alle bislang auf *n*-Alkanen isolierten Reinkulturen sind Proteobakterien (Widdel *et al.*, 2010).

Neben den Isolaten wurden auch Anreicherungskulturen beschrieben, die *n*-Alkane mit Nitrat (Bregnard *et al.*, 1997; Callaghan *et al.*, 2009) oder Sulfat (Caldwell *et al.*, 1998; Kniemeyer *et al.*, 2007; Savage *et al.*, 2010) oxidieren oder durch Methanogenese abbauen (Zengler *et al.*, 1999b; Anderson & Lovley, 2000; Jones *et al.*, 2008). Syntrophe Konsortien aus Archaeen und sulfatreduzierenden Bakterien oxidieren anaerob Methan (Boetius *et al.*, 2000; Nauhaus *et al.*, 2002). Vor kurzem wurde eine Anreicherungskultur beschrieben, in der das dominierende Bakterium *Methylomirabilis oxyfera* Methan durch Denitrifikation oxidiert (Ettwig *et al.*, 2008; Ettwig *et al.*, 2010).

Tab. 2 Bislang isolierte Bakterien, die anaerob *n*-Alkane bestimmter Kettenlängen abbauen.

Isolat	*n*-Alkane	Referenz
Denitrifizierer		
Stamm HdN1	C_{14}-C_{20}	Ehrenreich *et al.* (2000)
Stamm HxN1	C_6-C_8	Ehrenreich *et al.* (2000)
Stamm OcN1	C_8-C_{12}	Ehrenreich *et al.* (2000)
Marinobacter sp. (BC36, BC38, BP42)	C_{18}	Bonin *et al.* (2004)
Pseudomonas balearica Stamm BerOc6	C_{15}-C_{18}	Grossi *et al.* (2008)
Sulfatreduzierer		
Desulfococcus oleovorans Stamm Hxd3	C_{12}-C_{20}	Aeckersberg *et al.* (1991)
Stamm TD3	C_6-C_{16}	Rueter *et al.* (1994)
Stamm Pnd3	C_{14}-C_{17}	Aeckersberg *et al.* (1998)
Desulfatibacillum alkenivorans Stamm AK-01	C_{13}-C_{18}	So & Young (1999)
Desulatibacillum aliphaticivorans CV2803	C_{13}-C_{18}	Cravo-Laureau *et al.* (2004)
Desulfoglaeba alkanexedens (ALDC)	C_6-C_{12}	Davidova & Suflita (2005)
Desulfoglaeba alkanexedens (Lake)	C_6-C_{10}	Davidova & Suflita (2005)
Stamm Bus5	C_3, C_4	Kniemeyer *et al.* (2007)
Stamm PL12	C_6, C_{10}	Higashioka *et al.* (2009)
Chloratreduzierer		
Pseudomonas chloritidismutans	C_{10}	Mehboob *et al.* (2009)

3. Reaktionen, Proteine und Gene des anaeroben *n*-Alkanabbaus

Die Fähigkeit von Bakterien, *n*-Alkane unter anaeroben Bedingungen abzubauen, setzt einen Aktivierungsmechanismus voraus, der sich von der Aktivierung mittels Sauerstoff unterscheiden muss. Die Identifizierung der bislang bedeutendsten Aktivierungsreaktion sowie der dafür verantwortlichen Proteine baut auf den Erkenntnissen zur anaeroben Aktivierung des aromatischen Kohlenwasserstoffes Toluol auf.

3.1 *n*-Alkan-Aktivierung durch Addition an Fumarat

In dem denitrifizierenden Betaproteobakterium *Thauera aromatica* wurde gezeigt, dass die Bildung von Benzylsuccinat der erste Schritt im anaeroben Abbau von Toluol ist (Biegert *et al.*, 1996). Benzylsuccinat entsteht bei der Addition von Fumarat an die Methylgruppe des Toluols (Abb. 5). Diese Reaktion wurde in den folgenden Jahren für

weitere Nitrat- (Beller & Spormann, 1997b), Sulfat- (Beller & Spormann, 1997a; Morasch *et al.*, 2004) und Fe(III)-Reduzierer (Kane *et al.*, 2002), phototrophe Bakterien (Zengler *et al.*, 1999a) und eine methanogene Anreicherungskultur (Beller & Edwards, 2000) beschrieben. Ein alternativer Aktivierungsmechanismus für Toluol unter anaeroben Bedingungen ist bisher nicht bekannt.

Auch für andere monoaromatische Kohlenwasserstoffe, die vollständig von Bakterien mineralisiert werden, wurden Metabolite identifiziert, die auf eine Aktivierung mittels Addition an Fumarat hindeuten. Der sulfatreduzierende Stamm OX39 baut neben Toluol auch *m*- und *o*-Xylol vollständig ab (Morasch *et al.*, 2004). Der Denitrifizierer *Azoarcus* sp. Stamm T dagegen metabolisiert neben Toluol nur *m*-, aber nicht *o*-Xylol (Krieger *et al.*, 1999). *Desulfobacterium cetonicum* aktiviert *m*- und *p*-Cresol durch Addition an Fumarat und oxidiert beide Substrate vollständig zu CO_2 (Müller *et al.*, 1999, 2001). Für Ethylbenzol hingegen sind zwei verschiedene Aktivierungsmechanismen beschrieben worden. In dem sulfatreduzierenden Stamm EbS7 wird Ethylbenzol durch Addition der aromatenständigen Methylengruppe an Fumarat aktiviert (Kniemeyer *et al.*, 2003), in dem Nitratreduzierer *Aromatoleum aromaticum* Stamm EbN1 hingegen durch eine Dehydrogenierung (Kniemeyer & Heider, 2001).

Metabolitanalysen an *n*-Alkanabbauern, dem denitrifizierenden Stamm HxN1, den Sulfatreduzierern *D. alkenivorans* Stamm AK-01 und *D. aliphaticivorans* Stamm CV2803, und an sulfatreduzierenden Anreicherungskulturen, identifizierten die Additionsprodukte von Fumarat an das subterminale C-Atom eines *n*-Alkans, (Kropp *et al.*, 2000; Rabus *et al.*, 2001; Cravo-Laureau *et al.*, 2005; Davidova *et al.*, 2005; Callaghan *et al.*, 2006). So wird in Stamm HxN1 bei der Aktivierung von *n*-Hexan durch Addition an Fumarat (1-Methylpentyl)succinat gebildet (Abb. 5) (Rabus *et al.*, 2001).

Energetisch betrachtet ist die Aktivierung eines *n*-Alkans am sekundären C-Atom (395,7 kJ mol^{-1}) günstiger als am terminalen C-Atom (410 kJ mol^{-1}) (Tab.1). Es wird jedoch mehr Energie benötigt als für die Aktivierung von Toluol an dessen Methylgruppe (368 kJ mol^{-1}), da ein Benzylradikal durch sein π-Elektronensystem stabilisiert wird (Rabus *et al.*, 2001).

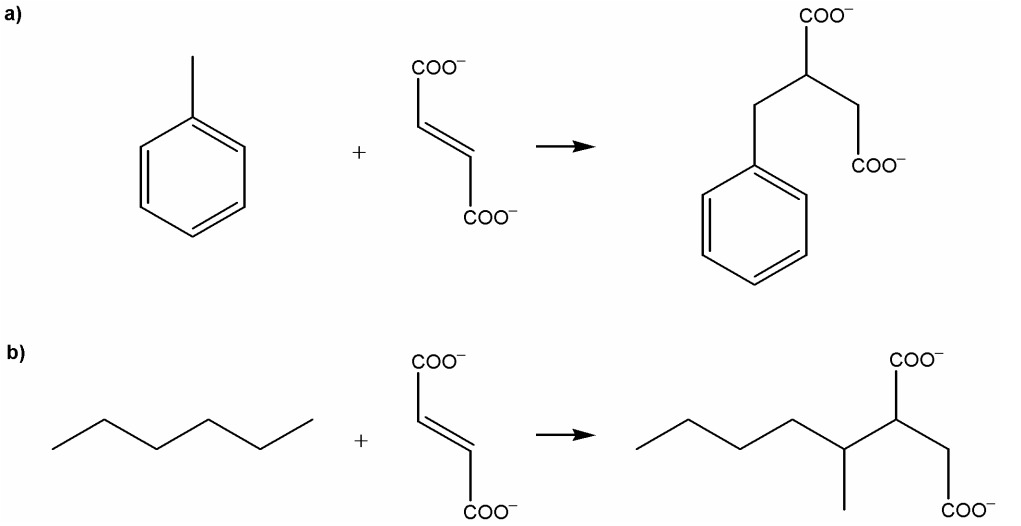

Abb. 5 Aktivierung von Aromaten und *n*-Alkanen durch Addition an Fumarat. **a)** Bei der Addition von Toluol an Fumarat entsteht Benzylsuccinat. **b)** Durch Addition von *n*-Hexan an Fumarat wird (1-Methylpentyl)succinat gebildet.

Nach der Aktivierung des *n*-Alkans zu einem (1-Methylalkyl)succinat wird dieses möglicherweise durch Coenzym A zu (1-Methylalkyl)succinyl-CoA aktiviert (Abb. 6) (Wilkes *et al.*, 2002). Die Oxidation zu CO_2 über die β-Oxidation erfordert zunächst eine intramolekulare Umlagerung des (1-Methylalkyl)succinyl-CoA zu (2-Methylalkyl)malonyl-CoA, das dann decarboxyliert wird (Wilkes *et al.*, 2002; Wilkes *et al.*, 2003). Das bei der Decarboxylation gebildete 4-Methylalkanoyl-CoA wurde in Form seines Methylesters in Zellen von Stamm HxN1, die auf *n*-Hexan gewachsen waren, detektiert (Wilkes *et al.*, 2002). Propionyl-CoA, das bei der β-Oxidation von methylverzweigten Fettsäuren gebildet wird, kann für die Regeneration von Fumarat, beispielsweise über den Methylmalonyl-CoA-Weg und den anschließenden Eintritt des Produktes dieser Reaktion, Succinyl-CoA, in den Citratzyklus genutzt werden (Abb. 6) (Wilkes *et al.*, 2002).

Die Addition an Fumarat wurde, basierend auf Metabolitstudien, auch für die Aktivierung zyklischer Alkane propagiert (Rios-Hernandez *et al.*, 2003; Musat *et al.*, 2010). Die Aktivierung findet bei Ethylcyclopentan, genau wie bei Cyclohexan, am Ring und nicht an der Alkylseitenkette statt (Rios-Hernandez *et al.*, 2003; Musat *et al.*, 2010). Für den polyzyklischen aromatischen Kohlenwasserstoff 2-Methylnaphthalin wurden ebenfalls Metabolite, die für eine Aktivierung mittels Addition an Fumarat an den Methylrest sprechen, analog zur Toluolaktivierung, identifiziert (Annweiler *et al.*, 2000; Musat *et al.*, 2009). Die Addition an Fumarat ist damit die bis heute am häufigsten dokumentierte Aktivierungsreaktion von Kohlenwasserstoffen unter anaeroben Bedingungen.

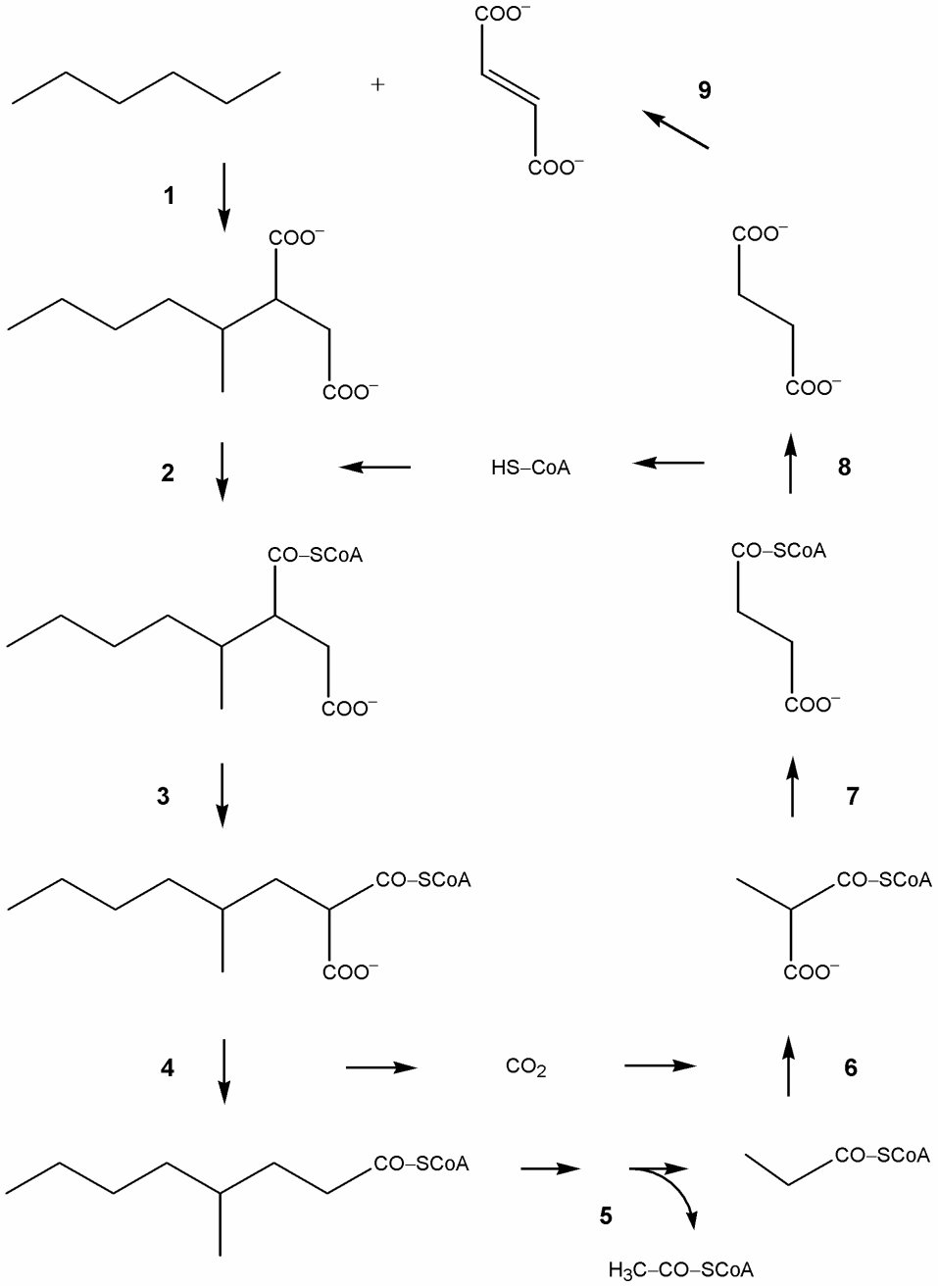

Abb. 6 Möglicher Abbauweg für *n*-Hexan unter anaeroben Bedingungen in Stamm HxN1. Nach Addition von *n*-Hexan an Fumarat wird das hierbei entstandene (1-Methylpentyl)succinat (**1**) durch Coenzym A aktiviert zu (1-Methylpentyl)succinyl-CoA (**2**). Durch eine intramolekulare Umlagerung wird (2-Methylhexyl)malonyl-CoA gebildet (**3**), welches decarboxyliert wird zu 4-Methyloctanoyl-CoA (**4**). Bei der anschließenden β-Oxidation werden Acetyl-CoA und Propionyl-CoA gebildet (**5**). Letzteres wird carboxyliert zu Methylmalonyl-CoA (**6**), während das Acetyl-CoA zu CO_2 oxidiert wird. Eine Umlagerung des Methylmalonyl-CoA bildet Succinyl-CoA (**7**), welches im Citratzyklus über Succinat (**8**) Fumarat als Co-Substrat der Aktivierung von *n*-Hexan regeneriert (**9**). Verändert nach: Wilkes *et al.* (2002).

3.2. Glycyl- und SAM-Radikalenzyme

Das Enzym, das die Additionsreaktion von Fumarat und Toluol katalysiert, wurde aus *T. aromatica* Stamm K172 isoliert (Leuthner *et al.*, 1998). Sequenzähnlichkeiten der α-Untereinheit dieser Benzylsuccinat-Synthase zu den bis dato einzigen Glycylradikal-enzymen Pyruvat-Formiat-Lyase (Knappe *et al.*, 1984) und anaerobe Ribonukleotid-Reduktase (Sun *et al.*, 1993) deuteten auf eine radikalische Aktivierung des Toluols hin (Leuthner *et al.*, 1998). Mittlerweile wurden als weitere Glycylradikalenzyme die 4-Hydroxyphenylacetat-Decarboxylase (Selmer & Andrei, 2001) und die Coenzym B_{12}-unabhängige Glycerin-Dehydratase beschrieben (Raynaud *et al.*, 2003; O´Brien *et al.*, 2004).

Für Glycylradikalenzyme ist ein konservierter Glycinrest mit dem Sequenzmotiv RVXG am C-Terminus der katalytischen Untereinheit charakteristisch (Sun *et al.*, 1993). Im aktiven Zustand des Enzyms ist ein Radikal an diesem Glycinrest lokalisiert (Abb. 7) (Wagner *et al.*, 1992; King & Reichard, 1995; Sun *et al.*, 1996). Bei einer Reaktion des Radikals mit Sauerstoff wird die Polypeptidkette an dieser Position irreversibel gespalten (Wagner *et al.*, 1992; King & Reichard, 1995). Das Glycylradikal liefert ein charakteristisches Elektronen-Paramagnetisches-Resonanz (EPR)-Signal (Unkrig *et al.*, 1989), welches in der aktiven, partiell aufgereinigten Benzylsuccinat-Synthase aus *Azoarcus* sp. Stamm T, in Zellextrakten von *T. aromatica* Stamm K172, angezogen auf Toluol und *m*-Xylol, und in Zellextrakten von Stamm HxN1, angezogen auf *n*-Hexan, nachgewiesen wurde (Krieger *et al.*, 2001; Rabus *et al.*, 2001; Verfürth *et al.*, 2004). Bei Wachstum von Stamm HxN1 auf der C_6-Fettsäure Capronat war dieses Radikal hingegen nicht nachweisbar (Rabus *et al.*, 2001).

Glycylradikalenzyme müssen durch andere Enzyme, die das Radikal auf den Glycinrest übertragen, aktiviert werden. Bei diesen Aktivierungsenzymen handelt es sich um S-Adenosylmethionin (SAM)-Radikalenzyme (Sofia *et al.*, 2001). SAM-Radikalenzyme zeichnet ein unkonventionelles Eisen-Schwefel-Zentrum aus, das nur durch drei anstatt vier Cysteinreste koordiniert wird (Layer *et al.*, 2004). Die Cysteine bilden ein konserviertes CxxxCxxC-Motiv in SAM-Radikalenzymen (Sofia *et al.*, 2001). Das Eisen-Schwefel-Zentrum transferiert ein Elektron von einem Elektronendonor mit niedrigem Potenzial, Flavodoxin oder Ferredoxin, auf S-Adenosylmethionin (Buckel & Golding, 2006), das dadurch homolytisch gespalten wird in Methionin und ein 5´-Desoxyadenosylradikal (Abb. 7) (Layer *et al.*, 2004). Das 5´-Desoxyadenosylradikal seinerseits abstrahiert ein Wasserstoffatom vom Glycinrest des Glycylradikalenzyms (Buckel & Golding, 2006).

Das aktivierte Glycylradikalenzym aktiviert sein Substrat nicht mit dem Glycylradikal, sondern mit einem Thiylradikal. Hierfür wird das Radikal innerhalb des Enzyms auf einen ebenfalls konservierten Cysteinrest übertragen (Knappe *et al.*, 1993). Das entstandene Thiylradikal vollzieht dann den Angriff auf das Substrat (Abb. 7). Das Radikal wird innerhalb des Enzyms regeneriert und steht dann für weitere Aktivierungen zur Verfügung.

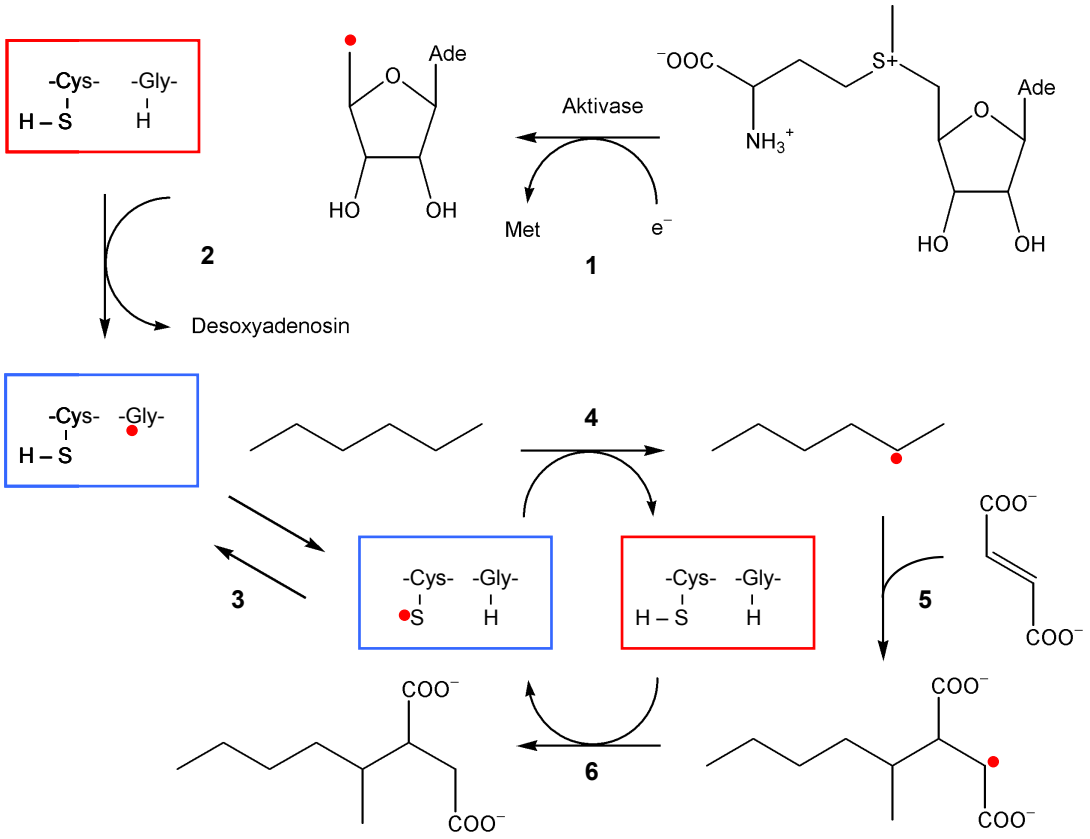

Abb. 7 Radikalischer Aktivierungsmechanismus von *n*-Hexan durch Addition an Fumarat. Die Aktivase überträgt ein Elektron auf S-Adenosylmethionin und spaltet dieses dadurch in Methionin und ein 5´-Desoxyadenosylradikal (**1**). Das Radikal abstrahiert ein Wasserstoffatom vom konservierten Glycinrest des Glycylradikalenzyms (**2**). Das Glycylradikal wiederum abstrahiert ein Wasserstoffatom vom konservierten Cysteinrest (**3**) und das hierbei entstehende Thiylradikal greift das *n*-Hexan am C-2 an, wobei ein *n*-Hexylradikal (**4**) gebildet wird. Hieran addiert das Fumarat unter Bildung eines (1-Methylpentyl)succinylradikals (**5**). Das Radikal wird dann im Glycylradikalenzym regeneriert (**6**,**3**), es entsteht das Additionsprodukt (1-Methylpentyl)succinat (**6**). Rot: inaktives Glycylradikalenzym; blau: aktives Glycylradikalenzym; roter Punkt: Radikal. Verändert nach: Widdel *et al.* (2006).

SAM-Radikalenzyme, die selber kein Substrat aktivieren sondern ein anderes Enzym, werden als Aktivasen bezeichnet (Layer *et al.*, 2004). Die Aktivasen der oben beschriebenen Glycylradikalenzyme nutzen SAM als Substrat, das irreversibel gespalten wird (Wang & Frey, 2007). Andere SAM-Radikalenzyme katalysieren direkt die Umwandlung eines Substrates. Ein Beispiel ist die Lysin-2,3-Aminomutase, die Wasserstoff vom 5´-Desoxyadenosylradikal auf Lysin und β-Lysin überträgt (Baraniak *et al.*, 1989). Hierbei wird SAM als Coenzym genutzt, das regeneriert wird (Buckel & Golding, 2006; Wang & Frey, 2007).

3.3 Benzylsuccinat- und (1-Methylalkyl)succinat-Synthase

Bei der Reinigung der Benzylsuccinat-Synthase aus *T. aromatica* Stamm K172, angezogen auf Toluol, wurden drei Untereinheiten des Enzyms identifiziert, eine große α-Untereinheit und zwei kleine β- und γ-Untereinheiten (Leuthner *et al.*, 1998). Für das Holoenzym wurde eine $\alpha_2\beta_2\gamma_2$-Zusammensetzung postuliert. Das kodierende *bss* Operon enthält vier offene Leserahmen, von denen drei den Untereinheiten des Enzyms zugeordnet wurden (Abb. 8) (Leuthner *et al.*, 1998). Die Sequenz der großen α-Untereinheit (BssA) ist ähnlich dem Glycylradikalenzym Pyruvat-Formiat-Lyase und auch die für Glycylradikalenzyme charakteristischen Glycin- und Cysteinreste wurden in dieser Untereinheit identifiziert (Leuthner *et al.*, 1998). Die Funktion der beiden kleinen Untereinheiten (β-Untereinheit: BssB; γ-Untereinheit: BssC) ist ungeklärt. In der Proteinsequenz dieser Untereinheiten wurden konservierte Cystein-Sequenzmotive identifiziert, die womöglich die in der Benzylsuccinat-Synthase detektierten Eisen-Schwefel-Zentren koordinieren (Li *et al.*, 2009; Hilberg *et al.*, 2012). Mögliche daraus abgeleitete Funktionen beinhalten die Vermittlung von struktureller Stabilität des Enzyms (Li *et al.*, 2009) oder ein Elektronentransfer bei der Bildung des Glycylradikals (Hilberg *et al.*, 2012). Das vierte Gen des Operons (*bssD*) kodiert die Aktivase der Benzylsuccinat-Synthase (Leuthner *et al.*, 1998).

Gene für die Benzylsuccinat-Synthase wurden auch in anderen Denitrifizierern (Coschigano *et al.*, 1998; Achong *et al.*, 2001; Kube *et al.*, 2004; Shinoda *et al.*, 2004; Shinoda *et al.*, 2005), sowie einem Fe(III)-Reduzierer (Kane *et al.*, 2002) und einer methanogenen Anreicherungskultur (Washer & Edwards, 2007), die Toluol abbauen, detektiert. Für Sulfatreduzierer stehen bislang nur partielle *bssA* Sequenzen zur Verfügung (Winderl *et al.*, 2007). *Georgfuchsia toluolica* metabolisiert Toluol mit Nitrat, Fe(III) oder Mn(IV) als Elektronenakzeptor (Weelink *et al.*, 2009). In *T. aromatica* Stamm T1 sind die *bss* Gene alternativ als *tut* Gene (für toluene utilization) benannt (Coschigano *et al.*, 1998). Zusätzlich zu den vier genannten Genen (*bssA* bis *bssD*)

kann das *bss* Operon in verschiedenen Organismen noch weitere Gene mit teilweise ungeklärter Funktion umfassen (Coschigano, 2000; Hermuth *et al.*, 2002; Kube *et al.*, 2004).

In dem anaerob *n*-Alkane abbauenden Stamm HxN1 wurden bei Wachstum auf *n*-Hexan Proteine identifiziert, die den Untereinheiten der Benzylsuccinat-Synthase ähnlich sind und womöglich die Untereinheiten einer (1-Methylalkyl)succinat-Synthase repräsentieren (Grundmann *et al.*, 2008). Die kodierenden Gene liegen in einem Operon mit insgesamt sieben offenen Leserahmen (Abb. 8) (Grundmann *et al.*, 2008). Sie wurden nach der postulierten Funktion des *n*-alkanaktivierenden Enzyms (1-Methylalkyl)succinat-Synthase als Gene *masA* bis *masG* benannt.

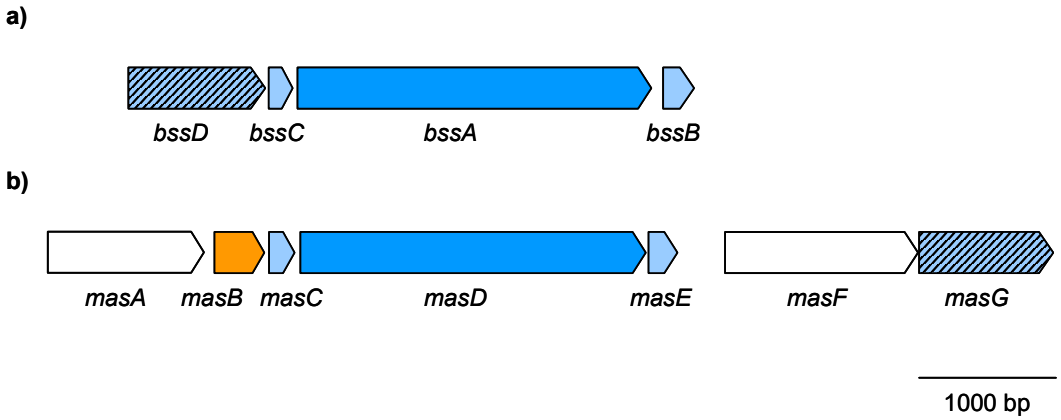

Abb. 8 Genetische Organisation der Gene für den anaeroben Kohlenwasserstoffabbau mittels Addition an Fumarat. **a)** *bss* Gene in Toluolabbauern. **b)** *mas* Gene in Stamm HxN1. Dunkelblau: Gene, die die katalytische Untereinheit des kohlenwasserstoffaktivierenden Enzyms (Bss, Mas) kodieren; hellblau: Gene, die die kleinen Untereinheiten des Enzyms kodieren; orange: Gen, das die zusätzliche vierte Untereinheit der Mas kodiert; blau-gestreift: Aktivase-kodierende Gene; weiß: zusätzliche Gene im *mas* Operon von Stamm HxN1 kodieren eine AcylCoA-Dehydrogenase (*masA*) und eine Transposase (*masF*).

Auf Proteinebene hat MasD eine Identität von 33,7% zur großen α-Untereinheit der Benzylsuccinat-Synthase (BssA) (Grundmann *et al.*, 2008). Die Sequenz weist auch die charakteristischen, konservierten Glycin- und Cysteinreste eines Glycylradikalenzyms auf. Die Genprodukte MasC und MasE wurden als die kleinen β- und γ-Untereinheiten des Enzyms charakterisiert, obwohl sie nur geringe (MasC) oder keine (MasE) Sequenzähnlichkeit zu den kleinen Untereinheiten der Benzylsuccinat-Synthase aufweisen (Grundmann *et al.*, 2008). MasC und MasE sind jedoch genauso wie BssB und BssC reich an Cysteinen und die kodierenden Gene sind im Operon vor und hinter

dem Gen für die große Untereinheit angeordnet, genauso wie im *bss* Operon (Grundmann *et al.*, 2008). Die Reinigung der (1-Methylalkyl)succinat-Synthase aus Stamm HxN1 identifizierte eine weitere Untereinheit, kodiert von *masB* (Werner, 2009). Die wenigen verfügbaren Homologe von MasB wurden alle in anaeroben *n*-Alkanabbauern gefunden (Werner, 2009). Auch MasB kennzeichnet das Vorkommen mehrerer Cysteine, seine Funktion ist jedoch, ebenso wie die der anderen kleinen Untereinheiten, ungeklärt. Die Aktivase, die für die Aktivierung der (1-Methylalkyl)succinat-Synthase notwendig ist, wird von *masG* kodiert (Grundmann *et al.*, 2008). MasG ist durch das für SAM-Radikalenzyme spezifische CxxxCxxC-Motiv charakterisiert. Anders als im *bss* Operon liegt *masG* nicht vor den Genen für das Glycylradikalenzym, sondern dahinter, separiert durch ein weiteres Gen (*masF*), welches eine Transposase kodiert (Abb. 8) (Grundmann *et al.*, 2008). Das erste Gen des Operons (*masA*) kodiert ein Protein, das Acyl-CoA-Dehydrogenasen ähnlich ist (Grundmann *et al.*, 2008). Eine Acyl-CoA-Dehydrogenase kann in den weiteren Abbau des aktivierten *n*-Alkans involviert sein (Wilkes *et al.*, 2002; Grundmann *et al.*, 2008). In dem Denitrifizierer Stamm OcN1 und den Sulfatreduzierern Stamm Pnd3 und Stamm TD3 wurden ebenfalls *mas* Gene identifiziert (Werner, 2009). In *D. alkenivorans* Stamm AK-01 wurden entsprechende *ass* Gene (für Alkylsuccinat-Synthase) annotiert (Callaghan *et al.*, 2008).

3.4 Alternative anaerobe Aktivierungsmechanismen für *n*-Alkane

Die Addition an Fumarat ist nicht die einzige Möglichkeit der anaeroben Aktivierung von *n*-Alkanen. Für *Desulfococcus oleovorans* Stamm Hxd3 und eine Anreicherungskultur wurde als Aktivierung eine Carboxylierung am C-3 Atom vorgeschlagen (So *et al.*, 2003; Callaghan *et al.*, 2006; Callaghan *et al.*, 2009). Nach Abspaltung einer C_2-Einheit wird die um ein C-Atom gegenüber dem *n*-Alkan verkürzte Fettsäure zu CO_2 oxidiert. In Stamm Hxd3 beeinflusst das verwertete *n*-Alkan die Zusammensetzung der zellulären Fettsäuren: *n*-Alkane mit einer geraden Anzahl an C-Atomen werden zu Fettsäuren mit einer ungeraden Anzahl an C-Atomen und umgekehrt abgebaut (Aeckersberg *et al.*, 1998). Dies spricht für die postulierte Carboxylierung, der Mechanismus bleibt jedoch hypothetisch, da verantwortliche Enzyme bisher nicht identifiziert wurden. Aber auch *bss/mas/ass* ähnlichen Sequenzen wurden im Genom von Stamm Hxd3 nicht annotiert (GenBank Acc.-Nr. NC 013939).

In Stamm HdN1 wurden ebenfalls weder im Genom *mas*- oder *bss*-ähnliche Sequenzen gefunden, noch wurden alkylsubstituierte Metabolite, die auf eine Addition an Fumarat hindeuten, detektiert (Zedelius *et al.*, 2011). Postuliert wird eine Dismutation von Nitrit

oder Stickstoffmonooxid zu molekularem Stickstoff und Sauerstoff. Der intramolekular gebildete Sauerstoff wird dann zur Aktivierung eines *n*-Alkans durch Alkanhydroxylasen, wie für den aeroben Abbau beschrieben, genutzt. Die Dismutation von Stickstoffmonooxid wurde auch für eine methanoxidierende Anreicherungskultur vorgeschlagen (Ettwig *et al.*, 2010). Die hierin dominierende Spezies *Methylomirabilis oxyfera* aktiviert Methan unter anaeroben Bedingungen durch eine Methan-Monooxygenase mit dem intramolekular produziertem Sauerstoff. Ähnliches wurde für den anaeroben Abbau von *n*-Decan mit Chlorat als Elektronenakzeptor in *Pseudomonas chloritidismutans* berichtet (Mehboob *et al.*, 2009). In diesem Fall wird das Chlorat zunächst zu Chlorit reduziert. Nach der Dismutation in Chlorid-Ionen und Sauerstoff wird der Sauerstoff zur Aktivierung des *n*-Alkans durch eine Oxygenase genutzt.

Die anaerobe Oxidation von Methan (AOM), katalysiert von Konsortien aus Archaeen und sulfatreduzierenden Bakterien, läuft vermutlich über reverse Methanogenese ab (Thauer, 2011). Das Schlüsselenzym der Methanogenese, die Methyl-CoM-Reduktase, katalysiert die exergone Umwandlung von Methyl-Coenzym M und Coenzym B zu Methan und dem Heterodisulfid CoM–S–S–CoB (Thauer, 1998). Ein homologes Enzym wurde aus methanotrophen Archaeen isoliert (Krüger *et al.*, 2003). Daher wurde vermutet, dass dieses Enzym in methanotrophen Archaeen die reverse Reaktion der Methanbildung, die Oxidation von Methan, katalysiert (Krüger *et al.*, 2003). In dem methanogenen Archaeon *Methanothermobacter marburgensis* wurde die Reversibilität diser Reaktion mittels eines Isotopen-Markierungs-Experimentes bestätigt (Scheller *et al.*, 2010). Dadurch wurde erstmalig gezeigt, dass die Aktivierung der starken C–H Bindung im Methan (439 kJ mol^{-1}) im Gegensatz zum aeroben Abbau von Methan mittels Methan-Monooxygenasen (Hanson & Hanson, 1996) auch ohne reaktive Sauerstoffspezies stattfinden kann. In AOM-Anreicherungskulturen wurde ebenfalls durch ein Markierungsexperiment die Reversibilität der AOM und somit vermutlich auch des methanogenen Stoffwechselweges bestätigt (Holler *et al.*, 2011). Die Kristallstruktur der Methyl-CoM-Reduktase aus methanotrophen Archaeen zeigte das Enzym in einem Komplex mit Coenzym M und Coenzym B, den gleichen Substraten, die die Methyl-CoM-Reduktase in methanogenen Archaeen zur Synthese von Methan nutzt (Shima *et al.*, 2011). Anders als bei der anaeroben Aktivierung von Alkanen und Aromaten durch Addition an Fumarat wird für die Aktivierung von Methan kein Glycylradikal benötigt. Möglicherweise ist jedoch ein Ni-Radikal darin involviert die Bindungsdissoziations-energie der C–H Bindung im Methan, die höher ist als die der Methyl- oder Methylengruppen in *n*-Alkanen, zu überwinden (Ragsdale, 2007).

4. Stamm HxN1 als Modellorgansimus für den anaeroben *n*-Alkanabbau

Eine Anreicherungskultur, die mit einem Gemisch von Grabenschlämmen des Kuhgrabens aus Bremen inokuliert worden war, verwertete unter denitrifizierenden Bedingungen in Süßwassermedium *n*-Alkane von C_5 bis C_{12} des als Kohlenstoffquelle eingesetzten Erdöls (Rabus *et al.*, 1999). Zur Isolierung der Denitrifizierer, die das *n*-Hexan in der Anreicherung abgebaut haben, wurde Süßwassermedium mit *n*-Hexan als Substrat und Nitrat als Elektronenakzeptor mit der Anreicherungskultur inokuliert. Die hieraus isolierte Reinkultur, Stamm HxN1, verwertet neben *n*-Hexan auch *n*-Heptan und *n*-Octan (Ehrenreich *et al.*, 2000). Zyklische Alkane, aromatische Kohlenwasserstoffe und Alkene werden von HxN1 nicht abgebaut (Behrends, 1999). Jedoch werden einige Alkohole, Aldehyde, Carbonsäuren und Fructose anaerob metabolisiert. Erwähnenswert ist die Verwertung der aromatischen Fettsäure Benzoat als einzige metabolisierbare aromatische Verbindung (Behrends, 1999). Aerobes Wachstum auf *n*-Alkanen, Carbonsäuren und Fructose ist ebenfalls möglich.

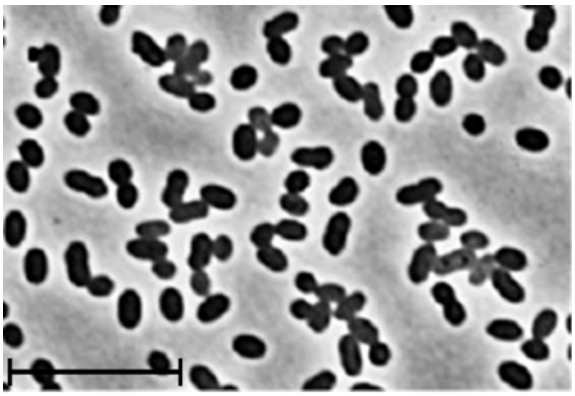

Abb. 9 Phasenkontrastmikroskopie-Aufnahme von Stamm HxN1, gewachsen auf *n*-Hexan. Balken = 10 µm. Aus: Ehrenreich *et al.* (2000).

Phylogenetisch wurde Stamm HxN1 aufgrund einer 16S rRNA-Analyse dem *Azoarcus/Thauera*-Cluster innerhalb der Betaproteobakterien zugeordnet (Behrends, 1999). Zur Gattung *Thauera* zählen u.a. Denitrifizierer, die fähig sind Toluol und andere Alkylbenzole abzubauen (Macy *et al.*, 1993; Anders *et al.*, 1995). Die Gattung *Azoarcus* umfasst zwei distinkte Untergruppen, zum einen die *Azoarcus indigens* Untergruppe, der pflanzenassoziierte, diazotrophe, aerobe Bakterien angehören und zum anderen die Untergruppe *Azoarcus evansii*, deren Mitglieder anaerob u.a. aromatische Kohlenwasserstoffe abbauen (Reinhold-Hurek & Hurek, 2006). Es wurde daher vorgeschlagen, die *A. evansii* Untergruppe als neue Gattung *Aromatoleum* zu klassifizieren, der aufgrund seines hohen Verwandtschaftsgrades zu den Mitgliedern dieser Gattung, Stamm EbN1 und Stamm PbN1, auch Stamm HxN1 zugeordnet werden soll (Wöhlbrand, 2008). Zellen des Stammes HxN1 sind unbeweglich und oval mit einer Größe von 1,0 - 1,5 µm x 1,8 - 2 µm (Abb. 9) (Ehrenreich *et al.*, 2000). Die Zellen wachsen homogen in der Flüssigkeit und adherieren im Gegensatz zu anderen Kohlenwasserstoffabbauern nicht an die kohlenwasserstoffhaltige Oberphase (Ehrenreich *et al.*, 2000). Diese Eigenschaft

und eine, verglichen mit Sulfatreduzierern, kurze Verdopplungszeit von ca. 11 Stunden auf *n*-Hexan machen Stamm HxN1 zu einem geeigneten Modellorganismus um den anaeroben *n*-Alkanabbau durch Addition an Fumarat weiter im Detail zu untersuchen.

5. Zielsetzung der vorliegenden Arbeit

Ziel dieser Arbeit war es, ein genetisches System für Stamm HxN1 zu entwickeln, das die Generierung von Mutanten dieses Stammes ermöglicht. Mithilfe dieses Systems sollte der *in vivo* Nachweis der bis dato nur postulierten Reaktion der (1-Methylalkyl)succinat-Synthase durch Deletion der kodierenden Gene erbracht werden. Anschließende physiologische Wachstumsversuche der generierten Mutanten würden dann die Effekte der Mutation auf die Fähigkeit *n*-Alkane abzubauen, aufzeigen.

Die Regulation des anaeroben *n*-Alkanabbaus ist noch unbekannt. Erste Hinweise auf die Regulation des *mas* Operons in Stamm HxN1 geben Studien zur Induktion und Inhibierung der Expression. Hierzu sollte die Anwesenheit der (1-Methylalkyl)succinat-Synthase nach Inkubation von Stamm HxN1 mit verschiedenen Kohlenwasserstoffen sowie weiteren Kohlenstoffquellen untersucht werden.

In vorangegangenen Arbeiten wurde für die (1-Methylalkyl)succinat-Synthase aus Stamm HxN1 ein Protokoll zur Reinigung entwickelt (Werner, 2009). In dieser Arbeit sollte die nach diesem Protokoll gereinigte (1-Methylalkyl)succinat-Synthase für Kristallisationsversuche eingesetzt werden. Ein Proteinkristall ermöglicht die Erstellung einer Röntgenstruktur der (1-Methylalkyl)succinat-Synthase und damit die Aufklärung der Funktion der verschiedenen Untereinheiten des Enzyms.

B Ergebnisse

Die Ergebnisse sind in Form von Manuskripten oder Berichten dargestellt.
Mein Anteil an den Manuskripten ist erläutert.

1. Manuskript

Purification of the (1-methylalkyl)succinate synthase from the Betaproteobacterium strain HxN1 revealed an unexpected fourth subunit that is conserved in all investigated fumarate dependent *n*-alkane activation enzymes

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Erstellung des Manuskriptes in Zusammenarbeit mit Olav Grundmann. Durchführung der Wachstumsversuche auf *n*-Pentan und Cyclopentan. Amplifikation der *masG* Sequenz von Stamm Pnd3 und der partiellen *masB* Sequenz von Stamm TD3 sowie Auswertung der generierten Sequenzen. Die weiteren Ergebnisse stammen aus der Promotion von Insa Schmitt, geb. Werner.

Abstract

The common mechanism for anaerobic *n*-alkane degradation is the activation by addition to fumarate yielding (1-methylalkyl)succinate in a first step. In the Betaproteobacterium strain HxN1 the tentative (1-methylalkyl)succinate synthase catalyzes this initial step in anaerobic *n*-alkane degradation. Here, we report for the first time the purification of an anaerobic *n*-alkane activating enzyme. Purification revealed the existence of four subunits of (1-methylalkyl)succinate synthase. In contrast, the functional homologous enzyme for the anaerobic degradation of toluene, benzylsuccinate synthase, consists of only three subunits. According to sequence analysis of bacteria degrading *n*-alkanes, toluene or 2-methylnaphthalene by addition to fumarate, homologues of the newly identified MasB subunit were found to be exclusively present in *n*-alkane degrading bacteria. In enzyme activity measurements the postulated catalyzed reaction of (1-methylalkyl)succinate synthase was displayed *in vitro.* The enzyme did not only activate the known growth substrates of strain HxN1, *n*-hexane, -heptane and -octane, but also *n*-pentane and cyclopentane. Whereas *n*-pentane was identified as growth substrate for strain HxN1, cyclopentane was oxidized incompletely.

Introduction

Saturated hydrocarbons (alkanes) are widespread in nature. They are major compounds of crude oil (Tissot & Welte, 1984), as well as produced by many plants and some microbes (Widdel & Rabus, 2001). The absence of functional groups or multiple C-bounds results in a chemical stability, which prevents alkanes from most common degradation mechanisms. Therefore, the degradation of alkanes requires a special activation mechanism to overcome their chemical inertness. Under aerobic conditions, oxygenases use free oxygen to introduce a hydroxyl group into the alkane (Rojo, 2009). The degradation of *n*-alkanes was also reported under anaerobic conditions, where no free oxygen is available: First isolates were obtained in the beginning of the 1990´s (overview in Widdel *et al.*, 2010). Recent publications also demonstrated an anaerobic activation of alkanes via oxygenases by using NO to build “intracellular” oxygen under anaerobic conditions (Ettwig *et al.*, 2010; Zedelius *et al.*, 2011).

A more common anaerobic activation mechanism is the radical involved addition of fumarate to the *n*-alkane, which results in a substituted succinate. Metabolites supporting this mechanism were identified in several denitrifying and sulfate-reducing bacteria (Kropp *et al.*, 2000; Rabus *et al.*, 2001; Cravo-Laureau *et al.*, 2005; Davidova *et al.*, 2005; Callaghan *et al.*, 2006). The postulated enzyme catalyzing this reaction is the (1-methylalkyl)succinate synthase (Mas) or alkylsuccinate synthase (Ass) (Callaghan *et al.*, 2008; Grundmann *et al.*, 2008). In the betaproteobacterial denitrifying strain HxN1 the proteins MasC, MasD and MasE are regarded as subunits of (1-methylalkyl)succinate synthase (Grundmann *et al.*, 2008) due to sequence similarities or characteristic features to the subunits of the well investigated benzylsuccinate synthase (Bss), the enzyme that activates toluene anaerobically by addition to fumarate (Leuthner *et al.*, 1998). Benzylsuccinate synthase, (1-methylalkyl)succinate synthase and alkylsuccinate synthase are supposed to be glycyl radical enzymes, because of conserved amino acid motifs in their large α-subunits (Leuthner *et al.*, 1998; Callaghan *et al.*, 2008; Grundmann *et al.*, 2008). Nevertheless, until now an experimental proof for the postulated catalyzed reaction of an anaerobic *n*-alkane activation enzyme is missing.

To demonstrate the predicted function of (1-methylalkyl)succinate synthase *in vitro* we purified the enzyme from strain HxN1 and measured enzyme activity for the addition of *n*-hexane to fumarate. Furthermore, purification allowed first insights into the structural composition of this enzyme, for which a configuration similar to benzylsuccinate synthase was proposed.

Material and Methods

Bacterial strains and growth conditions

Strain HxN1, OcN1, Pnd3 and TD3 are kept in the laboratory since their isolation from *n*-alkane-utilizing enrichment cultures (Rueter *et al.*, 1994; Aeckersberg *et al.*, 1998; Ehrenreich *et al.*, 2000). Growth conditions are described in detail elsewhere (Ehrenreich, 1996; Aeckersberg *et al.*, 1998; Ehrenreich *et al.*, 2000). Large scale cultivations of strain HxN1 were performed in an anaerobic 50 l fermenter. Culture mixing was achieved with a magnetic stirrer in a 28 °C water bath. In contrast to smaller cultures, nitrate was added continuously via a pump with a flow rate up to 1 mM nitrate h^{-1}. Nitrate and nitrite were measured with an ion chromatograph connected to an UV detector (Sykam, Fürstenfeldbruck, Germany) as described (Rabus & Widdel, 1995). Data analysis was performed with the Clarity HPLC software (DataApex, Praque, Czech Republic). *Escherichia coli* strain BL21 Star (DE3) (Invitrogen, Darmstadt, Germany) was cultivated in Luria Bertani medium at 37 °C. Kanamycin was added to a final concentration of 45 µg ml^{-1}.

Preparation of crude extract

Before harvesting, nitrate addition was stopped for at least 3 h to allow the culture to reduce remaining nitrate and nitrite. Cells were harvested anaerobically using a Heraeus Contifuge Stratos (Heraeus, Newport Pagnell, UK) with a flow through of 200 ml min^{-1} at 4 °C and 17000 rpm. During harvesting a pressure of 0.1 bar N_2 was applied to the culture to avoid oxygen input. Following centrifugation, the rotor was immediately transferred into an anoxic chamber. Cells were suspended in an equal volume of 100 mM Tris-HCl, pH 8.0, supplemented with 5 mM fumarate, 8 mM DTT, 4 mM sodium dithionite, 4 mM titanium (III) citrate and 20% (v/v) glycerol. The suspension was then transferred into a French press cell (SLM Aminco Spectronic Instruments, Rochester, USA), where cells were disrupted with a pressure of 1000 psig (70 bar) outside the anoxic chamber. Cell extract was transferred directly via a needle into an anoxic butyl stoppered bottle. 0.25 mg ml^{-1} DNaseA and small glass bullets (0.5 mm) were added for DNA disruption. The extract was agitated for 30 min at 28 °C and centrifuged anaerobically (20000 x *g*, 25 min) afterwards. The supernatant, in the following termed as crude extract, was then used for further investigations.

Purification of (1-methylalkyl)succinate synthase

Purification was performed in an anoxic chamber at 7 °C using an ÄKTA explorer FPLC system (GE Healthcare, Munich, Germany). Buffers were sterile filtered and degassed

before use and supplemented with 5 mM fumarate for protein stabilization and 0.5 mM sodium dithionite as reductant. Crude extract (12 ml) was loaded on five 5 ml HiTrap ANX FF columns (GE Healthcare) connected in series and equilibrated with 100 mM Tris-HCl, pH 8.0. The column was washed with 150 mM NaCl and a flow rate of 3 ml min^{-1}. Elution of (1-methylalkyl)succinate synthase was performed with 250 mM NaCl. To minimize the volume for the next column, the eluted fraction was concentrated by ultrafiltration with a cellulose membrane (Amicon Ultra-15 100K, Millipore, Billerica, USA). The concentrated ANX chromatography fraction was loaded on a sephadex G 25 column (GE Healthcare) equilibrated with 50 mM NaH_2PO_4/Na_2HPO_4, pH 8.0 at a flow rate of 3 ml min^{-1} for buffer exchange. The eluted protein fraction was loaded onto a 2 ml hydroxyapatite column (CHT2-1, Biorad, Munich, Germany). After washing with 50 mM NaH_2PO_4/Na_2HPO_4, pH 8.0 and a flow rate of 3.5 ml min^{-1}, the concentration was increased to 102 mM to elute (1-methylalkyl)succinate synthase. The presence of (1-methylalkyl)succinate synthase after each chromatography step was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot and enzymatic assay.

Molecular weight determination

A HiLoad 16/60 Superdex 200 prepgrade column (GE Healthcare) was calibrated with standard proteins from the High and Low Molecular Weight Gel Filtration Calibration Kit (GE Healthcare). The column was equilibrated with 100 mM Tris-HCl, pH 8.0 with 5 mM fumarate and a flow rate of 0.5 ml min^{-1}. The elution volumes of the standard proteins were used to calculate the partition coefficient (K_{AV}) of each protein and to generate a calibration line. To determine the molecular weight of (1-methylalkyl)succinate synthase 0.5 ml of the concentrated fraction after hydroxyapatite chromatography were loaded onto the HiLoad 16/60 Superdex 200 prepgrade column. Buffer and flow rate were identical to those of the calibration. With the resulting K_{AV} value the molecular weight of (1-methylalkyl)succinate synthase was determined from the calibration line.

SDS-PAGE and Western blotting

SDS-PAGE was performed on 12% (w/v) polyacrylamide gels as described (Laemmli, 1970). As molecular size marker the PageRuler Prestained Protein Ladder from Fermentas (St. Leon-Rot, Germany) was used. Proteins of interest were cut from the gel and analyzed by peptide mass fingerprinting (Toplab, Martinsried, Germany). Proteins were stained with Coomassie R250 and fixed with glacial acetic acid (0.25% (v/v) Coomassie R250, 40% (v/v) ethanol, 10% (v/v) glacial acetic acid).

For immunoblotting experiments, the proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane (Optitran BA-S 83 reinforced NC 0,2 µm, Whatman/GE Healthcare) by electroblotting. To generate antibodies against the α-, β- and γ-subunit of the (1-methylalkyl)succinate synthase, the genes *masC, masD* and *masE* of strain HxN1 were each cloned without start codon in frame into the expression vector pET-42a(+) (Novagen, Darmstadt, Germany) fused with the N-terminus to the GST-tag. Cloning was performed according to standard techniques using DNA modifying enzymes from Fermentas. Oligonucleotide primers and plasmids are depicted in table 1 and 2. The fusion proteins were expressed heterologously in *E. coli* BL21 Star (DE3) induced with 1 mM IPTG and purified via the GST-tag on a GSTrap HP column according to the instructions (GE Healthcare). The purified proteins were used to immunize rabbits for the production of antibodies (Pineda Antikörperservice, Berlin, Germany), which were applied in a non-purified form as immune serum in Western blot analysis. As secondary antibody goat anti-rabbit IgG-AP was used (Santa Cruz Biotechnology, Santa Cruz, USA). Hybridization signals were detected with NBT/BCIP ready-to-use-tablets (Roche, Darmstadt, Germany).

Table 1 Oligonucleotide primers for cloning of *mas* genes into the expression vector pET-42a(+). Restriction sites are underlined.

Primer	Target gene	Sequence (5´→ 3´)	Product length [bp]
*masC_Bam*HI_f	*masC*	CGGATCCTCTACATGCAAAGAGTGTC	183
*masC_Hind*III_r		GCCAAGCTTCTAATGCGCTTTTGCTGTTC	
*masD_Bam*HI_f	*masD*	GCGGATCCACTGCAACTTCAACACTATCCA	2520
*masD_Xho*I_r		CCGCTCGAGTTAGCCTAGCCCCTGGACGGT	
*masE_Hind*III_f	*masE*	CAGAAGCTTCCAAATGCACAGAATGTGGCCA	213
*masE_Xho*I_r		AGCTCGAGCTAACCTTCGGCCAAGTTTT	

Table 2 Plasmids for expression of Mas-GST fusion proteins.

Plasmid	Genotype and characteristics	Reference or source
pET-42a(+)	Km^R, GST-tag, His-tag, S-tag	Novagen
pET-42_*masC*	Km^R, GST-tag, His-tag, S-tag, *masC*	this study
pET-42_*masD*	Km^R, GST-tag, His-tag, S-tag, *masD*	this study
pET-42_*masE*	Km^R, GST-tag, His-tag, S-tag, *masE*	this study

(1-Methylalkyl)succinate synthase activity assay

The assay is based on the identification of (1-methylpentyl)succinate by gas chromatography coupled to mass spectrometry (GC-MS). Assay preparation and incubation was performed under anoxic conditions. The sample volume was 0.5 ml of protein fraction after each chromatography step respectively crude extract of strain HxN1. For stabilization of (1-methylalkyl)succinate synthase, the assay contained 50 mg ml^{-1} bovine serum albumin (BSA). As substrates, 6% (v/v) *n*-hexane and 40 mM fumarate were added. Alternative substrates (*n*-alkanes from C_5 to C_{12}, cyclopentane, cyclohexane and toluene) to determine the substrate range of (1-methylalkyl)succinate synthase in crude extract were added to a final concentration of 2% (v/v). In case of *n*-butane, the assay was flushed with the gas. The activity assays were incubated with agitation at 28 °C for 16 h and then stopped by adding 20 µl 50% sulfuric acid. Sebacic acid buffered in 200 mM Tris-HCl, pH 8.0 in a final concentration of 100 µM served as internal standard. Methylation of free carboxylic acid groups was performed by adding 100 µl 0.25 M trimethylsulfoniumhydroxide (TMSH) and incubation for 20 min at 99 °C in a water bath. After cooling, the ester of the (1-methylpentyl)succinate was extracted with *n*-hexane, concentrated to 100 µl and then analyzed by GC-MS on a type 5890 gas chromatograph (Hewlett Packard, Waldbronn, Germany) connected to a type 95SQ mass spectrometer (Finnigan MAT/Thermoquest, Egelsbach, Germany). For separation, 1 µl of sample was loaded splitless by means of an autosampler onto an OPTIMA 5MS capillary column (30 m long, 0.25 µm film thickness, Macherey-Nagel Düren, Germany). The temperature of the injector was set to 250 °C. Helium served as carrier gas. The GC program was as follows: The initial column temperature was 130 °C with a hold time of 3 min. At the first chute, the column temperature was set to 175° C with a heating rate of 4 °C min^{-1} and a hold time of 6 sec. At the second chute, the column temperature was set to 280 °C with a heating rate of 30 °C min^{-1} and a hold time of 1 min. The mass spectrometer was operated in electron impact mode. Identification of (1-methylpentyl)succinate was achieved by comparing spectra and retention time with those previously published (Rabus *et al.*, 2001).

Amplification of *mas* genes in the bacterial strains OcN1, Pnd3 and TD3

Chromosomal DNA was isolated with the Qiagen chromosomal DNA Kit according to the instructions (Qiagen, Hilden, Germany). For strain OcN1, a fosmid library was constructed with the CopyControl Fosmid Library Production Kit (Epicentre, Madison, USA). Obtained fosmid clones were transferred from solid medium onto nylon membranes (Hybond-N+, GE Healthcare). For the following steps the membranes were

in each case incubated for 5 min on soaked Whatman paper. Cells were lysed with 10% SDS. Denaturation of the DNA was performed with 1.5 M NaCl, 0.5 M NaOH, following neutralization with 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.4. Afterwards, the membranes were incubated with 2x SSPE (20 mM NaH_2PO_4, 0.3 M NaCl, 2 mM EDTA). The DNA was immobilized on the membrane by UV irradiation at 254 nm for 2 min at 1.5 J cm^{-2} (Biolink DNA Crosslinker, Biometra, Göttingen, Germany). A specific probe was obtained by polymerase chain reaction (PCR) with degenerated primers (table 3) on chromosomal DNA of strain OcN1. The probe was labeled with [α-^{33}P]-dATP using the HexaLabel DNA Labeling Kit (Fermentas). Hybridization was performed over night at 65 °C in Church buffer (1% (w/v) BSA, 7% (w/v) SDS, 1 mM EDTA, 250 mM $NaHPO_4$, pH 7.2). Radioactive signals were detected with a Storage Phosphor Screen (GE, Healthcare) and analyzed on a phosphoimager (Typhoon, GE Healthcare). One out of 56 positive clones was sequenced by GATC (Konstanz, Germany). For the strains Pnd3 and TD3, *mas* sequences were amplified with degenerated primers based on the *assA1* and *assA2* sequences of strain AK-01 (table 3). The obtained PCR products were sequenced with the Big Dye terminator cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) on a 3130XL Genetic Analyzer (Applied Biosystems). Sequence assembly was performed with the Lasergene software (DNASTAR, Konstanz, Germany).

Table 3 Oligonucleotide primers for amplification of (partial) *mas* genes in the strains OcN1, Pnd3, and TD3.

Primer	Target strain	Target gene	Sequence (5´→ 3´)	Product length [bp]
*masD*_OcN1_f	OcN1	*masD*	TWYGASGAKAAGAAGTACAC	~ 500
*masD*_OcN1_r			MMGTTGAACTGNAYRTGRTC	
*masD*_Pnd3_f	Pnd3	*masD*	AATGGTGGTGGRTSGCKGAA	2384
*masD*_Pnd3_r			AAAGTGKGCGCTGTADCCVG	
*masGD*_Pnd3_f	Pnd3	*masG* to *masD*	ATGGCCAATGCCTGCTTGAT	3246
*masGD*_Pnd3_r			AGGGCGTATTCCACCATCTT	
*masDE*_Pnd3_f	Pnd3	*masD* to *masE*	CTCGGCCGTTTTGAAATCCT	1127
*masDE*_Pnd3_r			GATTTCCAATCCGTGTTCCG	
*masB*_TD3_f	TD3	*masB*	GTBCCMGAGMAGGCRTGYGG	224
*masB*_TD3_r			TCGTKRCCRTCSGTATCRAT	
*masD*_TD3_f	TD3	*masD*	AATGGTGGTGGRTSGCKGAA	2379
*masD*_TD3_r			AAAGTGKGCGCTGTADCCVG	

Results and Discussion

Development of an *in vitro* activity assay for (1-methylalkyl)succinate synthase

The activation mechanism of (1-methylalkyl)succinate synthase was postulated based on metabolites identified in cells anaerobically grown with *n*-alkanes (Kropp *et al.*, 2000; Rabus *et al.*, 2001; Cravo-Laureau *et al.*, 2005; Davidova *et al.*, 2005; Callaghan *et al.*, 2006). An *in vitro* assay was developed to confirm the proposed *n*-alkane activation and the involvement of the genetically identified (1-methylalkyl)succinate synthase (Callaghan *et al.*, 2008; Grundmann *et al.*, 2008). The assay detects the activation product (1-methylalkyl)succinate by GC-MS analysis (Fig. 1). To verify the efficiency of the methylation, the C_{10} dicarboxylic acid sebacic acid served as internal standard. In addition, chemically synthesized (1-methylpentyl)succinate, kindly provided by Dr. Dauelsberg (University of Applied Science, Emden), was used to calibrate the GC-MS detection. Obviously, most of the (1-methylpentyl)succinate was incompletely methylated and eluted after 10.3 min (Fig. 1). Minor amounts were fully methylated, which eluted after 7.9 min.

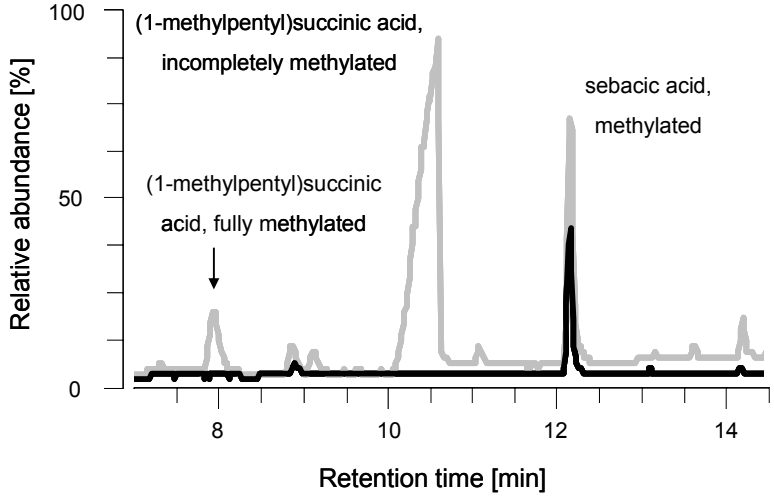

Fig. 1 Separation of methylated products of (1-methylalkyl)succinate synthase activity assay by gas chromatography. Grey: fully and incompletely methylated (1-methylpentyl)succinic acid after incubation with *n*-hexane; black: control without *n*-hexane. Sebacic acid was added as internal standard.

Typical *m/z* ions of single and double methylated (1-methylpentyl)succinate are depicted in Fig. 2. Most characteristic for (1-methylpentyl)succinic acid is the *m/z* 114 ion. Further abundant ions of the fully methylated form are the *m/z* 146, 157 and 199 ions, whereas in the incompletely methylated form the *m/z* 100, 143 and 185 ions are present. Attempts

to optimize the methylation efficiency of TMSH remained unsuccessful, although sebacic acid, as control in each sample, was always fully methylated. Anyhow, TMSH was taken as methylation reagent because of its advantages in handling and the possibility to specifically detect the partially methylated form.

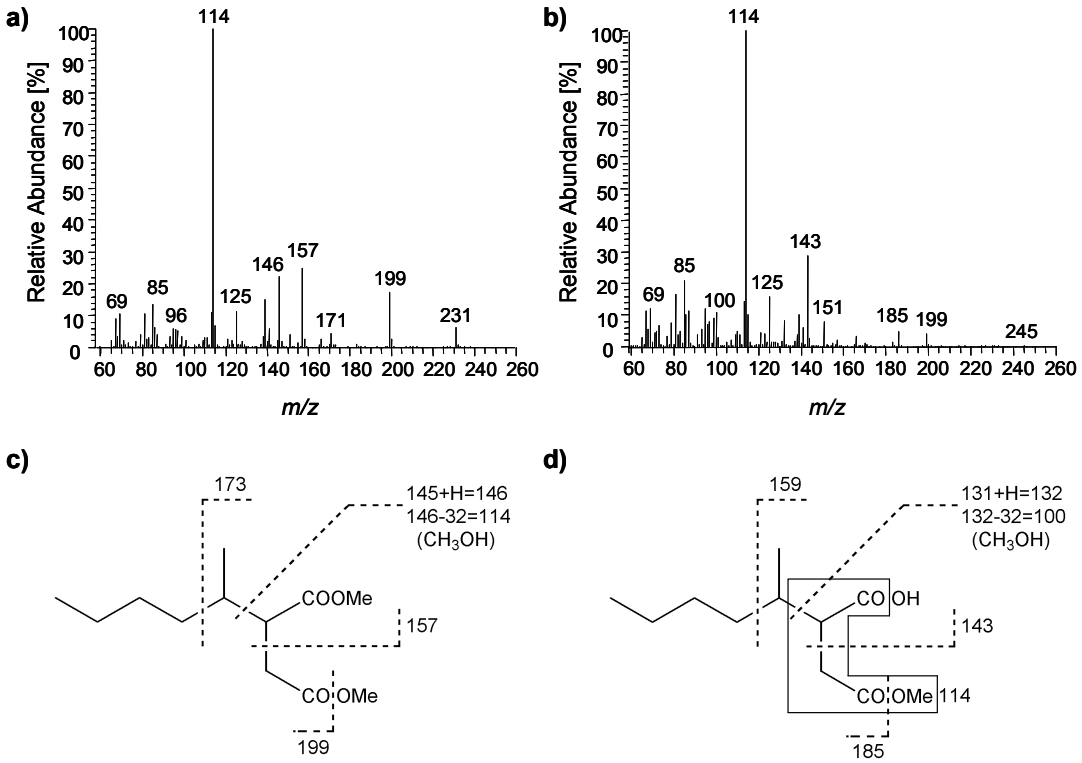

Fig. 2 Mass spectra of (1-methylpentyl)succinic acid. **a)** Characteristic peaks of the fully methylated (1-methylpentyl)succinic acid. **b)** Characteristic peaks of the incompletely methylated (1-methylpentyl)succinic acid. **c)** Fragment pattern of the fully methylated form. **d)** Fragment pattern of the incompletely methylated form.

The activity assay was optimized regarding temperature and time. In crude extracts of strain HxN1, the highest activity of (1-methylalkyl)succinate synthase was measured between 12 and 42 °C with a maximum at around 32 °C (Fig. 3a). This temperature was expected for an enzyme of a mesophilic organism with an optimal growth rate at about 28°C. As determined by an Arrhenius plot, the activation energy is 100 kJ mol $^{-1}$ (data not shown). Measurement of enzyme activity over time resulted in a stable production rate of (1-methylpentyl)succinate within the first five hours (Fig. 3b), independent of the starting activity (data not shown). After eight hours, about 95% of the product amount, which is produced within 24 hours of incubation, has already formed.

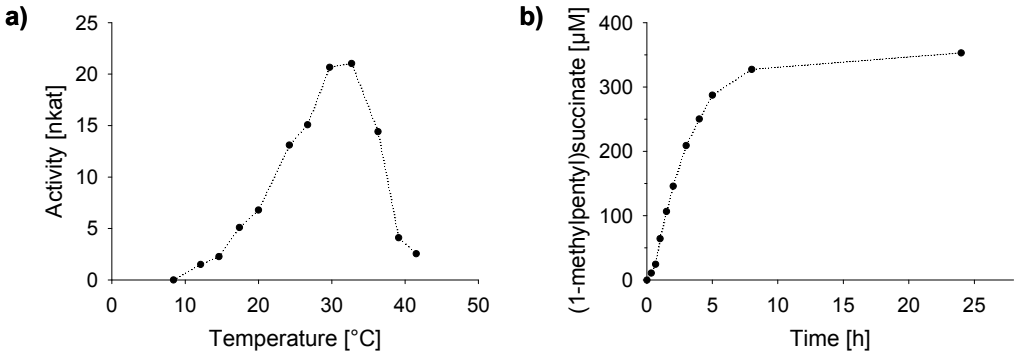

Fig. 3 Dependence of (1-methylalkyl)succinate synthase activity from temperature and time. **a)** Activity of (1-methylalkyl)succinate synthase plotted against temperature. **b)** Activity of (1-methylalkyl)succinate synthase expressed in the amount of formed product as a function of time.

Effect of nitrate and nitrite on *in vitro* enzyme activity

Usually, cells are most active in the logarithmic phase and thus, proteins are purified from log phase cells. However, (1-methylalkyl)succinate synthase was inactive in crude extracts of strain HxN1, when harvested in its log phase. In the beginning, the cells were harvested when the cultures had a doubling time of around 9 hours, which is on the level of optimal growth for strain HxN1 in batch culture (11 hours) as described by Ehrenreich *et al*. (2000). Additionally, nitrate and nitrite measurements were performed in the supernatant of the culture by high performance liquid chromatography. Within the log phase (OD_{600} 0.6-1.5), nitrate was added continuously up to a rate of 1 mM h^{-1}, but neither nitrate nor nitrite were detectable (detection limit is lower than 5 µM), supporting the hypothesis of a high metabolic activity of strain HxN1 at this state. However, no enzymatic activity was measured. The lack of enzymatic activity suggests inhibition or inactivation of (1-methylalkyl)succinate synthase during cell harvesting or disruption. Thus, harvesting conditions were modified to receive active (1-methylalkyl)succinate synthase. Enzyme activity was identified in crude extracts from cultures, which were starved for nitrate at least three hours before harvesting, but the obtained activity was with up to 0.45 fkat mg^{-1} protein more than 30 000 fold lower than expected from the nitrate consumption of the culture (14 nkat mg^{-1} protein). The very low measured *in vitro* activity is comparable to the low *in vitro* activities reported for benzylsuccinate synthases in crude extract of *T. aromatica* (0.3 - 3 fkat mg^{-1} protein) (Leuthner *et al.*, 1998) and in crude extract of *Azoarcus* sp. Strain T (98 fkat mg^{-1} protein) (Beller & Spormann, 1999), which are, compared to their growth rates, 200 - 1000 fold lower.

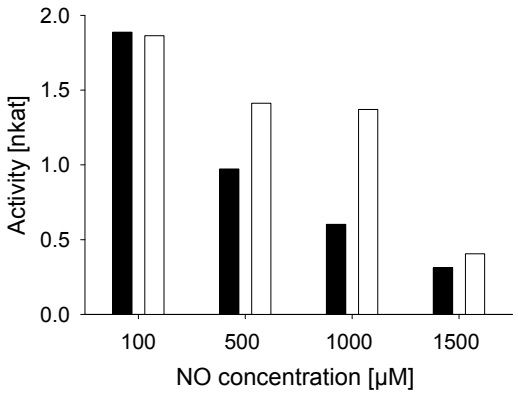

Fig. 4 Inhibition of (1-methylalkyl)succinate synthase by nitric oxide. Black: enzyme assays supplemented with NO; white: controls without NO, diluted with the equal amount of water.

The observation that nitrate starvation before cell harvesting results in measurable enzyme activity suggests an influence of nitrate reduction products to enzyme inhibition. For example, S-nitrosylation of proteins by nitric oxide is described as a known regulatory effect on protein activity (Broillet, 1999). Native benzylsuccinate synthase in cell extract of *T. aromatica* was shown to be completely inhibited by a 280 µM nitric oxide solution (Feil, 2006). Inhibition studies with nitric oxide on crude extract of strain HxN1 are shown in Fig. 4. The nitric oxide source was a fresh prepared water solution and as control only water was used. Nitric oxide concentrations of 500 to 1000 µM showed a clear inhibition of the (1-methylalkyl)succinate synthase activity. At higher concentrations (1500 µM) the effect interferes with the inhibition of the enzyme by dilution. One explanation for the higher nitric oxide resistance in comparison to the purified benzylsuccinate synthase is the high protein concentration (up to 50 mg ml^{-1}) in the crude extract, which traps the nitric oxide before reacting with (1-methylalkyl)succinate synthase. As consequence, log phase grown cultures were generally harvested after nitrate starvation for three to four hours to allow metabolization of possible inhibitory substances of the nitrate reduction pathway.

In vitro substrate range of (1-methylalkyl)succinate synthase

In growth experiments, strain HxN1 was able to grow with *n*-hexane, -heptane and -octane as sole carbon and energy source under denitrifying conditions (Ehrenreich *et al.*, 2000). Activity tests of crude extract from cells anaerobically grown with *n*-hexane identified additional to these three alkanes the succinated products of *n*-pentane and cyclopentane (Fig. 5), whereas *n*-butane, -nonane, -decane, -undecane, -dodecane, cyclohexane and toluene showed no corresponding activation products. However, the amount of the succinated products from *n*-pentane and cyclopentane are strongly reduced to 5% respectively 2.5% in comparison to *n*-hexane (Fig. 5).

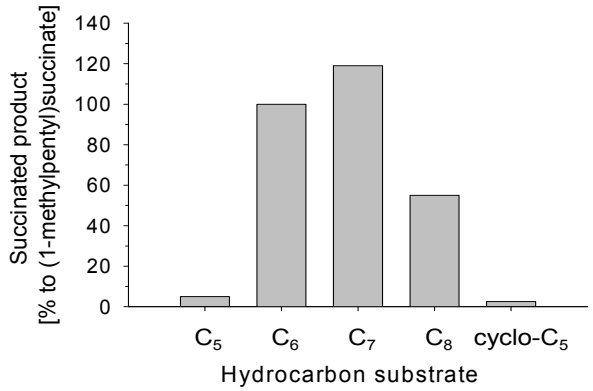

Fig. 5 Substrate range of (1-methylalkyl)succinate synthase of strain HxN1 in the activity assay. *n*-Pentane, -hexane, -heptane, -octane and cyclo-pentane were activated by addition to fumarate to a succinated product. The amount of formed methylpentylsuccinate (55 µM) was set as reference to 100%.

To investigate, whether activation of *n*-pentane and cyclopentane results in complete oxidation, growth of strain HxN1 on *n*-pentane respectively cyclopentane was tested. Strain HxN1 was able to grow with *n*-pentane but not with cyclopentane (Fig. 6). Growth with *n*-pentane is characterized by a longer lag phase and a doubling time, which is 3.5 to 4 fold lower than growth with *n*-hexane. Obviously, *n*-pentane is oxidized completely, but growth is retarded, compared to *n*-hexane, because (1-methylalkyl)succinate synthase has a lower affinity towards *n*-pentane.

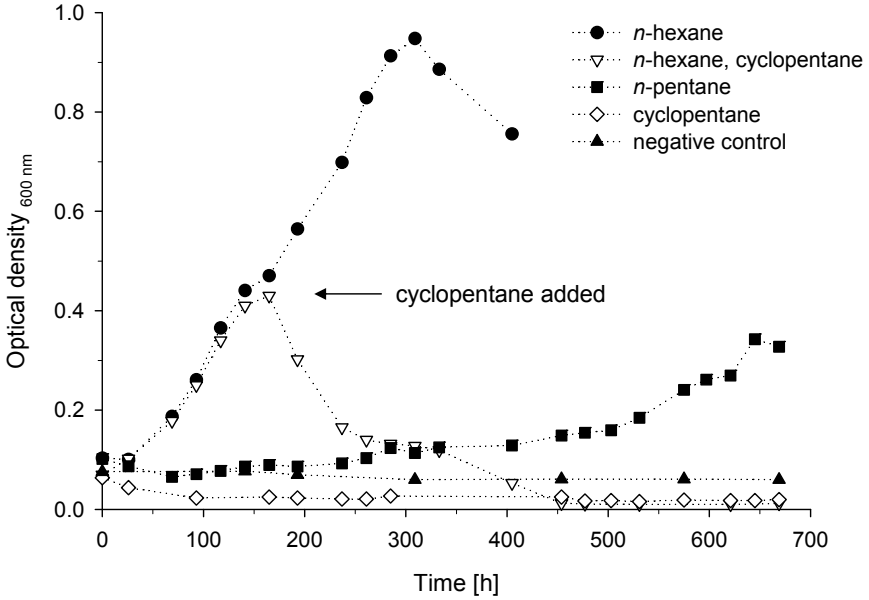

Fig. 6 Time course of growth of strain HxN1 with *n*-hexane, *n*-pentane and cyclopentane. *n*-Hexane is consumed after a lag phase of around 72 hours, but is inhibited by addition of cyclopentane (indicated with an arrow). Strain HxN1 is unable to grow with cyclopentane. Growth with *n*-pentane is characterized by a considerably longer lag phase of around ten days and a lower doubling time compared to grow with *n*-hexane.

Probably, activation of cyclopentane results in a death end product, which is not further oxidized to CO_2, due to a missing enzyme for cleavage of the cyclic structure. Interestingly, growth with *n*-hexane was inhibited by addition of cyclopentane after seven days (white dots in Fig. 6), whereas in a control experiment cyclopentane did not inhibit growth of strain HxN1 on caproate (data not shown). This control experiment excluded toxicity of cyclopentane onto growth in general. It rather seems that degradation of *n*-hexane is prohibited by a metabolite of cyclopentane, which blocks the active centre of an enzyme needed for *n*-hexane degradation. This might even be the (1-methylalkyl)succinate synthase itself. Thus, the substrate range of the (1-methylalkyl)succinate synthase is not the only limiting factor for growth of strain HxN1 with *n*-alkanes.

Purification of (1-methylalkyl)succinate synthase from strain HxN1

In contrast to the benzylsuccinate synthases for anaerobic toluene activation, anaerobic alkane activating proteins have not been purified so far. Here, a purification protocol for the (1-methylalkyl)succinate synthase from strain HxN1 is described. Exemplarily, a cell extract with a specific activity of 0.087 fkat mg^{-1} protein was applied to an ANX column. The eluted protein fraction showed no (1-methylalkyl)succinate synthase activity. After buffer exchange from 250 mM NaCl, 100 mM Tris-HCl, pH 8.0 to 50 mM NaH_2PO_4/Na_2HPO_4, pH 8.0 on a desalting column, the enzyme activity was recovered to 0.065 fkat mg^{-1} protein (Table 4). For further purification, the protein fraction was loaded onto a hydroxyapatite column, resulting in a specific activity of 0.03 fkat mg^{-1} protein in the eluted protein fraction. After purification 99.5% of the initial activity was lost. The obtained data for purification of the (1-methylalkyl)succinate synthase are comparable to the benzylsuccinate synthase from *T. aromatica* (Leuthner *et al.*, 1998) and from *Azoarcus* sp. Strain T (Beller & Spormann, 1999), where a loss of 98–99% respectively 99.3% was reported.

Table 4 Purification of (1-methylalkyl)succinate synthase from strain HxN1.

Purification step	Volume [ml]	Protein [mg]	Activity [fkat]	Specific activity [fkat mg^{-1} protein]	Yield [%]
Crude extract	12	533	46	0.086	100
ANX (+G25)	24	80	5.2	0.065	11.2
Hydroxyapatite	5	7.5	0.23	0.03	0.5

SDS-PAGE analysis of cell extract and ANX as well as hydroxyapatite chromatography fraction represents purification of a prominent band with a size of ~ 94 kDa (Fig. 7a). The

size corresponds well to the proposed MasD protein as α-subunit of (1-methylalkyl)succinate synthase (Grundmann *et al.*, 2008). This assumption was confirmed by Western blotting with immune serum against the α-subunit and peptide mass fingerprint analysis, which clearly identified the protein band as MasD (data not shown). During purification of the benzylsuccinate synthase from *T. aromatica* half the amount of the α-subunit was cleaved oxygenolytically, resulting in a truncated α´-subunit with a size of ~ 90 kDa (Leuthner *et al.*, 1998). Obviously, only one of the two catalytic subunits of the holoenzyme is present in its active state (Knappe & Sawers, 1990; Leuthner *et al.*, 1998). A truncated α´-subunit of the (1-methylalkyl)succinate synthase was, however, not observed.

The small β- and γ-subunits, which are proposed to be encoded by the genes *masE* (β) and *masC* (γ) (Grundmann *et al.*, 2008) were not clearly separated by SDS-PAGE (Fig. 7a) neither by tricine-SDS-PAGE (data not shown) due to their small size and low difference in mass (β-subunit: 8 kDa; γ-subunit: 7 kDa). Their presence in the purified fractions was hence displayed by Western blot using immune serum against the β- and γ-subunit (Fig. 7b, c). Signals were visible in cell extract of HxN1 cells grown with *n*-hexane as well as in ANX and hydroxyapatite fractions. The occurrence of a second band in the reaction of the immune serum against the γ-subunit with crude extract probably represents a cross-reaction with another protein (Fig. 7c).

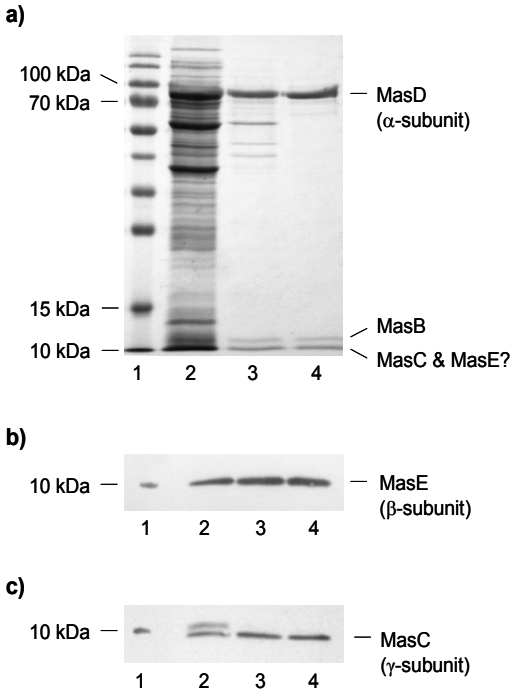

Fig. 7 Purification of (1-methylalkyl)succinate synthase from strain HxN1. **a)** SDS-PAGE of cell extract of HxN1 grown on *n*-hexane and fractions obtained during purification of (1-methylalkyl)succinate synthase. Lane 1: pre-stained protein ladder; lane 2: cell extract; lane 3: ANX fraction; lane 4: hydroxyapatite fraction. The subunits are labeled with MasD and MasB. The subunits MasC and MasE are not clearly separated since they run with the front. **b, c)** Western Blot for identification of MasE (**b**) and MasC (**c**) in extract of HxN1 cells grown with *n*-hexane and purification fractions containing (1-methylalkyl)succinate synthase. Lane 1: pre-stained protein ladder; lane 2: cell extract; lane 3: ANX fraction; lane 4: hydroxyapatite fraction.

(1-methylalkyl)succinate synthases contain an additional subunit compared to anaerobic aromatic hydrocarbon activating enzymes

In addition to MasD, a fourth protein with a size of ~ 13 kDa was displayed in SDS-PAGE analysis of purification steps (Fig. 7a). The close association of another protein to (1-methylalkyl)succinate synthase from strain HxN1 after its purification is a strong evidence that this protein represents an additional subunit of (1-methylalkyl)succinate synthase. Determination of the molecular weight by gel filtration chromatography revealed a mass of 240 kDa ± 10% for (1-methylalkyl)succinate synthase (data not shown). This is in between the masses so far determined for benzylsuccinate synthases. For *T. aromatica* a mass of 220 ± 20 kDa (Leuthner *et al.*, 1998) and for *Azoarcus* sp. Strain T a mass of 260 kDa is reported (Beller & Spormann, 1999). Based on the native molecular mass and the sizes of the three subunits an $\alpha_2\beta_2\gamma_2$ composition was proposed for benzylsuccinate synthase (Leuthner *et al.*, 1998). The mass of (1-methylalkyl)succinate synthase also fits in this holoenzyme composition. However, an $\alpha_2\beta_2\gamma_2\delta_2$ composition, assuming the existence of four different subunits, is in accordance with the molecular mass of 240 kDa ± 10% determined for (1-methylalkyl)succinate synthase of strain HxN1 as well.

The 13 kDa protein was identified as MasB by peptide mass fingerprint analysis. A function of MasB had not been proposed before. The encoding gene is localized within the *mas* operon upstream of the genes encoding the subunits of (1-methylalkyl)succinate synthase (Grundmann *et al.*, 2008). Despite two similar genes in the two *ass* operons of *Desulfatibacillum alkenivorans* strain AK-01 related sequences are unknown (Callaghan *et al.*, 2008). The MasB homologues AssB1 and AssB2 of strain AK-01 were annotated as β-subunits of the enzyme (Callaghan *et al.*, 2012). In comparison with the β-subunits of benzylsuccinate synthase (BssB), AssB1 and AssB2 show a similar distribution of cysteines, which are supposed to arrange a Fe-S cluster in benzylsuccinate synthases (Callaghan *et al.*, 2012; Hilberg *et al.*, 2012). Nevertheless, we stick to MasE as β-subunit because its size and the orientation of the encoding gene are consistent with the β-subunit of benzylsuccinate synthase (Grundmann *et al.*, 2008). The size of MasE (8 kDa) fits much better with the sizes of the β-subunits from the toluene and 2-methlynaphthalene activating enzymes (9 kDa) than MasB and AssB with a size of 13 kDa. In *bss* operons, the β-subunit encoding gene is located downstream of the α-subunit encoding gene. The same pattern is also present in the *mas* operon of strain HxN1 and the *nms* operon of 2-methylnaphthalene degrading strains (Fig. 8) (Selesi *et al.*, 2010). Thus, the gene downstream of *masD* was determined to code for the β-subunit in strain HxN1 (Grundmann *et al.*, 2008). Additionally, MasE contains four

cysteines as well. An alignment with protein sequences of BssB, MasB, MasE, AssB and potential AssE subunits did not elucidate a phylogenetic relationship of BssB to MasB/AssB or to MasE/AssE, because identities were only between 7% and 14.6% (data not shown). Thus, it remains unclear if MasB or MasE should be regarded as homologue of BssB.

So far, full sequence data for potential *n*-alkane activating enzymes were only available for the strains HxN1 and AK-01. To expand the number of sequence data, we constructed a fosmid library of strain OcN1, a nitrate reducer, which degrades *n*-alkanes with a chain length from C_8 to C_{12} (Ehrenreich *et al.*, 2000). By usage of a degenerated *masD* probe, a *mas* operon was identified in fosmid clones of strain OcN1. The assembly of the genes was similar to the *mas* operon of strain HxN1 (Fig. 8) and the gene product of one ORF showed 74% sequence identity to MasB of strain HxN1.

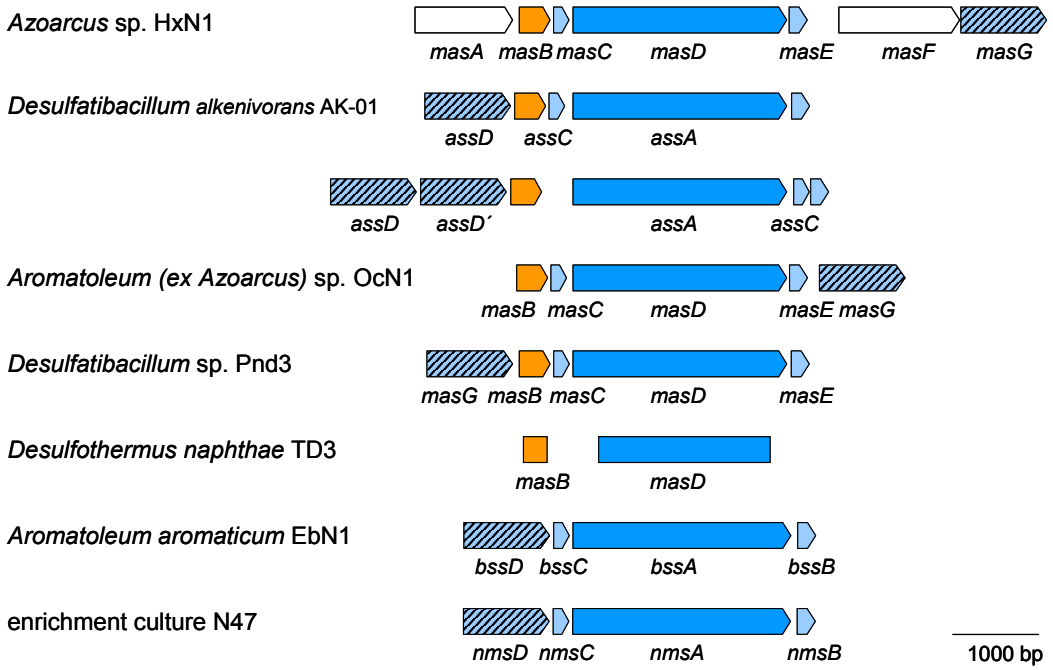

Fig. 8 Genes potentially involved in the anaerobic *n*-alkane activation in denitrifying (HxN1, OcN1) and sulfate-reducing bacteria (AK-01, Pnd3, TD3) compared to the genes of strain EbN1 as example for anaerobic toluene activation and the enrichment culture N47 as example for 2-methylnaphthalene activation. Orange: genes possibly encoding a fourth subunit of the *n*-alkane activating enzyme (*masB*); dark blue: genes encoding the catalytic subunit (*masD*, *assA*, *bssA, nmsA*); light blue: small subunits (*masC*, *assC*, *bssC, nmsC* and *masE*, *bssB, nmsB*); blue-striped: genes encoding the protein-activating SAM-enzyme (*masG*, *assD*, *bssD, nmsD*).

By PCR with degenerated primers, a full sequence *masB* homologue was also identified in the sulfate-reducing strain Pnd3 and a partial sequence was obtained for the

thermophilic sulfate-reducing strain TD3 (Fig. 8) (Rueter *et al.*, 1994; Aeckersberg *et al.*, 1998). The gene product of strain Pnd3 was 73% and of the partial MasB sequence of strain TD3 was 50% identical to the MasB protein of strain HxN1. In strain Pnd3 genes encoding the α-, β- and γ-subunit of the alkane activating enzyme and a gene for its activating enzyme were identified by PCR, revealing a similar organization of the genes as in the *ass1* operon of strain AK-01 (Fig. 8). An incomplete sequence of *masD* was amplified in strain TD3 with degenerated primers as well, while amplification of *masC* and *masE* sequences remained unsuccessful. Therefore, the position of the *masB* and *masD* genes to each other could not be resolved for strain TD3. A gene similar to *masB* is absent in all known *bss*/*tut* operons of anaerobic toluene degrading bacteria (Fig. 8).

Conclusion

Purification of (1-methylalkyl)succinate synthase from strain HxN1 revealed a new fourth subunit of this enzyme. Homologues of this subunit were identified in all other investigated alkane degrading bacteria, which activate *n*-alkanes by addition to fumarate, suggesting that (1-methylalkyl)succinate synthases are the first known glycyl radical enzymes with an $\alpha_2\beta_2\gamma_2\delta_2$ composition. The existence of a fourth subunit distinguishes (1-methylalkyl)succinate synthases from benzylsuccinate synthases. We conclude that MasB is needed specifically for the activation of *n*-alkanes by addition to fumarate, independently from the electron acceptor and the length of *n*-alkanes which are degraded.

Acknowledgement

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References

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

Beller, H.R. & Spormann, A.M. (1999) Substrate range of benzylsuccinate synthase from *Azoarcus* sp. strain T. *FEMS Microbiol. Lett.* **178**: 147–153.

Broillet, M.C. (1999) S-nitrosylation of proteins. *Cell. Mol. Life Sci.* **55**: 1036–1042.

Callaghan, A.V., Gieg, L.M., Kropp, K.G., Suflita, J.M. & Young, L.Y. (2006) Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two bacterial isolates and a bacterial consortium. *Appl. Environ. Microbiol.* **72**: 4274–4282.

Callaghan, A.V., Wawrik, B., Ni Chadhain, S.M., Young, L.Y. & Zylstra, G.J. (2008) Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. *Biochem. Biophys. Res. Commun.* **366**: 142–148.

Callaghan, A.V., Morris, B.E., Pereira, I.A., McInerney, M.J., Austin, R.N., Groves, J.T. *et al.* (2012) The genome sequence of *Desulfatibacillum alkenivorans* AK-01: a blueprint for anaerobic alkane oxidation. *Environ. Microbiol.* **14**: 101–113.

Cravo-Laureau, C., Grossi, V., Raphel, D., Matheron, R. & Hirschler-Réa, A. (2005) Anaerobic *n*-alkane metabolism by a sulfate-reducing bacterium, *Desulfatibacillum aliphaticivorans* strain CV2803T. *Appl. Environ. Microbiol.* **71**: 3458–3467.

Davidova, I.A., Gieg, L.M., Nanny, M., Kropp, K.G. & Suflita, J.M. (2005) Stable isotopic studies of *n*-alkane metabolism by a sulfate-reducing bacterial enrichment culture. *Appl. Environ. Microbiol.* **71**: 8174–8182.

Ehrenreich, P. (1996) Anaerobes Wachstum neuartiger sulfatreduzierender und nitratreduzierender Bakterien auf *n*-Alkanen und Erdöl. Dissertation, Universität Bremen.

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

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M. *et al.* (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543–548.

Feil, C. (2006) Biochemie des anaeroben Toluol-Stoffwechsels von *Thauera aromatica*. Dissertation, Universität Darmstadt.

Grundmann, O., Behrends, A., Rabus, R., Amann, J., Halder, T., Heider, J. & Widdel, F. (2008) Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN1. *Environ. Microbiol.* **10**: 376–385.

Hilberg, M., Pierik, A.J., Bill, E., Friedrich, T., Lippert, M.L. & Heider, J. (2012) Identification of FeS clusters in the glycyl-radical enzyme benzylsuccinate synthase via EPR and Mossbauer spectroscopy. *J. Biol. Inorg. Chem.* **17**: 49–56.

Knappe, J. & Sawers, G. (1990) A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. *FEMS Microbiol. Rev.* **6**: 383–398.

Kropp, K.G., Davidova, I.A. & Suflita, J.M. (2000) Anaerobic oxidation of *n*-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. *Appl. Environ. Microbiol.* **66**: 5393–5398.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

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

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

Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A.J. & Widdel, F. (2001) Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: Evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. *J. Bacteriol.* **183**: 1707–1715.

Rojo, F. (2009) Degradation of alkanes by bacteria. *Environ. Microbiol.* **11**: 2477–2490.

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

Selesi, D., Jehmlich, N., von Bergen, M., Schmidt, F., Rattei, T., Tischler, P. *et al.* (2010) Combined genomic and proteomic approaches identify gene clusters involved in anaerobic 2-methylnaphthalene degradation in the sulfate-reducing enrichment culture N47. *J. Bacteriol.* **192**: 295–306.

Tissot, B.P. & Welte, D.H. (1984) Petroleum formation and occurence. Berlin, Germany: Springer Verlag.

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

Widdel, F., Knittel, K. & Galushko, A. (2010) Anaerobic hydrocarbon-degrading microorganisms: an overview. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 1998–2021.

Zedelius, J., Rabus, R., Grundmann, O., Werner, I., Brodkorb, D., Schreiber, F. *et al.* (2011) Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. *Environ. Microbiol. Rep.* **3**: 125–135.

2. Bericht

Attempts to crystallize (1-methylalkyl)succinate synthase of strain HxN1 and alternative strategies for protein purification

Dieser Bericht enthält zusätzliche Ergebnisse für die (1-Methylalkyl)succinat-Synthase, die nicht Bestandteil eines Manuskriptes sind.

Summary

The (1-methylalkyl)succinate synthase of strain HxN1, which was purified according to the protocol established by Schmitt *et al*. (unpublished results), crystallized to many thin needles. Modifications of the crystallization conditions did not lead to the formation of larger crystals for the generation of an X-ray structure of (1-methylalkyl)succinate synthase. Alternative purification strategies comprised immunoaffinity purification of the native enzyme as well as the purification of tagged (1-methylalkyl)succinate synthase from *Escherichia coli* and from strain HxN1. By immunoaffinity, so far only one subunit of the enzyme was purified. The expression of tagged (1-methylalkyl)succinate synthase in *E. coli* resulted in aggregation of most of the enzyme in inclusion bodies. The amount of (1-methylalkyl)succinate synthase in the soluble fraction was insufficient for its purification. In strain HxN1 the tagged (1-methylalkyl)succinate synthase was not expressed.

Introduction

The (1-methylalkyl)succinate synthase catalyzes the activation of *n*-alkanes by addition to fumarate (Rabus *et al.*, 2001; Grundmann *et al.*, 2008). The inert *n*-alkane molecules are activated by insertion of a radical, which is transferred from the catalytic α-subunit (MasD) of the (1-methylalkyl)succinate synthase to the *n*-alkane. Oxidative cleavage at the radical harboring residue inactivates the enzyme irreversibly (Wagner *et al.*, 1992). In addition to the catalytic subunit MasD, the enzyme consists of three small subunits (MasB, MasC and MasE), whose function is still unclear (Grundmann *et al.*, 2008; Schmitt *et al*., unpublished results). The presence of four subunits is a unique feature of (1-methylalkyl)succinate synthase, since all other known glycyl radical enzymes do not exceed three subunits (Schmitt *et al*., unpublished results). The benzylsuccinate synthase, catalyzing the addition of toluene to fumarate, consists of only three subunits, one large catalytic subunit (BssA) and two small subunits (BssB, BssC) (Leuthner *et al.*, 1998). Due to the presence of Fe-S cluster in the small subunits of benzylsuccinate synthase, it has been speculated that the small subunits are required for electron transfer or for structural stabilization of the holoenzyme (Li *et al.*, 2009; Hilberg *et al.*, 2012).

This study describes attempts to crystallize (1-methylalkyl)succinate synthase that has been purified from cell extracts of strain HxN1 grown with *n*-hexane. A crystal structure of (1-methylalkyl)succinate synthase will illustrate the association of the subunits to each other and thus, may explain the function of the small subunits. Of special interest is the fourth subunit MasB, which is not necessary for anaerobic toluene activation. Besides, alternative purification strategies were developed in order to obtain a more stable protein for crystallization.

Material and Methods

Bacterial strains and growth conditions

Strain HxN1 was cultivated under denitrifying conditions in defined mineral medium with *n*-hexane or caproate as described previously (Rabus & Widdel, 1995; Ehrenreich *et al.*, 2000). Cultivation in a 50 l fermenter was performed as described by Schmitt *et al*. (unpublished results), cultivation on solid medium as described in Webner *et al*. (unpublished results). The bacterial *Escherichia coli* strains used in this study are described in table 1. They were cultivated at 37 °C in Luria Bertani medium. Antibiotics

were added at the following concentrations: ampicillin (50 µg ml^{-1}), chloramphenicol (20 µg ml^{-1}), kanamycin (45 µg ml^{-1}).

Table 1 Strains used in this study.

Strain	Genotype	Reference or source
HxN1	wild type	Ehrenreich *et al*. (2000)
E. coli BL21 Star (DE3)	F- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm rne131* (DE3)	Invitrogen (Darmstadt, Germany)
E. coli Lemo21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS/ p*Lemo(Cam^R) *λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5* *p*Lemo = pACYC184-*PrhaBAD-lysY*	New England Biolabs (Ipswich, USA)
E. coli S17-1	*thi recA pro hsdR* RP 4-2-Tc::MU-Km::Tn7	Simon *et al.* (1983)

Cloning of *mas* genes

Genomic DNA of strain HxN1 was isolated with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The genes *masB, masC, masD* and *masE* were amplified by polymerase chain reaction (PCR) with Phusion High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland). For cloning, the oligonucleotide primers (table 2) contained recognition sites for restriction enzymes, which were applied according to the instructions (Fermentas, St. Leon-Rot, Germany). Restricted DNA was purified with the QIAquick Gel Extraction Kit (Qiagen) from agarose gels or with the QIAquick PCR Purification Kit (Qiagen). PCR products and plasmids (table 3) were mixed in a 2:1 molar ratio and ligated with T4 DNA Ligase (Fermentas).

For purification of the (1-methylalkyl)succinate synthase with the glutathione S-transferase (GST)-tag, the genes were cloned into pET-42a(+) and for purification with the Strep-tag, they were cloned into pET-51b(+). The genes *masBC* including the ribosomal binding site of *masB* were cloned upstream of the tag-coding sequence, *masD* was amplified without its start codon together with *masE* and cloned in frame behind the tag to enable purification as a fusion protein (Fig. 1a). To generate pBBR1MCS-2_*masBCDE*-Strep for purification of tagged (1-methylalkyl)succinate synthase from strain HxN1, *masBCDE* were amplified together with the sequence coding

for the Strep-tag from pET-51b_*masBCDE* and ligated into pBBR1MCS-2 under the control of the T7 promoter (Fig. 1b).

The plasmids pET-42a_*masBCDE* and pET-51b_*masBCDE* were transformed into chemically competent *E. coli* BL21 Star (DE3) or Lemo21 (DE3) cells according to Inoue *et al.* (1990) and cultivated as described above. pBBR1MCS-2_*masBCDE*-Strep was transferred by conjugation from *E. coli* S17-1 into strain HxN1 as described by Webner *et al*. (unpublished results). To confirm the correct insert, the plasmids were sequenced with the BigDye v3.0 terminator cycle sequencing kit on an ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Sequencing data were analyzed with the Lasergene software (DNASTAR, Konstanz, Germany).

Table 2 Sequences of oligonucleotide primers used in this study; restriction sites are underlined.

Primer	Target gene	Sequence (5´→ 3´)	Product length [bp]
*masB_Hind*III_f	*masB*	TGACAAGCTTATAGTCGACGCGATGAGTG	360
*masB_Xho*I_r		CGCTCGAGTCAGGATTTCTTGATGCTTGA	
*masBC_Xba*I_f	*masBC*	GATCTAGACAGTGAAGAAGAAGCCAC	647
*masBC_Xba*I_r		CGTCTAGATCAATGCGCTTTTGCTGT	
*masDE_Hind*III_f	*masDE*	GTCAAGCTTGCACTGCAACTTCAACACTA	2758
*masDE_Xho*I_r		ATGACTCGAGCTAACCTTCGGCCAAGTTT	
*masDE_Bam*HI_f	*masDE*	CAGGATCCTACTGCAACTTCAACACTATCC	2758
*masDE_Hind*III_r		CTGAAGCTTCTAACCTTCGGCCAAGTTTTC	
*masB_Sac*II_*f*	*masBCDE*	ATCCGCGGCAGTGAAGAAGAAGCCAC	3453
*masE_Hind*III_r		CTGAAGCTTCTAACCTTCGGCCAAGTTTTC	

Table 3 Plasmids used in this study.

Plasmid	Characteristics	Reference or source
pET-42a(+)	Km^R, GST-tag, His-tag, S-tag	Novagen (Darmstadt, Germany)
pET-42a_*masB*	Km^R, GST-tag, His-tag, S-tag, *masB*	this study
pET-42a_*masBC*	Km^R, GST-tag, His-tag, S-tag, *masBC*	this study
pET-42a_*masBCDE*	Km^R, GST-tag, His-tag, S-tag, *masBCDE*	this study
pET-51b(+)	Ap^R, Strep-tag II, His-tag	Novagen
pET-51b_*masBC*	Ap^R, Strep-tag II, His-tag, *masBC*	this study
pET-51b_*masBCDE*	Ap^R, Strep-tag II, His-tag, *masBCDE*	this study
pBBR1MCS-2	Km^R, *mob*	Kovach *et al.* (1995)
pBBR1MCS-2_ *masBCDE*-Strep	Km^R, *mob*, Strep-Tag II, *masBCDE*	this study

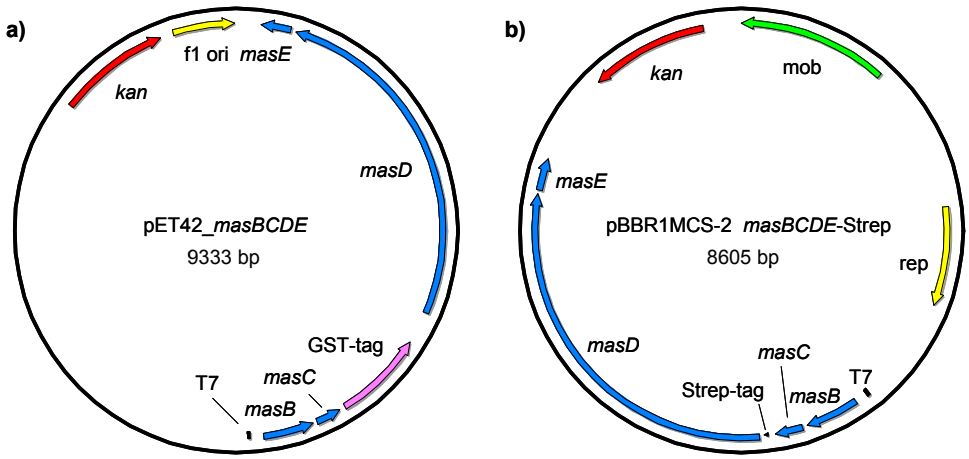


Fig. 1 Plasmids for purification of tagged (1-methylalkyl)succinate synthase. **a)** Genes *masB*, *masC*, *masD* and *masE* cloned into pET-42a(+) for purification of the holoenzyme by the GST-tagged MasD subunit from *E. coli*. For purification via the Strep-tag the *mas* genes were cloned into pET-51b(+) in an analogous manner. **b)** pBBR1MCS-2_*masBCDE*-Strep for expression of tagged (1-methylalkyl)succinate synthase in strain HxN1. Blue: *mas* genes; red: antibiotic resistance genes; yellow: origin of replication; green: origin of transfer; purple: GST- and Strep-Tag.

Purification of (1-methylalkyl)succinate synthase

Purification of (1-methylalkyl)succinate synthase from cell extracts of strain HxN1 and determination of molecular weight and of enzymatic activity was performed as described in Schmitt *et al*. (unpublished results). Purification of (1-methylalkyl)succinate synthase from strain HxN1 by immunoaffinity chromatography was performed in an anaerobic chamber. Columns with crosslinked antibodies against MasB or MasD were prepared with the Pierce Crosslink Immunoprecipitation Kit (Pierce Protein, Rockford, USA). The production of immune serum against MasD is described in Schmitt *et al*. (unpublished results). Immune serum against MasB was performed analogously. Oligonucleotide primers for cloning of *masB* into pET-42a(+) are listed in table 2. Each 100 µl of immune serum was used to crosslink the antibodies to the column. Cell lysates of strain HxN1, which were applied to the columns, were adjusted to a concentration of 500 to 1000 µg protein. The protein concentration was determined with the Bio-Rad Protein Assay based on the method of Bradford (1976). The protein was eluted from the column according to the instructions of the Crosslink Immunoprecipitation Kit.

The recombinant (1-methylalkyl)succinate synthase fused to GST- or Strep-tag was purified from *E. coli* harboring the appropriate plasmid. Protein expression was induced by addition of 1 mM IPTG in the exponential growth phase (optical density at 600 nm = 0.6). For expression of the (1-methylalkyl)succinate synthase in *E. coli* Lemo21 (DE3), 2 mM rhamnose were added at inoculation. The cells were harvested by centrifugation after 3 h of induction (4410 x *g*, 15 min, 4 °C). 2 g of wet weight cell pellet were re-suspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) or binding buffer (100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 8), both supplemented with 1 mg ml^{-1} DNase. Cells were disrupted with 10 mg ml^{-1} lysozyme, incubated for 10 min at room temperature, followed by ultrasonification on ice. Afterwards, the cell lysate was centrifuged to remove cell debris (16100 x *g*, 15 min, 4 °C) and the obtained supernatant was filtered (0.2 µm, Whatman/GE Healthcare, Munich, Germany) before purification with an ÄKTA purifier FPLC system (GE Healthcare). Buffers were sterile filtered (0.45 µm, Sarstedt, Nümbrecht, Germany) and degassed. The flow rate for all steps was set to 1 ml min^{-1}. The GST-tag fusion protein was purified on a 1 ml GSTrap HP column (GE Healthcare) equilibrated with PBS. Following washing with 5 ml PBS, the GST-tagged protein was eluted with 5 ml of 10 mM reduced glutathione in 50 mM Tris-HCl, pH 8. The Strep-tag fusion protein was purified on a 1 ml StrepTrap HP column (GE Healthcare), equilibrated with binding buffer. After washing with 10 ml binding buffer the Strep-tagged protein was eluted with

6 ml 2.5 mM desthiobiotin in binding buffer. Eluted protein from both columns was sampled in five fractions of 1 ml.

SDS-PAGE and Western Blot

Protein purification was displayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970). The PageRuler Prestained Protein Ladder (Fermentas) was used for size determination. Immunoblotting experiments were performed as described in Schmitt *et al*. (unpublished results), with immune sera against MasB, MasC, MasD and MasE and goat anti-rabbit IgG-AP as secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA).

Protein crystallization

The (1-methylalkyl)succinate synthase purified from strain HxN1 was concentrated through a cellulose membrane (Amicon Ultra-15 100K, Millipore, Billerica, USA) to a concentration of 4 to 20 mg ml^{-1}. The protein was dissolved in 102 mM NaH_2PO_4/Na_2HPO_4, pH 8.0 or 100 mM Tris-HCl, 5 mM fumarate, pH 8.0. Crystallization experiments were performed with the sitting drop method using comboplates and crystal bridges (Greiner Bio-One, Kremsmünster, Austria) in an anaerobic chamber at 20 °C. Each drop consisted of 5 µl protein solution and 5 µl reservoir solution. The volume of solution in the reservoir was 1 ml. Reservoir solutions were obtained from the JBScreen Classic 1 to 10 Crystal Screening Kits (Jena Bioscience, Jena, Germany). Newly developed solutions based on those from the Screening Kit were designed with chemicals of analytical grade and sterilized by filtration. All solutions were adapted to anaerobic conditions before protein crystallization was started. As control, crystals of lysozyme were grown with the JBS Starter Kit (Jena Bioscience). Crystal structures were recorded inside the anaerobic chamber with the Moticam 2300 (Motic, Wetzlar, Germany) connected to a binocular and a monitor.

Results and Discussion

Attempts to crystallize (1-methylalkyl)succinate synthase

For a first screening, protein purified from crude extract of strain HxN1 at a concentration of 4 mg ml^{-1}, stored in 102 mM NaH_2PO_4/Na_2HPO_4, pH 8.0 was used. The crude extract had an enzyme activity of 140 µM of (1-methylpentyl)succinate, indicating that the (1-methylalkyl)succinate synthase was active before purification. However, the enzyme activity might be affected during purification. The enzyme activity was not determined

again after purification, because the remaining protein solution would not have been enough for a complete screening. Crystallization experiments were performed under 240 different conditions with the JBScreen Classic 1 to 10 Crystal Screening Kits. Each kit is based on another primary precipitant and consists of 24 solutions (A1 to D6), which differ in precipitant concentration, added secondary precipitants, buffer and pH. One hour later, the protein had been precipitated in 122 of the tested conditions, while in the remaining 118 conditions precipitation did not occur (table 4).

Table 4 Screening for ideal conditions to crystallize (1-methylalkyl)succinate synthase of strain HxN1 with the JBScreen Kits. Conditions, which did not lead to protein precipitation after one hour, are marked with a +.

Number \ Kit		1	2	3	4	5	6	7	8	9	10
A	1			+			+	+	+	+	+
	2	+	+	+			+	+			+
	3	+			+					+	+
	4	+		+	+		+	+	+	+	+
	5		+	+			+	+		+	
	6	+	+				+	+	+		+
B	1		+				+	+			+
	2				+		+	+	+		+
	3	+			+			+	+	+	
	4					+	+	+	+		+
	5		+				+		+		+
	6	+		+		+	+		+		+
C	1	+			+		+	+	+		
	2				+						+
	3	+		+	+		+		+		+
	4	+					+	+	+		+
	5		+			+	+	+	+		
	6	+			+	+	+	+	+		+
D	1	+					+	+		+	+
	2					+		+	+	+	+
	3							+	+		
	4							+		+	
	5				+			+		+	+
	6		+		+	+		+	+		+

Dark, fast evolving precipitates usually indicate denaturation of the protein. Clear approaches were checked daily for the formation of crystals. Crystals develop by reducing the solubility of the protein molecule. In case this happens too fast, precipitation occurs. Reduction of protein solubility is achieved by addition of salts, organic solvents or polymers. The solubility is also influenced by the pH. Crystal formation lasts up to several weeks. Two conditions, 100 mM Tris-HCl, pH 8.5, 10% isopropanol, 10 mM $MgCl_2$ (solution 9A4) and 100 mM Tris-HCl, pH 8.5, 2% isopropanol, 10 mM $MgSO_4$ (solution 8D6) led to the formation of crystal needles after one day (Fig. 2a). Slipknot-like structures represent a large number of thin needles growing from a single nucleation centre (http: xray.bmc.uu/se/terese/tutorial). Nucleation is the first step in crystallization, followed by crystal growth (Garcia-Ruiz, 2003). Under optimal conditions, a single crystal develops from one nucleation centre. However, the formation of larger, single crystals failed. The slipknots appeared to be dark coloured, while other needles were colourless (Fig. 2a). The purified (1-methylalkyl)succinate synthase is also dark brown coloured, possibly due to Fe-S cluster. The small subunits of (1-methylalkyl)succinate synthase are expected to coordinate Fe-S cluster, because they have, like the small subunits of benzylsuccinate synthase (Hilberg *et al.*, 2012), conserved cysteine residues, which might coordinate Fe-S cluster.

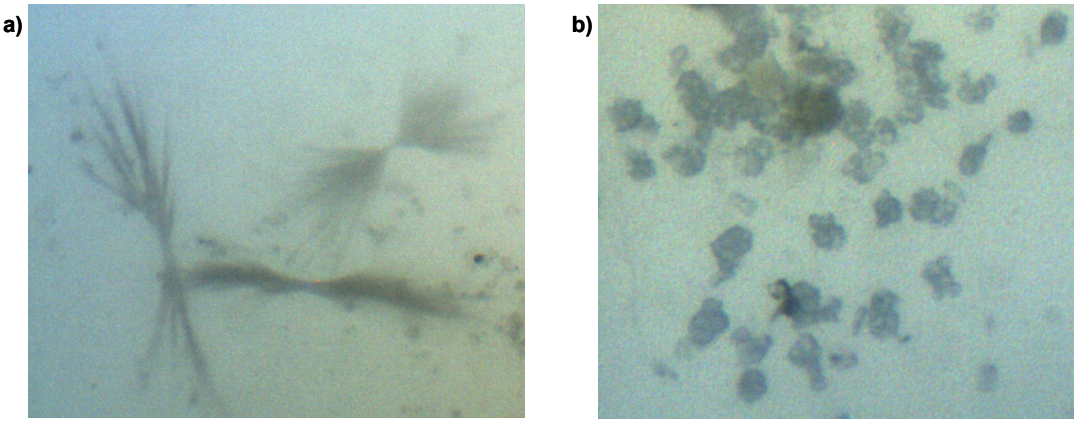


Fig. 2 Crystals of (1-methylalkyl)succinate synthase. **a)** Slipknot-like structures after one day of incubation in 100 mM Tris-HCl, pH 8.5, 10% isopropanol, 10 mM $MgCl_2$. **b)** Small crystals after 15 days of incubation in 100 mM Tris-HCl, pH 8.5, 1% isopropanol, 10 mM $MgCl_2$. Pictures were taken with 40x magnification.

The complete screening was performed a second time in a slightly different way: The protein concentration was raised to 10 mg ml^{-1} and the protein was purified from inactive crude extract. In its inactive state the (1-methylalkyl)succinate synthase does not carry a radical at its catalytic subunit. It was assumed that the inactive protein is more stable

during crystallization, because it cannot be cleaved at the radical harboring site by accidentally exposure to oxygen. The truncated catalytic subunit may destabilize the composition of the holoenzyme by loosing association of the different subunits to each other. Losses in activity during purification have been reported for (1-methylalkyl)succinate synthase and benzylsuccinate synthase (Leuthner *et al.*, 1998; Schmitt *et al*., unpublished results). The correct composition of the inactive holoenzyme was confirmed by gel filtration, which determined the molecular weight (data not shown). The new screening confirmed the previously obtained results. The same conditions mentioned above showed crystal-like structures, while formation of larger crystals failed. To obtain larger crystals, new solutions were prepared, based on those, which yielded initial crystal formation. The buffer (100 mM Tris-HCl, pH 8.5) remained the same, while for the two precipitants different concentrations were prepared, in total two times 24 new conditions (table 5 and 6).

Table 5 Composition of new solutions for crystallization of (1-methylalkyl)succinate synthase, based on solution 9A4 (100 mM Tris-HCl, pH 8.5, 10% isopropanol, 10 mM $MgCl_2$) of the JBScreen Kit. Conditions, which led to the formation of crystal structures, are marked with a +.

$MgCl_2$ \ Isopropanol		1 2%	2 5%	3 10%	4 15%	5 20%	6 25%
A	10 mM	+	+	+	+		
B	50 mM	+					
C	100 mM	+					
D	200 mM						

Table 6 Composition of new solutions for crystallization of (1-methylalkyl)succinate synthase, based on solution 8D6 (100 mM Tris-HCl, pH 8.5, 2% isopropanol, 10 mM $MgSO_4$) of the JBScreen Kit. Conditions, which led to the formation of crystal structures, are marked with a +.

$MgSO_4$ \ Isopropanol		1 2%	2 5%	3 10%	4 15%	5 20%	6 25%
A	10 mM	+	+	+			
B	50 mM	+	+				
C	100 mM	+					
D	200 mM	+					

The new experiments were performed with inactive protein, concentrated to 20 mg ml^{-1}. In parallel, the 102 mM NaH_2PO_4/Na_2HPO_4 buffer was used alone as control, to exclude formation of salt crystals. Conditions marked with a + in table 5 and 6 led to the formation of crystalline structures (needles and slipknots) within one to five days, while crystals were not observed in the control experiment with NaH_2PO_4/Na_2HPO_4 buffer. As evident, combinations of high salt and high isopropanol concentrations caused protein precipitation (table 5 and 6). For a further improvement of the obtained results, solutions with lower concentrations of salt and isopropanol were prepared (table 7 and 8). The same protein with a concentration of 20 mg ml^{-1} was used. This time, more approaches led to the formation of crystals (marked with a + in table 7 and 8).

Table 7 Composition of new solutions for crystallization of (1-methylalkyl)succinate synthase, based on the solutions of table 5. Conditions, which led to the formation of crystal structures, are marked with a +.

Isopropanol / $MgCl_2$		1 1% (A,C) 6% (B,D)	2 2% (A,C) 7% (B,D)	3 3% (A,C) 8% (B,D)	4 4% (A,C) 6% (B,D)	5 5% (A,C) 6% (B,D)	6 1% (A,C) 6% (B,D)
A	10 mM	+	+	+	+	+	+
B	10 mM	+	+	+	+	+	+
C	20 mM	+	+	+	+	+	+
D	20 mM	+	+	+	+		

Table 8 Composition of new solutions for crystallization of (1-methylalkyl)succinate synthase, based on the solutions of table 6. Conditions, which led to the formation of crystal structures, are marked with a +.

Isopropanol / $MgSO_4$		1 1%	2 2%	3 3%	4 5% (A-C) 1% (D)	5 7.5% (A-C) 2% (D)	6 10% (A-C) 3% (D)
A	10 mM	+	+	+	+	+	+
B	50 mM	+	+	+	+	+	+
C	100 mM	+	+	+	+	+	+
D	150 mM (1-3) 200 mM (4-6)	+	+	+	+	+	+

After incubation of two weeks, needles had disappeared and small crystals appeared (Fig. 2b). Small size crystals develop, if growth happens too fast, because of suboptimal conditions and they are unsuitable for X-ray analysis. Furthermore, the obtained crystals were not brownish anymore. They turned out to be salt crystals, which probably developed from the buffer in the crystallization solution (Tris-HCl) and the NaH_2PO_4/Na_2HPO_4 buffer, in which the protein was dissolved, yielding NaCl. In the control experiments with buffer instead of protein solution, no salt crystals developed within the first five days, as mentioned above. Unfortunately, these approaches were not further analyzed for a later crystal development. It is assumed that the initially observed crystal structures were of protein origin, which did not develop to a larger crystal and instead were destroyed due to suboptimal conditions. By contrast, the salt crystals developed later, after evaporation of the solution.

The transfer of a crystal into fresh solutions with a thin hairloop needle, which is called seeding, might allow further growth of this crystal, because the conditions for nucleation are not the ideal ones for subsequent growth (Bergfors, 2003). So far, application of this method remained unsuccessful for (1-methylalkyl)succinate synthase. To circumvent formation of salt crystals, the (1-methylalkyl)succinate synthase was buffered in 20 mM Tris-HCl, pH 8 and a new screening was performed with the Screen Kits 1 to 10. Nearly the same conditions depicted in table 4 stayed clear, but further improvements were not undertaken. Simply to get an idea about the shape of protein crystals, lysozyme was crystallized according to standard protocols. After one day, large and structurally diverse crystals had formed (Fig. 3).

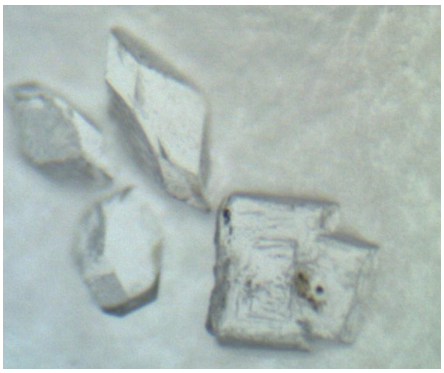
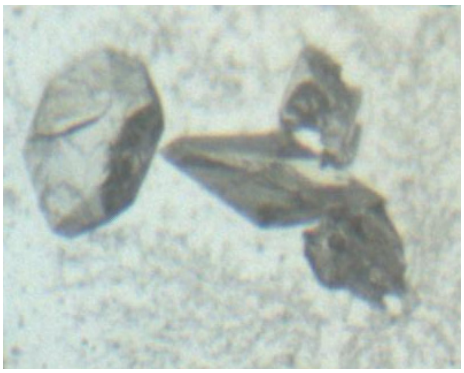
a)  **b)** 

Fig. 3 Crystals of lysozyme one day after preparation. **a)** Conditions: 7% NaCl, 250 mM sodium acetate, pH 4.8. **b)** Conditions: 8% NaCl, 250 mM sodium acetate, pH 4.8. Pictures were taken with 40x magnification.

Immunoaffinity purification of (1-methylalkyl)succinate synthase

To minimize purification steps and coupled loss in activity, an alternative purification strategy was investigated for (1-methylalkyl)succinate synthase of strain HxN1. Purification was attempted with columns, to which antibodies against MasD or MasB were bound covalently. The native holoenzyme is purified, if the quaternary structure is maintained and if the antibody binding site of the protein is accessible. In first attempts, MasB was purified with the MasB immune serum (Fig. 4), while the other subunits were not. A reason for unsuccessful purification of the other three subunits along with MasB might be inconvenient conditions (e.g. buffer, pH) during purification, which caused dissoziation of the subunits from each other. Purification with the MasD immune serum even failed for the MasD subunit. However, this purification strategy might be valuable after effective optimization of adequate conditions for (1-methylalkyl)succinate synthase to purify related enzymes. The immune sera against MasB and MasD have been successfully applied in Western blot analysis to detect MasB and MasD of strain OcN1 (Fig. 5). In strain OcN1, which degrades *n*-alkanes with a chain length of C_8–C_{12} anaerobically (Ehrenreich *et al.*, 2000), a *mas* operon has been identified, assuming activation of the *n*-alkanes by addition to fumarate as well (Werner, 2009). Sequence identity of MasB from strains HxN1 and OcN1 is 71% and of MasD it is 86%. This indicates that the antibodies of the immune sera possess relaxed specificity towards target polypeptides. Thus, it might be possible to purify the (1-methylalkyl)succinate synthase of strain OcN1 and as well as of other strains with the antisera against the subunits of strain HxN1. Hereby, the presence of a MasB subunit, which has been proposed to be an exclusive feature of anaerobic *n*-alkane activating enzymes, can be analyzed (Schmitt *et al*., unpublished results).

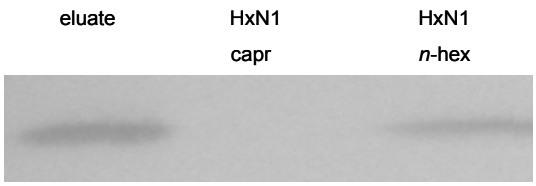


Fig. 4 Purification of (1-methylalkyl)succinate synthase with immune serum against MasB. Strain HxN1 grown with caproate or *n*-hexane served as negative respectively positive control.

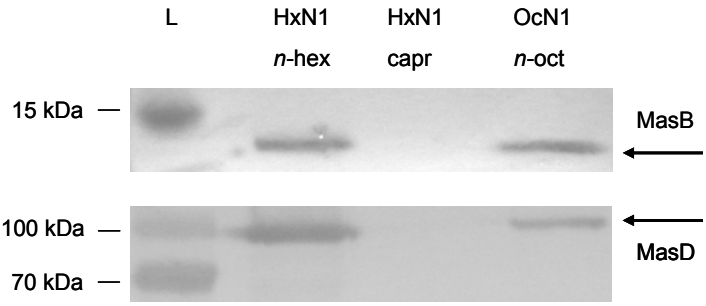


Fig. 5 Detection of MasB and MasD in cell extracts of strain OcN1 grown with *n*-octane by Western blot with immune serum against MasB and MasD of strain HxN1. Strain HxN1 grown with *n*-hexane or caproate served as positive or negative control, respectively. L: pre-stained ladder.

Purification of recombinant (1-methylalkyl)succinate synthase from *E. coli*

(1-Methylalkyl)succinate synthase is purified from *E. coli* in its inactive form, due to the missing activating enzyme, which transfers a radical generated by cleavage of S-adenosylmethionine from the resulting 5´-deoxyadenosylradical to the catalytic subunit of (1-methylalkyl)succinate synthase (Layer *et al.*, 2004). Thus, cleavage of a radical-bearing polypeptide chain by reaction with oxygen (Wagner *et al.*, 1992) is precluded and the enzyme is supposed to be more stable. The (1-methylalkyl)succinate synthase expressed in *E. coli* BL21 Star (DE3) was purified under aerobic conditions. Purification from *E. coli* was also applied for the benzylsuccinate synthase of *Thauera aromatica* strain T1 (Li *et al.*, 2009). Additionally to the generation of a stable protein, *E. coli* enables the production of higher amounts of protein in a shorter time. Fusion of the desired protein to a tag further simplifies purification. The N- and C-termini of proteins are rarely located inside the three-dimensional structure and therefore, fused tags should be accessible for affinity purification. Affinity purification gives high yields of pure protein, but is anyway often combined with a second purification step. The Strep-tag consists of only eight amino acids and, due to his small size, does not hamper crystallization (Terpe, 2003). The larger GST-tag (26 kDa) needs to be removed before crystallization, which is achieved by cleavage of the protein during purification, while it is bound to the column, or afterwards with the proteases thrombin or Factor Xa (Terpe, 2003). Recognition sites for these proteases are encoded in the plasmid backbone of pET-42a(+) between the GST-coding sequence and the multiple cloning site. For purification of tagged (1-methylalkyl)succinate synthase only the large subunit MasD was fused with its N-terminus either to the GST- or Strep-tag. If the correct quaternary structure of (1-methylalkyl)succinate synthase is formed in *E. coli*, the small subunits should be co-purified together with the tagged MasD subunit. Co-purification of the two small subunits

of benzylsuccinate synthase together with the his_6-tagged large subunit was shown by Li *et al*. (2009). In this case, all three subunits were expressed as soluble proteins in *E. coli* BL21 Star (DE3), while expression of the tagged large subunit alone caused inclusion bodies.

For the tagged (1-methylalkyl)succinate synthase of strain HxN1 expression in *E. coli* was successful (Fig. 6a). In denaturing gel electrophoresis the tagged MasD subunit with a size of 94 kDa or 120 kDa (94 kDa MasD + 26 kDa GST-Tag), respectively, is clearly visible after induction with IPTG. Expression of the small subunits was verified by Western blot, as exemplarily shown for pET51_*masBCDE* in Fig. 6b.

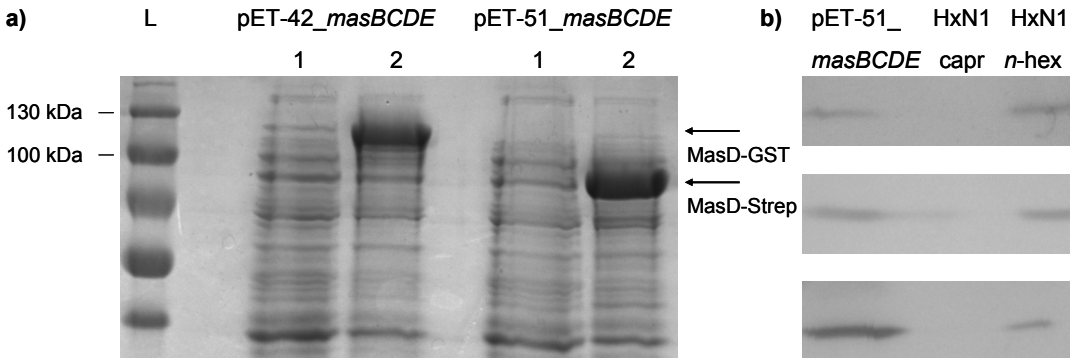


Fig. 6 Expression of recombinant (1-methylalkyl)succinate synthase in *E. coli*. **a)** SDS-PAGE of cell extracts before (1) and three hours after induction (2). GST-tagged MasD expressed from pET-42_*masBCDE* is apparent with a size of 120 kDa, Strep-tagged MasD expressed from pET-51_*masBCDE* has a size of 94 kDa. L: ladder. **b)** Western blot for detection of MasB (top), MasC (center) and MasE (bottom) in cell extract of *E. coli* + pET-51_*masBCDE*, which was obtained three hours after induction with IPTG. Cell extract of strain HxN1 either grown with caproate or with *n*-hexane served as negative or positive control, respectively.

Most of the Mas-GST-tag and Mas-Strep-tag fusion proteins aggregated as inclusion bodies (Fig. 7), which are present after cell lysis in the cell debris rather than in the soluble fraction because of their high density. Inclusion bodies consist of incorrectly folded protein, which are stabilized by hydrophobic interactions (Mukhopadhyay, 1997). Especially hydrophobic proteins tend to fold incorrectly. Proteins, which require formation of disulfide bonds to obtain their correct structure, need to be transported into the periplasm, because under the reducing conditions in the cytoplasm disulfide bonds are not formed. The lack of a proper secretory systems leads to accumulation of incorrect folded proteins in the cytoplasm as inclusion bodies (Mukhopadhyay, 1997). Sometimes, cofactors required by the protein are missing in *E. coli*. Another reason for formation of inclusion bodies is protein expression at high rates (Mukhopadhyay, 1997). Expression

vectors like the pET-series are optimized for high expression levels by using the T7 promoter to obtain a large amount of the desired protein. With this system the induced protein accounts for up to 50% of the entire cell protein. A disadvantage of high expression rates is insufficient time for protein folding (Mukhopadhyay, 1997). To circumvent high expression rates, it might be advantageous to perform growth below the optimal temperature of 37 °C. For expression of (1-methylalykl)succinate synthase in *E. coli* BL21 Star (DE3) incubation at room temperature did not lead to the formation of less inclusion bodies (data not shown). Protein expression was also tried in *E. coli* strain Lemo21 (DE3). The Lemo strain allows tuning of expression by varying the level of lysozyme, the natural inhibitor of T7 RNA polymerase, which is achieved by adding rhamnose during growth. Rhamnose regulates expression of lysozyme. However, a concentration of 2 mM rhamnose resulted in insufficient amounts of expressed (1-methylalykl)succinate synthase (data not shown). Further optimization regarding the concentration of rhamnose was not performed. Overall, the amount of protein purified from the soluble fraction of *E. coli* BL21 Star (DE3) was insufficient to initiate crystallization (Fig. 7). The GST-tagged MasD subunit is barely visible in the eluted fractions 3 and 4 after purification with glutathione. The same result was obtained for the Strep-tagged enzyme (data not shown). The small subunits were not visible in SDS-PAGE. Their presence in the elution fraction was, however, not confirmed by Western blot. Thus, it is presently unclear, if the low amount of soluble holoenzyme, which was purified, has been folded correctly in *E. coli* and if all four subunits were purified.

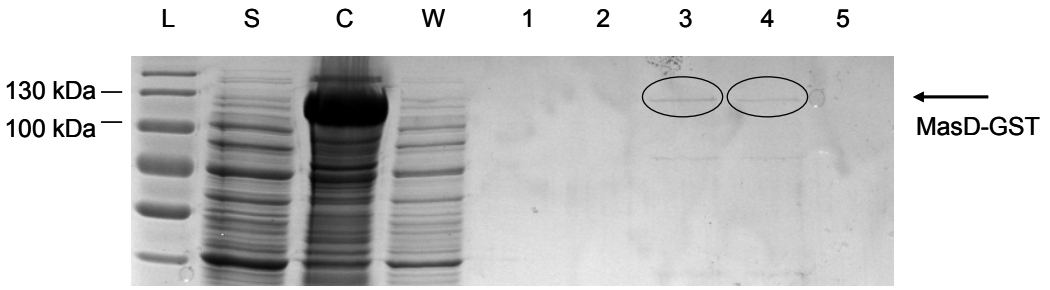


Fig. 7 Purification of GST-tagged (1-methylalkyl)succinate synthase from *E. coli*. L: ladder; S: supernatant after cell lysis, which was used for purification; C: cell debris after cell lysis; W: non-bound protein eluted after column wash; 1–5: elution fractions from the column. GST-tagged MasD with a size of 120 kDa is dominant in the cell debris, small amounts of purified MasD-GST are in fraction 3 and 4.

For purification of the benzylsuccinate synthase holoenzyme from *E. coli*, it was shown to be advantageous to supplement the growth medium with $Fe(NH_4)_2(SO_4)_2$, to enable

formation of Fe-S cluster within the small subunits (Li *et al.*, 2009). Fe-S clusters are supposed to be important for assembly of the holoenzyme. Correct folding avoids precipitation in form of inclusion bodies, as mentioned above. Therefore, the effect of $Fe(NH_4)_2(SO_4)_2$ onto the formation of soluble (1-methylalykl)succinate synthase in *E. coli* should be tested in the future.

In principle, protein purification from inclusion bodies is possible. Inclusion bodies are separated by ultracentrifugation from cell debris due to their dense clustering. However, solubilization of inclusion bodies requires denaturing agents. Thus, the protein has to refold under convenient conditions afterwards. Purification from inclusion bodies was successfully shown for the large subunit of benzylsuccinate synthase fused to a His_6 tag (Li *et al.*, 2009), but was not taken into account for the (1-methylalykl)succinate synthase holoenzyme, because the success to refold four subunits and to retain the correct quaternary structure was estimated to be marginal.

A plasmid for purification of tagged (1-methylalkyl)succinate synthase from strain HxN1

To combine the advantages of purification of the inactive, radical-free, tagged (1-methylalkyl)succinate synthase with the expression in the natural environment to simplify correct folding, it was thought to express the tagged protein encoded on a plasmid in strain HxN1 grown with caproate. In pBBR1MCS-2_*masBCDE*-Strep (Fig. 1b) the *mas* genes are under the control of the T7 promoter and thus, are expressed constitutively, whereas the wild type *mas* operon is not induced during growth with caproate. Expression of the wild type *mas* operon requires the presence of a hydrocarbon such as *n*-hexane (Grundmann *et al.*, 2008). Consequently, the activating enzyme of (1-methylalkyl)succinate synthase encoded by *masG* will not be expressed during growth with caproate. MasG is needed to deliver a radical to the catalytic subunit. Thus, the (1-methylalkyl)succinate synthase will be produced in its inactive form in strain HxN1 in the absence of MasG.

The recently established genetic system allows introduction of foreign DNA by conjugation into strain HxN1 (Webner *et al.*, unpublished results). The broad-host-range plasmid pBBR1MCS-2 was shown to be stably maintained in the strain. Therefore, pBBR1MCS-2_*masBCDE*-Strep was transferred into strain HxN1 and kanamycin-resistant clones were obtained. Regrettably, expression of the plasmid encoded *mas* genes was not confirmed in Western Blot analysis (data not shown). For future analysis, *masBCDE*-Strep should be cloned the other way round into the multiple cloning site of pBBR1MCS-2. Then, expression would be under the control of the T3 and *lac* promoter.

Expression of *masD* cloned into pBBR1MCS in this direction was successful in a mutant of strain HxN1 grown with caproate (Webner *et al*., unpublished results).

Conclusion

Purification of native (1-methylalkyl)succinate synthase by immunoaffinity or purification of Strep-tagged protein from strain HxN1 should be further tracked as alternative purification strategies. The results obtained so far for these strategies do not exclude them as appropriate method, yet. Still, there are possibilities for modifications to improve the outcoming results. In case of successful purification by application of these alternative strategies, another attempt to crystallize the enzyme should be started as well as with the “traditionally” purified enzyme.

References

Bergfors, T. (2003) Seeds to crystals. *J. Struct. Biol.* **142**: 66–76.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.

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

Garcia-Ruiz, M.J. (2003) Nucleation of protein crystals. *J. Struct. Biol.* **142**: 22–31.

Grundmann, O., Behrends, A., Rabus, R., Amann, J., Halder, T., Heider, J. & Widdel, F. (2008) Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN1. *Environ. Microbiol.* **10**: 376–385.

Hilberg, M., Pierik, A.J., Bill, E., Friedrich, T., Lippert, M.L. & Heider, J. (2012) Identification of FeS clusters in the glycyl-radical enzyme benzylsuccinate synthase via EPR and Mossbauer spectroscopy. *J. Biol. Inorg. Chem.* **17**: 49–56.

Inoue, H., Nojima, H. & Okayama, H. (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**: 23–28.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd & Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**: 175–176.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Layer, G., Heinz, D.W., Jahn, D. & Schubert, W.D. (2004) Structure and function of radical SAM enzymes. *Curr. Opin. Chem. Biol.* **8**: 468–476.

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

Li, L., Patterson, D.P., Fox, C.C., Lin, B., Coschigano, P.W. & Marsh, E.N. (2009) Subunit structure of benzylsuccinate synthase. *Biochemistry* **48**: 1284–1292.

Mukhopadhyay, A. (1997) Inclusion bodies and purification of proteins in biologically active forms. *Adv. Biochem. Eng. Biotechnol.* **56**: 61–109.

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

Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A.J. & Widdel, F. (2001) Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: Evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. *J. Bacteriol.* **183**: 1707–1715.

Simon, R., Priefer, U. & Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Nat. Biotechnol.***1**: 784–791.

Terpe, K. (2003) Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Appl. Microbiol. Biotechnol.* **60**: 523–533.

Wagner, A.F., Frey, M., Neugebauer, F.A., Schäfer, W. & Knappe, J. (1992) The free radical in pyruvate formate-lyase is located on glycine-734. *Proc. Natl. Acad. Sci. U S A* **89**: 996–1000.

Werner, I. (2009) Untersuchungen zum Stoffwechsel des anaeroben Alkanabbaus. Dissertation, Universität Bremen

3. Manuskript

Identification of a second functional *mas* operon in the anaerobic *n*-alkane degrader strain HxN1 by a newly developed genetic system

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Erstellung des Manuskriptes, Planung und Durchführung der Versuche. Die Plasmide pCM184_∆*masD_aacC1* und pBBR1MCS_*mas* operon wurden von Olav Grundmann hergestellt.

Abstract

Strain HxN1 is able to degrade *n*-alkanes anaerobically with nitrate as terminal electron acceptor. The *n*-alkanes are activated by addition to fumarate, presumably catalyzed by the (1-methylalkyl)succinate synthase (Mas). The enzyme and its encoding *mas* genes were recently identified in strain HxN1. To study the anaerobic *n*-alkane activation in more detail, strain HxN1 was chosen as a model organism. Here, we report on the introduction of foreign DNA into strain HxN1 by conjugation and gene deletion by homologous recombination. The applicability of this genetic system was demonstrated by deletion of *masD*, encoding the tentative catalytic subunit of (1-methylalkyl)succinate synthase. Physiological characterization of the *masD* deletion mutant confirmed its proposed function of being necessary for the anaerobic *n*-alkane degradation. By complementation the phenotype was restored. The present study proofs for the first time the function of an anaerobic *n*-alkane activating enzyme *in vivo*. In addition, deletion of *masD* revealed a second, identical copy of the *mas* operon in strain HxN1.

Introduction

The isolation of bacterial strains with the capability to degrade *n*-alkanes with nitrate (e.g. Ehrenreich *et al.*, 2000) or sulfate (e.g. Rueter *et al.*, 1994) as terminal electron acceptor with the beginning of the 1990s (reviewed in Widdel *et al.*, 2010) opened a new research field focused on the identification of underlying activation mechanisms and responsible enzymes and genes. Previously, *n*-alkanes were regarded as to be unable to be degraded under anaerobic conditions, because they have no functional groups and contain exclusively apolar σ-bonds, which make them chemically unreactive. Under aerobic conditions *n*-alkanes are activated by monooxygenases, which introduce a functional hydroxyl group into the molecule (van Beilen & Funhoff, 2005).

Metabolite analyses in the denitrifying strain HxN1 (Rabus *et al.*, 2001) and sulfate-reducing strains and enrichments (Kropp *et al.*, 2000; Cravo-Laureau *et al.*, 2005; Davidova *et al.*, 2005; Callaghan *et al.*, 2006) demonstrated activation of the *n*-alkanes at the secondary carbon atom by addition to fumarate yielding (1-methylalkyl)succinates. Further studies with strain HxN1 and *D. alkenivorans* strain AK-01 identified the potential enzymes catalyzing this activation reaction as well as their encoding genes (Callaghan *et al.*, 2008; Grundmann *et al.*, 2008). In strain HxN1 proteins especially formed during growth on *n*-hexane were supposed to be involved in the anaerobic activation of *n*-alkanes (Grundmann *et al.*, 2008). Four proteins, MasC, MasD, MasE and MasG, were assigned as homologues to the subunits of the glycyl radical enzyme benzylsuccinate synthase (BssABC) and its activating enzyme (BssD), which were first identified in *Thauera aromatica* strain K172 (Leuthner *et al.*, 1998). The benzylsuccinate synthase catalyzes the addition of fumarate to the aromatic hydrocarbon toluene (Leuthner *et al.*, 1998). The large α-subunit BssA harbors a conserved glycine and cysteine residue. These residues transiently carry the radical necessary for toluene activation and are characteristic for glycyl radical enzymes (Selmer *et al.*, 2005). The two small β- (BssB) and γ-subunits (BssC) contain Fe-S cluster, but their function is still unknown (Li *et al.*, 2009; Hilberg *et al.*, 2012). The necessitiy of the benzylsuccinate synthase for the anaerobic degradation of toluene was confirmed in *Azoarcus* sp. strain T by deletion of *bssA* and in *Thauera aromatica* strain T1 by deletion of *tutF, tutD* and *tutG* (Achong *et al.*, 2001; Coschigano, 2002).

MasC and MasD are regarded as structural homologues of BssC and BssA, respectively and thus, are supposed to be the α- and γ-subunit of a potential (1-methylalkyl)succinate synthase (Grundmann *et al.*, 2008). Corresponding genes are encoded in a *mas* operon, containing seven open reading frames (*masA* to *masG*). The gene product of another ORF, MasE, was designated as homologue of BssB, representing the β-subunit

(Grundmann *et al.*, 2008). Other publications postulated MasB as homologue of BssB (Callaghan *et al.*, 2012; Hilberg *et al.*, 2012). Recently, purification of the (1-methylalkyl)succinate synthase from strain HxN1 revealed the existence of four subunits, namely MasB, MasC, MasD and MasE (Schmitt *et al.*, unpublished results). MasG represents the activating enzyme of (1-methylalkyl)succinate synthase, homologous to BssD (Grundmann *et al.*, 2008). The two remaining ORFs (*masA, masF*) in the *mas* operon of strain HxN1 are not directly linked to the anaerobic *n*-alkane activation. MasA is similar to acyl-CoA dehydrogenases and might be involved in the further downstream β-oxidation of *n*-alkanes, whereas MasF was designated as transposase (Grundmann *et al.*, 2008). In *D. alkenivorans* strain AK-01 two operons (*ass1* and *ass2*) coding for proteins of the anaerobic *n*-alkane activation were identified (Callaghan *et al.*, 2008). The Ass proteins showed homologies to the Bss proteins as well.

A definite proof of the predicted function of (1-methylalkyl)succinate synthase requires the analysis of *mas* deletion mutants. For an anaerobic *n*-alkane degrading strain a genetic approach was not available so far. Herein, we describe the development of a genetic system for strain HxN1 and the exemplary deletion of gene *masD*, presumably encoding the catalytic subunit of the *n*-alkane activating enzyme.

Material and Methods

Bacterial strains and plasmids

The bacterial *Escherichia coli* and HxN1 strains and the plasmids used in this study are described in table 1. Strain HxN1 has been maintained in the laboratory since its isolation from a nitrate-reducing, *n*-hexane degrading enrichment culture (Ehrenreich *et al.*, 2000). Chemically competent *E. coli* cells were prepared according to the calcium chloride method as described by Cohen *et al.* (1972).

Table 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Marker	Genotype and characteristics	Reference or source
Strains			
E. coli S17-1		*thi recA pro hsdR* RP 4-2-Tc::MU-Km::Tn7	Simon *et al.* (1983)
E. coli TOP10	Str^R	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen (Darmstadt, Germany)
HxN1		wild type	Ehrenreich *et al.* (2000)
HxN1 Δ*masD*	Km^R	Δ*masD*	this study
HxN1 Δ*masD*, Δ*masD´*	Km^R, Gm^R	Δ*masD*, Δ*masD´*	this study
Plasmids			
pCM184	Ap^R, Km^R, Tc^R		Marx & Lidstrom (2002)
pCM184_*masABC*	Ap^R, Km^R, Tc^R	*masA*, *masB, masC*	this study
pCM184_Δ*masD*	Ap^R, Km^R, Tc^R	*masA, masB, masC, masE, masF*	this study
pCM184_Δ*masD*_ *aacC1*	Ap^R, Gm^R, Tc^R	*masA, masB, masC, masE, masF*	this study
pBBR1MCS	Cm^R		Kovach *et al.* (1994)
pBBR1MCS_*masD*	Cm^R	*masD* + ribosomal binding site	this study
pBBR1MCS_ *mas* operon	Cm^R	*mas* operon + 1kb upstream sequence	this study
pBBR1MCS-2	Km^R		Kovach *et al.* (1995)
pBBR1MCS-5	Gm^R		Kovach *et al.* (1995)

Cultivation and growth media

E. coli strains were cultivated at 37 °C in Luria Bertani (LB) medium. Strain HxN1 was cultivated in defined anoxic mineral medium at 28 °C under a head space of an N_2/CO_2 mixture as described (Rabus & Widdel, 1995; Ehrenreich *et al.*, 2000). As carbon sources *n*-hexane, provided as a 5% (v/v) solution in heptamethylnonane, or caproate at a final concentration of 5 mM were supplied. Nitrate was added regularly in portions ≤ 5 mM.

Anaerobic growth experiments with *n*-hexane or caproate were performed in tubes with 10 ml of anoxic medium. HxN1 cultures grown with caproate served as inoculum. Prior inoculation, the cultures were washed once with anoxic medium to remove remaining caproate. An approach without added carbon source served as negative control. The optical density (OD) was monitored at 600 nm directly in the tubes (UV-1202, Shimadzu, Duisburg, Germany). The amount of nitrate and nitrite in the cultures was determined with Merckoquant test stripes (Merck, Darmstadt, Germany). Nitrate was re-added in portions of 5 mM after it has been consumed.

The solid mineral medium contained per liter 0.1 g $CaCl_2$ and 0.3 g NH_4Cl. Following autoclaving in a smaller volume, the following components were added from sterile stock solutions: 8 ml HCl (1 M), 6.67 ml $MgSO_4$ (15 g 100 ml^{-1}), 8 ml KH_2PO_4 (1 M), 32 ml K_2HPO_4 (1 M), each 1 ml of vitamins, an EDTA-chelated mixture of trace elements and selenate/tungsten solution (Widdel & Bak, 1992), 5 ml fructose (1 M) as carbon source and 5 ml $NaNO_3$ (1 M) as electron acceptor for anoxic incubation. The pH was adjusted to 7.0–7.2. Finally, separately autoclaved agar was added to a concentration of 1.5% (v/v). The same medium without agar served as oxic liquid mineral medium.

Antibiotics in liquid and solid medium were added at the following concentrations: ampicillin (50 µg ml^{-1}), chloramphenicol (20 µg ml^{-1}), gentamycin (10 µg ml^{-1}), kanamycin (45 µg ml^{-1}), neomycin (30 µg ml^{-1}), rifampicin (200 µg ml^{-1}), streptomycin (50 µg ml^{-1}), tetracycline (20 µg ml^{-1}).

DNA techniques

Genomic DNA was isolated with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Plasmid DNA was isolated by alkaline lysis with SDS as described (Sambrook & Russell, 2001). Amplification by polymerase chain reaction (PCR) was performed with *Taq* DNA Polymerase (Fermentas, St. Leon-Rot, Germany) or Phusion High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) according to the instructions. Oligonucleotide sequences are listed in table 2. DNA restriction enzymes were applied according to the instructions (Fermentas). For analysis or further

purification, PCR-products and restricted plasmid DNA were run on 1 to 1.5% agarose gels. DNA size marker (GeneRuler 100 bp Plus DNA Ladder and GeneRuler 1 kb DNA Ladder) were obtained from Fermentas. Restricted DNA was purified with the QIAquick Gel Extraction Kit (Qiagen) from agarose gels or with the QIAquick PCR Purification Kit (Qiagen). PCR products and plasmids were ligated in a 2:1 ratio with T4 DNA Ligase (Fermentas). Sequencing was performed with the BigDye v3.0 terminator cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) on an ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems). Sequencing data were analyzed with the Lasergene software (DNASTAR, Konstanz, Germany).

Construction of a *masD* deletion cassette in pCM184

The upstream and downstream regions of *masD* were cloned consecutively into the vector pCM184 (Marx & Lidstrom, 2002). First, the upstream region beginning with the start codon of *masA* and ending directly before the start codon of *masD* was amplified from genomic DNA of strain HxN1 with primers containing recognition sites. The PCR-product was cloned into the first MCS of pCM184 upstream of the *kan* gene. The obtained plasmid was named pCM184_*masABC*. Second, the downstream region beginning directly behind the stop codon of *masD* and ending with the stop codon of *masF* was amplified from genomic DNA of strain HxN1 with one primer containing a recognition site and a second primer generating a blunt end. The PCR-product was cloned into the second MCS of pCM184_*masABC* downstream of the *kan* gene. The *Hpa*I recognition site of the plasmid was used to generate a blunt end. The obtained plasmid was named pCM184_Δ*masD*. A second deletion cassette with an alternative antibiotic resistant marker was obtained by exchanging the *kan* gene in pCM184_Δ*masD* with the *aacC1* gene. The *kan* gene and its ribosomal binding site were cut from pCM184_Δ*masD* with *Sbf*I. The *aacC1* gene including its ribosomal binding site was amplified from pBBR1MCS-5 (Kovach *et al.*, 1995) with primers containing *Pst*I recognition sites and cloned into the *Sbf*I-cut pCM184_Δ*masD*. *Sbf*I and *Pst*I generate the same overhangs. This plasmid was named pCM184_Δ*masD_aacC1*.

Table 2 Oligonucleotide primers used in this study; restriction sites are underlined.

Primer	Target gene or region	Sequence (5´→ 3´)	Product length [bp]
masD deletion construct in pCM184			
*masABC_Eco*RI_f	upstream *masD*	CTGAATTCATGAATCGCGCGCACTTT	1842
*masABC_Nco*I_r		ATCCATGGGATTCAATCCTCCTAAGG	
*masEF_Apa*I_f	downstream *masD*	CAGGGCCCAAAATTCATTTAATTAGG	1991
*masEF*_r		ATGGATCCCTAATTGGACTGTGTCATTAA	
*aacC1_Pst*I_f	*aacC1*	ATGCTGCAGCCGATCTCGGCTTGAACGA	674
*aacC1_Pst*I_r		ATGCTGCAGCAGTGGCGGTTTTCATGGC	
Confirmation of double crossover			
*masD*_f	*masD*	CTGCAACTTCAACACTATCC	2438
*masD*_r		ACCAGCCACACGAACGATA	
*bla*_f	*bla*	ACATTTCCGTGTCGCCCTTA	837
*bla*_r		ATCAGTGAGGCACCTATCTC	
*kan*_f	*kan*	ATGAGCCATATTCAACGGGA	708
*kan*_r		GAGGCAGTTCCATAGGATG	
*aacC1*_f	*aacC1*	ATGTTACGCAGCAGCAAC	525
*aacC1*_r		GGTACTTGGGTCGATATCA	
*masA*_f	*masA to masG*	CGGCTACACGTCGATTGAAG	6534[a]/
*masG*_r		ATGACACCAAGCACATCGCA	5417[b]
masD complementation			
*masD_Xho*I_f	*masD*	GACTCGAGAGCATTCACCATCTA	2520
*masD_Hind*III_r		CTGAAGCTTCCACATTCTGTGCAT	
mas operon_f	*mas* operon	GGTACAGCGCCAACCACTCGTAGAT	8356
mas operon_*Spe*I_r		ATTACTAGTGTTAATAGAAGACGCCGCTAT	
Probe for southern blot			
southern probe_f	*masA*	TTCAGAGCTATTGACCCGTG	505
southern probe_r	*masB*	GACAGTACTTGGCGTCACTA	

[a] referring to HxN1 wild type; [b] referring to HxN1 Δ*masD*

Construction of complementation plasmids

The *masD* gene including its ribosomal bindung site and the complete *mas* operon including around 1 kb of upstream sequence were amplified from genomic DNA of strain HxN1 and cloned into the broad-host-range vector pBBR1MCS under the control of the *lac* and the T3 promoter (Kovach *et al.*, 1994). The plasmids were designated as pBBR1MCS_*masD* and pBBR1MCS_*mas* operon.

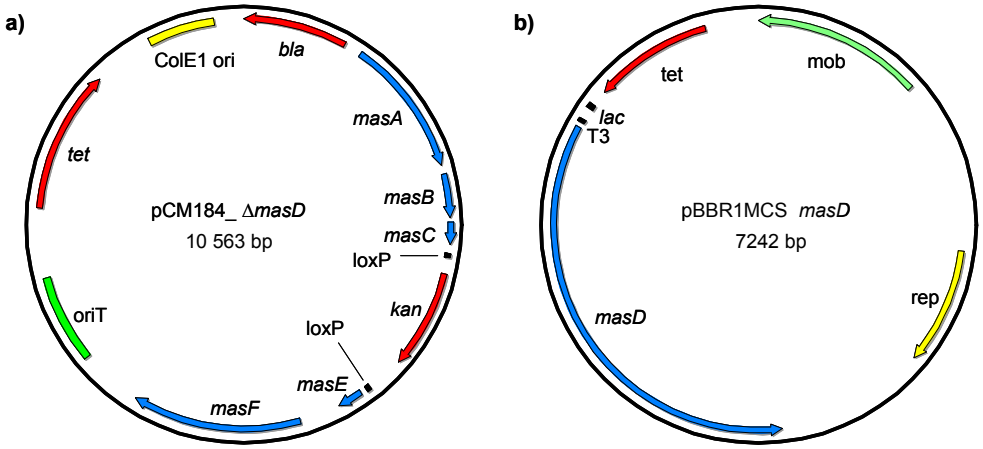

Fig. 1 Schematic depiction of the constructed plasmids used in this study. **a)** pCM184_Δ*masD* for generation of a Δ*masD* deletion mutant of strain HxN1. **b)** pBBR1MCS_*masD* for complementation of the Δ*masD* deletion in strain HxN1. Blue: *mas* genes; red: antibiotic resistance marker; yellow: origin of replication; green: origin of transfer; black: loxP sites in pCM184 and promoters T3 and *lac* in pBBR1MCS.

Plasmid transfer

Ligation reactions were transformed into chemically competent *E. coli* TOP10 as described by Inoue *et al.* (1990). For conjugation, the appropriate plasmids were transformed into the donor *E. coli* S17-1. For conjugational plasmid transfer, overnight cultures of strain *E. coli* S17-1 containing the appropriate plasmid served as donor. The recipient, strain HxN1, was used in its exponential growth phase on caproate. Cultures of *E. coli* S17-1 and strain HxN1 were adjusted to an optical density at 600 nm of 1, washed once with oxic mineral medium and then mixed in a 2:1 (recipient : donor) ratio in a final volume of 1 ml. This mixture was concentrated by centrifugation (16 000 x *g*, 5 min) to 50 µl and spotted as single drop onto solid mineral medium without antibiotics.

Incubation was performed aerobically at 28 °C for 9 h. The drop was washed from the solid medium with 1 ml of oxic mineral medium and serial dilutions were plated onto solid mineral medium containing the convenient antibiotics. Incubation was carried out at 28 °C for 5 days in anaerobic jars under an N_2-atmosphere.

Identification of HxN1 Δ*masD* clones

Colonies obtained on solid medium with kanamycin after conjugation of HxN1 with pCM184_Δ*masD* were streaked onto new solid mineral medium containing kanamycin to enable a double crossover. Transconjugants of HxN1 Δ*mas*::*kan* (= HxN1 Δ*masD*) with pCM184_Δ*masD_aacC1* were streaked onto new solid mineral medium containing kanamycin and gentamycin to select for a double crossover. Afterwards, single colonies were transferred into 150 µl oxic mineral medium with antibiotics. Cultures were allowed to grow for three days before being transferred into 5 ml of anoxic mineral medium with antibiotics and caproate as carbon source. All incubations were performed at 28 °C. For PCR analysis, 0.5 ml of the cultures was centrifuged (10 000 x *g*, 5 min, 4 °C). The pellet was suspended in 50 µl PCR-H_2O and 1 µl of this suspension served as template in the PCR-reaction. PCR was performed to verify a double crossover by applying primer pairs which target i) *masD*, ii) *bla*, iii) *kan*, iv) *aacC1* and v) the *mas* operon. Colonies obtained from conjugations of HxN1 Δ*mas*::*kan*, Δ*masD´*::*aacC1* (= HxN1 Δ*masD*, Δ*masD´*) with the complementation plasmids were directly transferred into liquid mineral medium with convenient antibiotics.

Southern blot

1 µg of chromosomal DNA of HxN1 wild type, Δ*masD* and Δ*masD*, Δ*masD´* mutants was digested with *Pst*I and separated on 0.75% agarose gels. Prior transfer, the DNA was partially hydrolyzed (0.25 M HCl) to simplify transfer, denatured (0.5 M NaOH, 1 M NaCl) and neutralized (1 M NH_4ClAc, 0.02 M NaOH). Afterwards, the DNA was transferred onto a positively charged nylon membrane (Amersham Hybond-N+ 0.45 µm, GE Healthcare, Munich, Germany) by dry capillary blotting. Following transfer, the DNA was immobilized on the membrane by UV irradiation at 254 nm for 2 min at 1.5 J cm^{-2} (Biolink DNA Crosslinker, Biometra, Göttingen, Germany). A specific probe derived from PCR was labelled with biotin-dUTP using the Biotin DecaLabel DNA Labeling Kit (Fermentas). The biotinylated hybridized probe was detected with a streptavidin-HRP conjugate and the chemiluminescent substrate luminol. Hybridization and detection was performed with the North2South Chemiluminescent Hybridization and Detection Kit according to the instructions (Pierce Protein, Rockford, USA). Hybridization signals were

visualized with an ECL camera system (BIS 303 PC Bioimaging, Amersham Pharmacia Biotech/GE Healthcare). For molecular weight determination the Biotinylated DNA Molecular Weight Marker (Vector Laboratories, Burlingame, USA) was used.

SDS-PAGE and Western blot

Cell extracts of HxN1 wild type and mutants adjusted to an optical density at 600 nm of 4 were run on 12% polyacrylamide gels as described by Laemmli (1970). The PageRuler Prestained Protein Ladder from Fermentas served as molecular weight marker. Immunoblotting experiments for the detection of MasC, MasD and MasE are described in Schmitt *et al*. (unpublished results).

Results and Discussion

Establishment of conjugational DNA transfer into strain HxN1

Characterization of deletion mutants requires the possibility to isolate genetically modified clones. Hence, a solid growth medium was developed, because strain HxN1 was unable to grow on standard solid LB medium. The originally described defined anoxic mineral medium (Rabus & Widdel, 1995; Ehrenreich *et al.*, 2000) was modified as follows: the medium was buffered with phosphate instead of bicarbonate and fructose was added as carbon source instead of caproate. On this newly developed solid mineral medium, white colonies with a size of 0.5 to 1 mm in diameter grew after four to five days of incubation at 28 °C both under oxic and anoxic conditions.

Since electroporation of strain HxN1 remained unsuccessful, conjugation was chosen for the introduction of DNA into strain HxN1. Conjugation has been effectively applied in *Aromatoleum aromaticum* strain EbN1, an anaerobic aromatic hydrocarbon degrading strain, which is related to strain HxN1 (Wöhlbrand & Rabus, 2009). A common method to select between donor and recipient strain in conjugation-mediated DNA transfer is to generate an antibiotic resistant mutant of the recipient strain (Schultheiss & Schuler, 2003; Wöhlbrand & Rabus, 2009). However, all attempts to generate an antibiotic resistant mutant of strain HxN1 failed. Growth of strain HxN1 was completely inhibited in anoxic liquid and solid mineral medium by ampicillin (50 µg ml^{-1}), chloramphenicol (20 µg ml^{-1}), gentamycin (10 µg ml^{-1}), kanamycin (45 µg ml^{-1}), neomycin (30 µg ml^{-1}), rifampicin (200 µg ml^{-1}), streptomycin (50 µg ml^{-1}) and tetracycline (20 µg ml^{-1}). Thus, the disability of *E. coli* S17-1 to grow on the solid mineral medium served as selection marker.

Uptake of foreign DNA was established by conjugational transfer of the broad-host-range cloning vector pBBR1MCS-2 (Kovach *et al.*, 1995) from the donor *E. coli* S17-1 to strain HxN1. The pBBR1MCS- series of vectors are relatively small (~ 5 kb), have an extensive multiple cloning site and are available in variants with different antibiotic marker resistance genes (Kovach *et al.*, 1994; Kovach *et al.*, 1995). These features make them an appropriate tool for the establishment of conjugational transfer and for later complementation of the deleted gene. The pBBR1MCS vectors are not self-transmissible. Instead they are transferred by the *tra* functions of plasmid RP4 that are integrated into the chromosome of *E. coli* S17-1 (Simon *et al.*, 1983). The vector pBBR1MCS-2 carries a kanamycin resistance gene (*kan*) for screening purposes. Transfer of this vector from *E. coli* S17-1 to strain HxN1 yielded kanamycin resistant colonies after 5 days of anoxic incubation. Conjugation frequencies varied between 1.1×10^{-3} and 1.8×10^{-4}. For the transfer of pBBR1MCS-4 from *E. coli* S17-1 to strain EbN1 a conjugation frequency of 1.3×10^{-5} was reported (Wöhlbrand & Rabus, 2009). Within the same range conjugation frequencies were also reported for the proteobateria *Bartonella henselae* (2×10^{-5}), *Chromatium vinosum* (3.6×10^{-4} to 7.5×10^{-2}) and *Eikenella corrodens* (2.5×10^{-7}) with *E. coli* S17-1 as donor (Rao *et al.*, 1993; Pattaragulwanit & Dahl, 1995; Dehio & Meyer, 1997). The presence of pBBR1MCS-2 in the HxN1 transconjugants was verified by PCR targeting the *kan* gene, indicating replication of the plasmid in strain HxN1 (data not shown).

Generation of a Δ*masD* deletion mutant of strain HxN1

Deletion of *masD* was performed by homologous recombination and gene replacement using the *cre-lox* system of Marx & Lidstrom (2002). This system is based on a positive screening for antibiotic resistant mutants and further allows the generation of multiple mutants by antibiotic marker recycling in a second step. For homologous recombination with the bacterial DNA of strain HxN1, the narrow host range vector pCM184 was used (Marx & Lidstrom, 2002). The pCM184 vector carries three antibiotic resistance marker genes (*bla, kan, tet*). The *kan* gene is flanked by two loxP sites and two multiple cloning sites and replaces the deleted gene after homologous recombination. The loxP sites are recognition sites for the Cre recombinase, encoded on vector pCM157, which enable the generation of an unmarked deletion mutant (Marx & Lidstrom, 2002). The two additional antibiotic genes encoded in pCM184, *bla* and *tet*, are markers to screen for a double crossover event.

Due to its narrow host range, limited to *E. coli* and closely relatives, the plasmid pCM184 is only maintained in HxN1 cells if the DNA integrates into the genome. Consequentially,

the transfer of pCM184 without homologous insertions from *E. coli* S17-1 to strain HxN1 did not yield any transconjugants. Additionally, this served as control for the efficiency of the selection criteria: *E. coli* S17-1 harboring pCM184 should be able to grow in the presence of kanamycin. Since *E. coli* S17-1 is unable to grow on the solid mineral medium no colonies were obtained.

For deletion of *masD*, the vector pCM184_Δ*masD* (Fig. 1a) was transferred by conjugation from *E. coli* S17-1 into strain HxN1. Following crossover of the homologous regions of pCM184_Δ*masD* with their counterparts in strain HxN1, *masD* should be replaced by *kan*, resulting in a marked deletion mutant. The integration frequency of pCM184_Δ*masD* into the genome of strain HxN1 was 7.06×10^{-6}, as displayed by kanamycin resistant colonies. Similar integration frequencies (2.2×10^{-6}) with the *cre-lox* system were reported for *Magnetospirillum gryphiswaldense* (Gärdes, 2005). Obtained kanamycin resistant colonies were streaked onto fresh solid medium containing kanamycin to promote a double crossover in the absence of ampicillin and tetracycline. The generation of a marked deletion mutant and the following screening in the presence of an antibiotic has the advantage over the generation of an unmarked mutant that a reversion to the wild type genotype is omitted. Accordingly, the number of Δ*masD* mutants should be higher with the *cre-lox* system than with a system, which generates unmarked deletion mutants as for instance the *sacB* system (Schäfer *et al.*, 1994). 20% of the screened clones of strain HxN1 showed a deletion of *masD*, whereas the remaining clones still contained the plasmid backbone, indicating a single crossover. It is assumed that a double crossover will also happen in these clones after longer incubation without selective pressure towards ampicillin and tetracycline, yielding a Km^R, Ap^S, Tc^S phenotype. In comparison, the generation of an unmarked deletion mutant of *A. aromaticum* strain EbN1 with the *sacB* system yielded less than 4% of double crossover mutants (Wöhlbrand & Rabus, 2009).

Identification of a second *mas* operon in strain HxN1

The potential deletion mutants were analyzed by PCR. Unexpectedly, the *masD* and the *kan* gene were both present, although removal of the plasmid DNA backbone was confirmed in a PCR targeting the *bla* gene (product size 837 bp), which was negative for the mutant (Fig. 2). To elucidate these contradictory results, another PCR reaction was performed. The used primers were localized outside the regions, which are part of the deletion cassette in pCM184_Δ*masD* to exclude binding of the primers to the cassette, in case it has integrated elsewhere in the genome. The forward primer hybridizes 73 bases upstream of *masA*, the reverse primer within *masG*. The expected length of the PCR

product for the deletion mutant was 5417 bp, whereas for the wild type a length of 6534 bp was calculated.

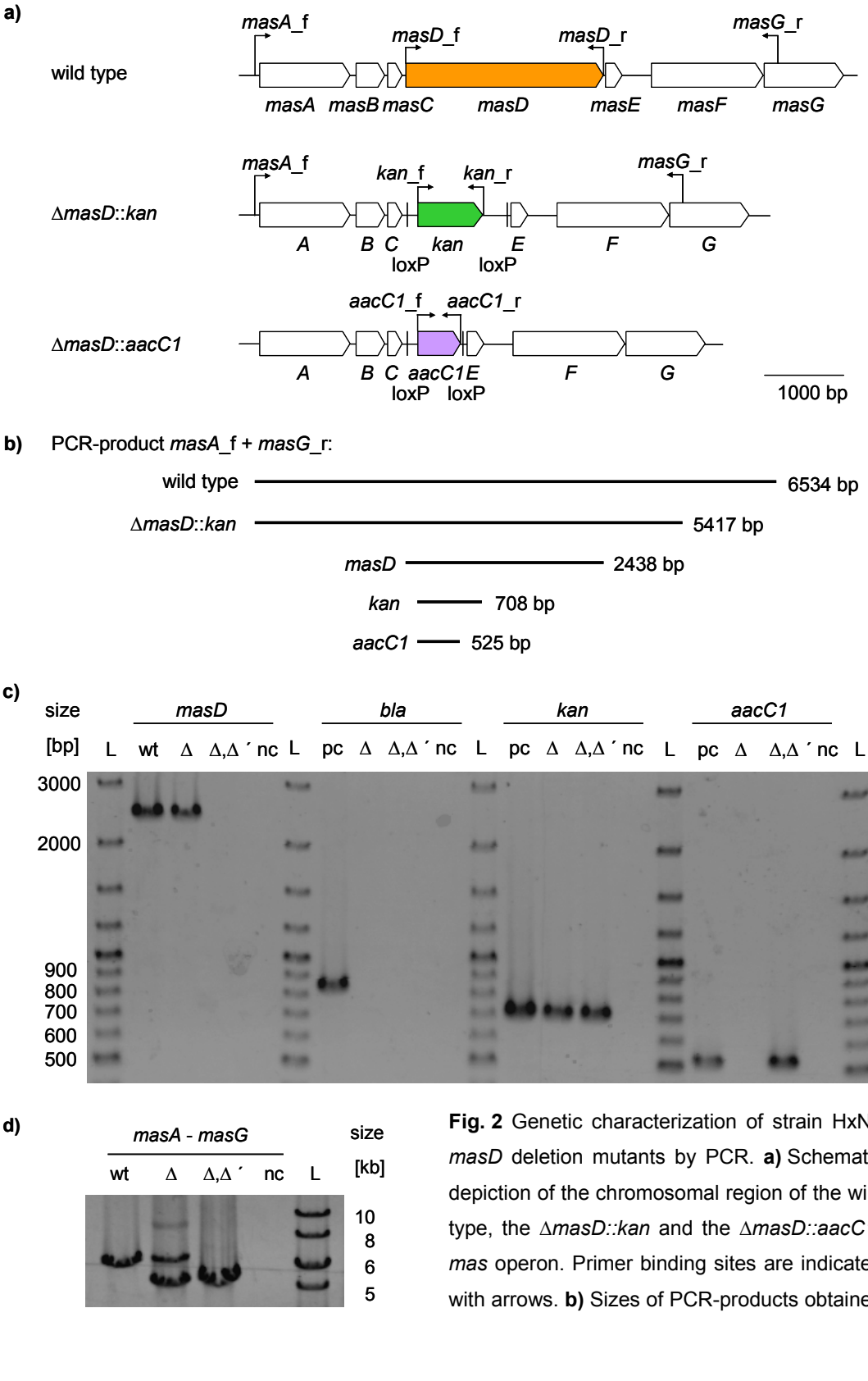

Fig. 2 Genetic characterization of strain HxN1 *masD* deletion mutants by PCR. **a)** Schematic depiction of the chromosomal region of the wild type, the Δ*masD::kan* and the Δ*masD::aacC1´ mas* operon. Primer binding sites are indicated with arrows. **b)** Sizes of PCR-products obtained

with the applied primers. **c)** Electropherogram of PCR-products obtained for the HxN1 wild type (abbreviated as wt), the Δ*masD* (Δ) and the Δ*masD*, Δ*masD´* mutant (Δ,Δ´). A negative control (nc) was performed without template. As positive controls (pc) plasmid-DNA of pCM184 for *bla* and *kan* and of pBBR1MCS-5 for *aacC1* was used. L: molecular weight ladder. **d)** Electropherogram of the PCR-product obtained with the primer pair targeting the *mas* operon in the HxN1 wild type (wt), the Δ*masD* (Δ) and the Δ*masD*, *masD´* (Δ,Δ´) mutant. A negative control (nc) was performed without template.

Unexpectedly, the mutant strain showed both signals (Fig. 2). This pointed to the presence of at least two *mas* operons in strain HxN1. Following conjugational transfer of pCM184_Δ*masD*, homologous recombination occurred with one *mas* operon, while the other *mas* operon(s) were not affected due to transfer of only one plasmid molecule during conjugation.

For further investigation of the obtained Δ*masD* mutant, Southern blot analysis was performed. The applied probe hybridizes with parts of the genes *masA* and *masB* (Fig. 3). Following restriction digest of genomic DNA with *Pst*I, the probe bound in the wild type to a DNA fragment with a size of 7300 bp (Fig. 3). In a mutant, where *masD* is replaced by *kan*, the probe should hybridize with a 6150 bp fragment, due to the smaller size of the *kan* gene as compared to *masD*. The present mutant strain yielded two hybridization signals (Fig. 3), both for the wild type and for the Δ*masD*::*kan mas* operon, which further supports the hypothesis of two or more *mas* operons in the genome of strain HxN1. Assuming another *mas* operon to be present in strain HxN1, a second deletion cassette with another marker gene (*aacC1*) for resistance against gentamycin was constructed for homologous recombination with the remaining wild type *mas* operon. The vector pCM184_Δ*masD_aacC1* was introduced into HxN1 Δ*masD* by conjugation, which resulted in kanamycin and gentamycin resistant colonies. The presence of kanamycin prevents from a double crossover with the already mutated *mas* operon, where *masD* is replaced by *kan*. Only those colonies, which harbor two modified *mas* operons were able to grow on mineral medium containing kanamycin and gentamycin. PCR analysis targeting the *masD, bla* and *aacC1* genes was performed to verify a double crossover in potential mutants (Fig. 2). Concurrent with the absence of *bla*, *masD* was not detected anymore, which confirmed a double crossover and excluded the presence of more than two *mas* operons in strain HxN1 (Fig. 2). In the Δ*masD,* Δ*masD´* mutant, both antibiotic marker genes, *kan* and *aacC1*, which have been integrated into the genome, were detected. The primer pair, which amplifies the *mas* operon did not yield the 6534 bp PCR-product for the wild type *mas* operon anymore, but only the 5417 bp PCR-product for the Δ*masD::kan mas* operon. A second PCR-product

for the Δ*masD::aacC1 mas* operon with a calculated size of 4858 bp was not obtained, probably due to suboptimal conditions of the PCR conditions (Fig. 2d).

Integration of *aacC1* into the *mas* operon also results in a different restriction pattern with *Pst*I in comparison to the wild type and the Δ*masD::kan mas* operon. Accordingly, in Southern blot analysis the probe should hybridize in the *mas* operon where *masD* is replaced by *aacC1* with a 5600 bp fragment. Consequentially, the HxN1 mutant strain with two modified *mas* operons showed two signals: the 6510 bp signal for the Δ*masD::kan mas* operon and the 5600 bp signal for the Δ*masD::aacC1 mas* operon, whereas the 7300 bp signal of the wild type *mas* operon was absent (Fig. 3).

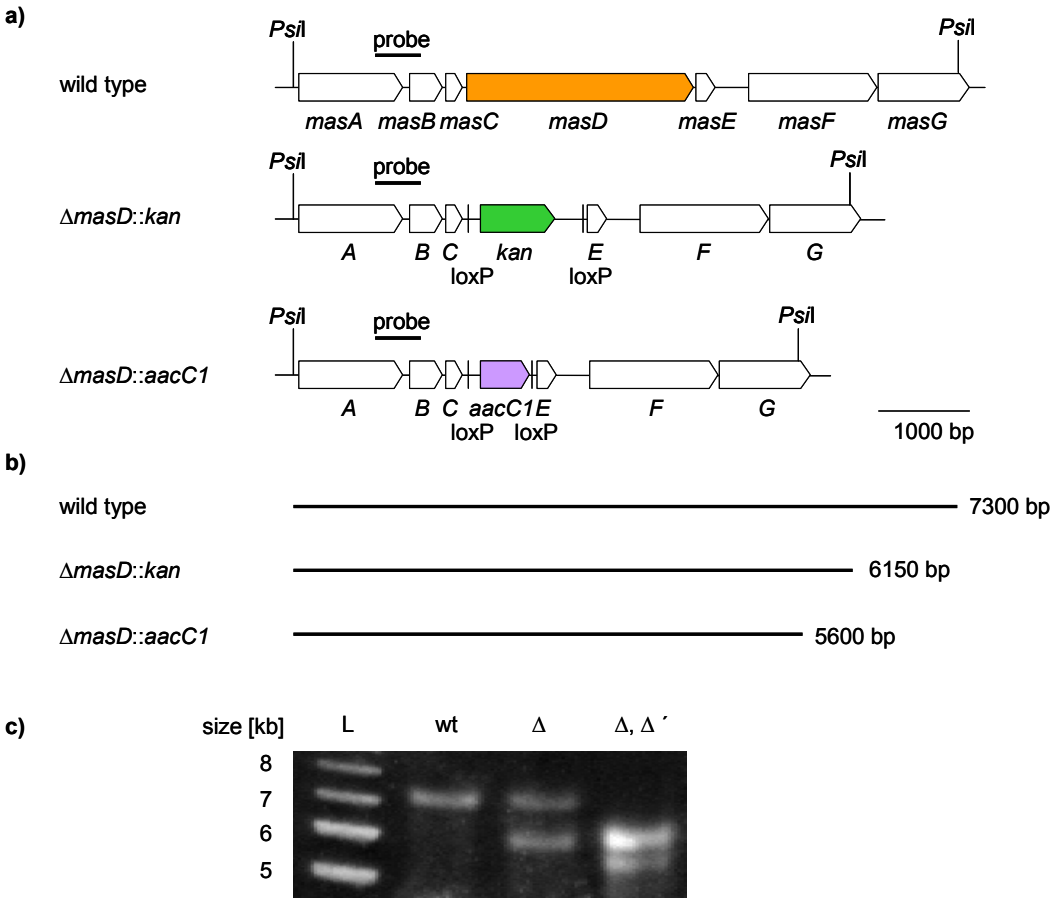

Fig. 3 Genetic characterization of strain HxN1 *masD* deletion mutants by Southern blot. **a)** Schematic depiction of the chromosomal region of the wild type *mas* operon, the Δ*masD::kan mas* operon and the Δ*masD*::*aacC1 mas* operon. Restriction sites of *Pst*I and the hybridization site of the probe are indicated. **b)** Sizes of fragments obtained by restriction with *Pst*I in the wild type *mas* operon, the Δ*masD::kan* and the Δ*masD*::*aacC1 mas* operon of strain HxN1. **c)** Detection of the *Pst*I restriction fragments in the HxN1 wild type (abbreviated as wt), the Δ*masD* (Δ) and the Δ*masD*, Δ*masD´* (Δ,Δ´) mutant by Southern blot. L: biotinylated molecular weight ladder.

Sequencing of the remaining *masD* gene of the Δ*masD* mutant revealed a sequence completely identical to the one, which has been deposited in GenBank under Accession No. AM748709 (Grundmann *et al.*, 2008). We assume that both *mas* operons are identical in their sequence because sequencing always resulted in only one sequence and not in ambiguous chromatograms. In addition, the *mas* operons need to be identical in the 73 bases upstream of *masA*, otherwise the forward primer for the amplification of the *mas* operon could not have bound to the mutated and to the wild type *mas* operon (Fig. 2). Apart from the sequence of the *mas* operon, around 3 kb of upstream sequence were retrieved in a clone library. However, the upstream sequence is covered by only one clone completely and partially by two more clones. Downstream of the *mas* operon, the sequence is unknown at all. As long as more sequence information is not available, it remains unclear, where the sequences upstream and downstream of the *mas* operons start to differ from each other. Accordingly, the location of the two *mas* operons in the genome of strain HxN1 is presently unknown. Therefore, the antibiotic marker genes in the mutants were not recycled by Cre recombinase, because Cre-mediated recombination might result in the removal of the sequence between the two *mas* operons, which both harbor loxP recognition sites.

The presence of two *mas* operons is not unique to strain HxN1. The anaerobic *n*-alkane degrading sulfate reducer *D. alkenivorans* strain AK-01 contains two *ass* operons as well, but in contrast to strain HxN1 the *ass* operons are not identical to each other (Callaghan *et al.*, 2008). Two or more non-identical copies of the genes encoding an *n*-alkane activating enzyme have not only been identified in anaerobic but also in aerobic *n*-alkane degrading bacteria (van Beilen *et al.*, 2001; van Beilen *et al.*, 2004). As the given examples indicate, multiple copies of *n*-alkane activating enzyme encoding genes are widespread. However, strain HxN1 differs from other known strains with more than one gene for an *n*-alkane activating enzyme by having two completely identical copies of an entire operon.

Physiological characterization of the HxN1 Δ*masD* and Δ*masD*, Δ*masD´* mutants

The obtained Δ*masD* and Δ*masD*, Δ*masD ´*mutants were analyzed regarding their ability to grow anaerobically with *n*-hexane. The Δ*masD* mutant was, after a prolonged lag-phase as compared to the wild type, still able to grow with *n*-hexane (Fig. 4). Growth was only slightly impaired in the presence of the two antibiotics, kanamycin and gentamycin. The ability of the Δ*masD* mutant to grow with *n*-hexane as well as the difference in the lag phase between wild type and mutant confirms the functionality of both *mas* operons. In the Δ*masD* mutant the lag phase is prolonged, because with less amount of

(1-methylalkyl)succinate synthase it takes longer to reach substrate saturation and the maximal reaction rate of the enzyme. Thus, a duplication of the *mas* operon optimizes growth with *n*-alkanes in the beginning, while later on the metabolic rate is limited by substrate saturation. Therefore, the doubling time (23 hours) and the growth rate (μ = 0.029) of the wild type and the Δ*masD* mutant were identical. Under optimal conditions the doubling time of strain HxN1 is 9 to 11 hours (Ehrenreich *et al*., 2000; Schmitt *et al*., unpublished results). The higher doubling times observed in this study are the result of stepwise addition of small amounts of nitrate (≤ 5 mM) instead of supply with 8.5 to 9 mM (Ehrenreich *et al.*, 2000) or continuous supply in a fermenter (Schmitt *et al*., unpublished results). The proposed function of the *mas* encoded (1-methylalkyl) succinate synthase was confirmed by the inability of the Δ*masD*, Δ*masD´* mutant to grow with *n*-hexane (Fig. 4). Control experiments with caproate as carbon source showed no effect of the *masD, masD´* deletion on the capability of strain HxN1 to degrade fatty acids (data not shown).

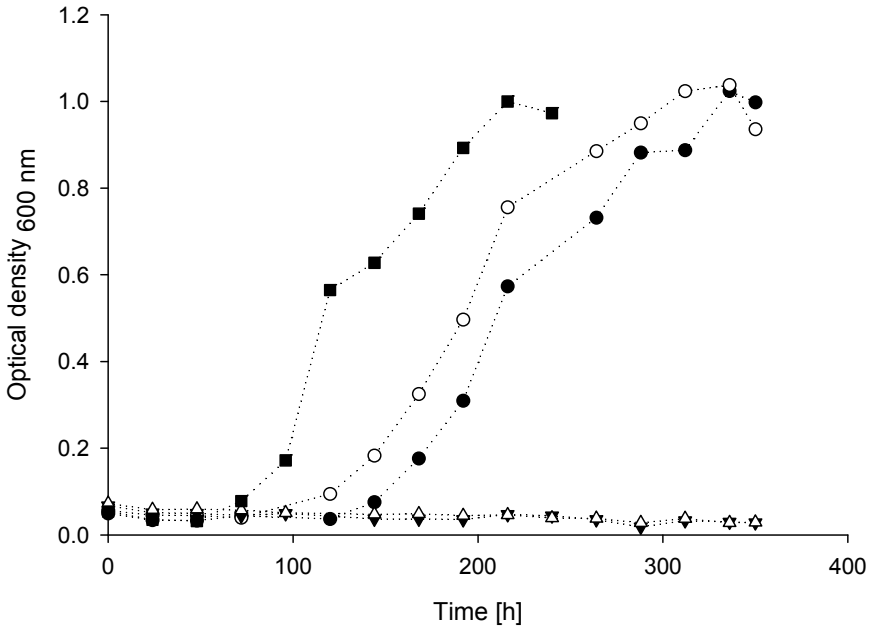

Fig. 4 Time course of anaerobic growth of strain HxN1 wild type, Δ*masD* and Δ*masD,* Δ*masD´* with *n*-hexane. Growth of the wild type (black squares) starts after three days, whereas the lag phase in the Δ*masD* mutant (black and white circles) is prolonged. With antibiotics (black circles) growth is slightly inhibited. The Δ*masD,* Δ*masD´* mutant (black and white triangles) is unable to grow with *n*-hexane.

In trans expression of *masD* by complementation

To restore the capability of the Δ*masD*, Δ*masD´* mutant to grow with *n*-alkanes anaerobically, *masD* was introduced into strain HxN1 on the vector pBBR1MCS (Fig. 1b) (Kovach *et al.*, 1994). Despite *in trans* expression of *masD* was confirmed in Western blot (Fig. 5), the complemented mutant failed to grow with *n*-hexane, independent of the presence or absence of antibiotics. As revealed by Western blot, the signal obtained for MasD of the complemented mutant is weaker, compared to the wild type of strain HxN1 grown with *n*-hexane (Fig. 5). Probably, expression of *masD* under the control of the *lac* and the T3 promoter of the vector is suboptimal, but constitutively. However, reduced growth should also be possible with low amounts of (1-methylalkyl)succinate synthase.

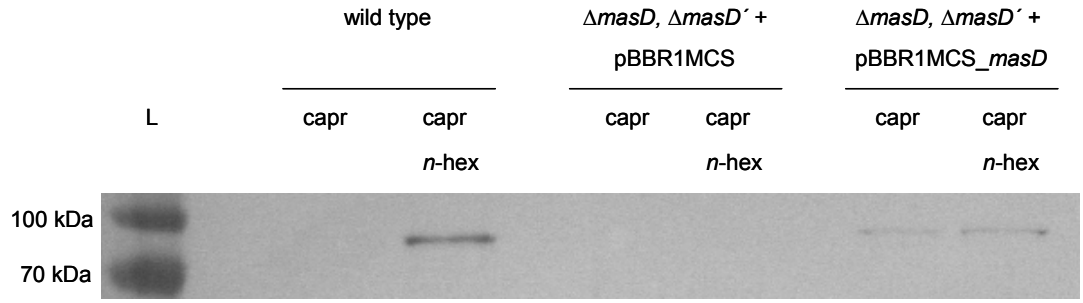

Fig. 5 Western blot for verification of *masD* expression in the complemented Δ*masD,* Δ*masD´* mutant. The HxN1 wild type and the Δ*masD,* Δ*masD´* mutants were grown with caproate either alone or in addition with *n*-hexane. Obtained signals in the Δ*masD,* Δ*masD´* mutant complemented with pBBR1MCS_*masD* during growth with caproate and *n*-hexane indicate constitutive expression of *masD* under the control of the plasmid promoters. The Δ*masD,* Δ*masD´* mutant with the empty pBBR1MCS plasmid served as negative control. The HxN1 wild type served as positive control. For the wild type, a signal for MasD was only obtained during growth with *n*-hexane, because the *mas* operon needs to be induced. L: pre-stained ladder.

As the deletion and insertion of genes can cause polar effects on the adjacent genes, expression of *masC* and *masE* was analyzed on the protein level in Western blot. MasC and MasE probably represent the small subunits of (1-methylalkyl)succinate synthase and thus, are needed for enzyme activity. For this purpose, the mutants were grown in the presence of *n*-hexane and caproate to promote expression of the *mas* operon, which is only induced in the presence of *n*-alkanes (Grundmann *et al.*, 2008), while caproate served as carbon source to enable growth of the mutants. Expression of the *mas* operon was shown to be not inhibited in the presence of a second carbon source (Webner *et al.*, unpublished results). Western blot confirmed polar effects, because MasC and MasE were not detectable in the mutant strain (data not shown). Polar effects were also

observed in *T. aromatica* strain T1 after partial deletion of *tutD*, which encodes the catalytic subunit of the benzylsuccinate synthase (Coschigano, 2002). By introducing a plasmid harboring the entire *tut* gene cluster with an in-frame deletion in the upstream gene of *tutD*, the phenotype was restored (Coschigano, 2002). This proved that only the genes downstream of *tutD* are affected by its deletion. However, in strain HxN1 the downstream as well as the upstream genes are affected by the deletion of *masD*. A possible recombination between the chromosomal and the plasmid encoded *tut* operon of strain T1 was excluded and thus, the ability of the complemented mutant to grow with toluene, was due to complementation rather than recombination (Coschigano, 2002). In *Azoarcus* sp. strain T complementation of the deletion of 96% of the *bssA* gene was achieved by introducing a plasmid harboring the genes *bssA*, *bssB* and *bssC* without any modifications (Achong *et al.*, 2001). The complemented mutant grew again with a reduced growth rate on toluene. However, possible recombination events and polar effects were not analyzed in this case.

Based on the results obtained for the complementation of the benzylsuccinate synthase catalytic subunit, a plasmid harboring the entire *mas* operon plus 1 kb of upstream sequence to include its elements for transcription was constructed for complementation of *masD* in the HxN1 mutant strain. One week later, the complemented mutant started to grow with *n*-hexane in the absence of antibiotics, whereas growth in the presence of all three antibiotics together (kanamycin, gentamycin, chloramphenicol) was not observed (data not shown). In further approaches, the culture, which was grown in the absence of antibiotics, was transferred into new tubes with *n*-hexane, each containing only one of the three antibiotics. In the presence of kanamycin or chloramphenciol the mutant grew, but with gentamycin growth started not until a considerably longer lag phase of at least two weeks (data not shown). Obviously, gentamycin inhibits growth with *n*-hexane by an unknown mechanism, whereas it has no effect onto growth with caproate. Growth might be possible after around two weeks, because gentamycin has become ineffective over time. However, the successful reconstitution of the phenotype proofs the necessity of *masD* for the anaerobic degradation of *n*-alkanes. A possible recombination of the wild type *mas* operon of the plasmid with the mutated *mas* operons in the genome was not investigated, but even in this case, the necessity of *masD* for the degradation of *n*-hexane was confirmed. Re-introduction of *masD* into the genome would demonstrate the effort of the cells to restore optimal conditions for the degradation of *n*-alkanes.

Conclusion

The development of a genetic system for strain HxN1 will allow the investigation of proteins with so far unknown function. Of special interest is MasB, the fourth subunit of (1-methylalkyl)succinate synthase, which is unique to anaerobic *n*-alkane degrading bacteria (Schmitt *et al*., unpublished results). The established genetic system will further be a useful tool to study the proteins of other anaerobic *n*-alkane degrading strains, which differ in their substrate range. Strain HxN1 might represent a convenient model organism due to its relatively fast growth with *n*-alkanes (doubling time of around 11 hours (Ehrenreich *et al.*, 2000)) compared to sulfate-reducing bacteria. Another interesting aspect of strain HxN1 revealed in this study is the identification of two completely identical *mas* operons. As soon as the genome sequence of strain HxN1 will be available, the genetic system will be useful to identify the regulating system of these two *mas* operons.

Acknowledgement

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References

Achong, G.R., Rodriguez, A.M. & Spormann, A.M. (2001) Benzylsuccinate synthase of *Azoarcus* sp. strain T: cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization. *J. Bacteriol.* **183**: 6763–6770.

Callaghan, A.V., Gieg, L.M., Kropp, K.G., Suflita, J.M. & Young, L.Y. (2006) Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two bacterial isolates and a bacterial consortium. *Appl. Environ. Microbiol.* **72**: 4274–4282.

Callaghan, A.V., Wawrik, B., Ni Chadhain, S.M., Young, L.Y. & Zylstra, G.J. (2008) Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. *Biochem. Biophys. Res. Commun.* **366**: 142–148.

Callaghan, A.V., Morris, B.E., Pereira, I.A., McInerney, M.J., Austin, R.N., Groves, J.T. *et al.* (2012) The genome sequence of *Desulfatibacillum alkenivorans* AK-01: a blueprint for anaerobic alkane oxidation. *Environ. Microbiol.* **14**: 101–113.

Cohen, S.N., Chang, A.C. & Hsu, L. (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc. Natl. Acad. Sci. U S A* **69**: 2110–2114.

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

Cravo-Laureau, C., Grossi, V., Raphel, D., Matheron, R. & Hirschler-Réa, A. (2005) Anaerobic *n*-alkane metabolism by a sulfate-reducing bacterium, *Desulfatibacillum aliphaticivorans* strain CV2803T. *Appl. Environ. Microbiol.* **71**: 3458–3467.

Davidova, I.A., Gieg, L.M., Nanny, M., Kropp, K.G. & Suflita, J.M. (2005) Stable isotopic studies of n-alkane metabolism by a sulfate-reducing bacterial enrichment culture. *Appl. Environ. Microbiol.* **71**: 8174–8182.

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

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

Gärdes, A. (2005) Deletionsmutagenese eines Magnetosomenproteins in *Magnetospirillum gryphiswaldense* unter Etablierung des *Cre-loxP*-Systems. Diplomarbeit, Fachhochschule Emden.

Grundmann, O., Behrends, A., Rabus, R., Amann, J., Halder, T., Heider, J. & Widdel, F. (2008) Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN1. *Environ. Microbiol.* **10**: 376–385.

Hilberg, M., Pierik, A.J., Bill, E., Friedrich, T., Lippert, M.L. & Heider, J. (2012) Identification of FeS clusters in the glycyl-radical enzyme benzylsuccinate synthase via EPR and Mossbauer spectroscopy. *J. Biol. Inorg. Chem.* **17**: 49–56.

Inoue, H., Nojima, H. & Okayama, H. (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**: 23–28.

Kovach, M.E., Phillips, R.W., Elzer, P.H., Roop, R.M., 2nd & Peterson, K.M. (1994) pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16**: 800–802.

Kovach, M.E., Elzer, P.H., Hill, D.S., Robertson, G.T., Farris, M.A., Roop, R.M., 2nd & Peterson, K.M. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* **166**: 175–176.

Kropp, K.G., Davidova, I.A. & Suflita, J.M. (2000) Anaerobic oxidation of *n*-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. *Appl. Environ. Microbiol.* **66**: 5393–5398.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

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

Li, L., Patterson, D.P., Fox, C.C., Lin, B., Coschigano, P.W. & Marsh, E.N. (2009) Subunit structure of benzylsuccinate synthase. *Biochemistry* **48**: 1284–1292.

Marx, C.J. & Lidstrom, M.E. (2002) Broad-host-range *cre-lox* system for antibiotic marker recycling in gram-negative bacteria. *Biotechniques* **33**: 1062–1067.

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

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

Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A.J. & Widdel, F. (2001) Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: Evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. *J. Bacteriol.* **183**: 1707–1715.

Rao, V.K., Whitlock, J.A. & Progulske-Fox, A. (1993) Development of a genetic system for *Eikenella corrodens*: transfer of plasmids pFM739 and pLES2. *Plasmid* **30**: 289–295.

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

Sambrook, J. & Russell, D.W. (2001) Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G. & Pühler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**: 69–73.

Schultheiss, D. & Schuler, D. (2003) Development of a genetic system for *Magnetospirillum gryphiswaldense*. *Arch. Microbiol.* **179**: 89–94.

Selmer, T., Pierik, A.J. & Heider, J. (2005) New glycyl radical enzymes catalysing key metabolic steps in anaerobic bacteria. *Biol. Chem.* **386**: 981–988.

Simon, R., Priefer, U. & Pühler, A. (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. *Nat. Biotechnol.***1**: 784–791.

van Beilen, J.B. & Funhoff, E.G. (2005) Expanding the alkane oxygenase toolbox: New enzymes and applications. *Curr. Opin. Biotechnol.* **16**: 308–314.

van Beilen, J.B., Panke, S., Lucchini, S., Franchini, A.G., Rothlisberger, M. & Witholt, B. (2001) Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. *Microbiol.* **147**: 1621–1630.

van Beilen, J.B., Marin, M.M., Smits, T.H., Rothlisberger, M., Franchini, A.G., Witholt, B. & Rojo, F. (2004) Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis*. *Environ. Microbiol.* **6**: 264–273.

Widdel, F. & Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. *In* The Prokaryotes. Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K.-H. (ed). Berlin: Springer Verlag, pp. 3352–3389.

Widdel, F., Knittel, K. & Galushko, A. (2010) Anaerobic hydrocarbon-degrading microorganisms: an overview. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 1998–2021.

Wöhlbrand, L. & Rabus, R. (2009) Development of a genetic system for the denitrifying bacterium '*Aromatoleum aromaticum*' strain EbN1. *J. Mol. Microbiol. Biotechnol.* **17**: 41–52.

4. Bericht

Construction and characterization of Δ*masBCDE* deletion mutants of strain HxN1

In diesem Bericht ist die Herstellung weiterer Mutanten von Stamm HxN1 beschrieben, die nicht Bestandteil des Manuskriptes “Identification of a second functional *mas* operon in the anaerobic *n*-alkane degrader strain HxN1 by a newly developed genetic system” sind.

Herstellung und Charakterisierung der Mutanten. Die verwendeten Plasmide wurden von Olav Grundmann hergestellt.

Summary

The generation of a Δ*masBCDE,* Δ*masBCDE´* mutant of strain HxN1 confirmed the results obtained for the Δ*masD,* Δ*masD´* mutant, namely the presence of two *mas* operons and the necessity of the deleted genes for the anaerobic degradation of *n*-alkanes.

Introduction

The successful establishment of a genetic system for strain HxN1 and the *in vivo* verification of the necessity of *masD*, encoding the catalytic subunit of (1-methylalkyl)succinate synthase (Webner *et al*., unpublished results), enables the investigation of the necessity of the small subunits MasB, MasC and MasE for the anaerobic activation of *n*-alkanes. For this purpose a Δ*masBCDE* mutant of strain HxN1 was generated, instead of deletion of each gene alone. The aim was to re-introduce all subunit encoding genes except of one on a plasmid into the mutant and to analyze the effect of the one missing subunit onto growth of strain HxN1 with *n*-hexane.

Material and methods

For a detailed description of the applied methods the reader is referred to the manuscript "Identification of a second functional *mas* operon in the anaerobic *n*-alkane degrader strain HxN1 by a newly developed genetic system". Bacterial strains and plasmids used in this study are depicted in table 1, sequences of oligonucleotide primers are listed in table 2. The *masBCDE* deletion cassette in pCM184 was constructed analogously by subsequent cloning of the upstream region of *masB* (2940 bp) and of the downstream region of *masE* (2753 bp) into pCM184. The resulting plasmid pCM184_Δ*masBCDE* has a size of 12 453 bp.

Table 1 Bacterial strains and plasmids used in this study.

Strain or plasmid	Marker	Genotype and characteristics	Reference or source
Strains			
E. coli S17-1		*thi recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7	Simon *et al.* (1983)
E. coli TOP10	Str^R	F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen (Darmstadt, Germany)
HxN1			Ehrenreich *et al.* (2000)
HxN1 Δ*masBCDE*	Km^R	Δ*masBCDE*	this study
HxN1 Δ*masBCDE*, Δ*masBCDE´*	Km^R, Gm^R	Δ*masBCDE,* Δ*masBCDE´*	this study
Plasmids			
pCM184	Ap^R, Km^R, Tc^R		Marx & Lidstrom (2002)
pCM184_upstream-*masB*	Ap^R, Km^R, Tc^R	*masA,*	this study
pCM184_Δ*masBCDE*	Ap^R, Km^R, Tc^R	*masA, masF, masG*	this study
pCM184_Δ*masBCDE_aacC1*	Ap^R, Gm^R, Tc^R	*masA, masF, masG*	this study
pBBR1MCS_*mas* operon	Cm^R	*mas* operon + 1kb upstream sequence	Webner *et al*., unpublished

Table 2 Oligonucleotide primers used in this study; restriction sites are underlined.

Primer	Target gene or region	Sequence (5´→ 3´)	Product length [bp]
masBCDE deletion construct in pCM184			
upstream_*Bgl*II_f	upstream *masBCDE*	ATCAGATCTTAGCCAGAATTGCATGGTCAT	2940
upstream_*Kpn*I_r		ATCGGTACCCATAACATATTAGGATTTTTAC	
downstream_*Apa*I_f	downstream *masBCDE*	TCGGGCCCTAGGCAGCAAGTAGCCTCCCTT	2753
downstream_*Mlu*I_r		ATCACGCGTGAAGACGCCGCTATCAGTCAG	
*aacC1_Pst*I_f	*aacC1*	ATGCTGCAGCCGATCTCGGCTTGAACGAA	674
*aacC1_Pst*I_r		ATGCTGCAGCAGTGGCGGTTTTCATGGC	
Confirmation of double crossover			
*masD*_f	*masD*	CTGCAACTTCAACACTATCC	2438
*masD*_r		ACCAGCCACACGAACGATA	
*bla*_f	*bla*	ACATTTCCGTGTCGCCCTTA	837
*bla*_r		ATCAGTGAGGCACCTATCTC	
*kan*_f	*kan*	ATGAGCCATATTCAACGGGA	708
*kan*_r		GAGGCAGTTCCATAGGATG	
*aacC1*_f	*aacC1*	ATGTTACGCAGCAGCAAC	525
*aacC1*_r		GGTACTTGGGTCGATATCA	
masBCDE complementation			
mas operon_f	*mas* operon	GGTACAGCGCCAACCACTCGTAGAT	8356
mas operon_*Spe*I_r		ATTACTAGTGTTAATAGAAGACGCCGCTAT	
Probe for southern blot			
southern probe_f	*masA*	TTCAGAGCTATTGACCCGTG	505
southern probe_r	*masB*	GACAGTACTTGGCGTCACTA	

Results and Discussion

Generation of a Δ*masBCDE,* Δ*masBCDE´* deletion mutant of strain HxN1

Following conjugational transfer of pCM184_Δ*masBCDE* into strain HxN1, the obtained kanamycin resistant colonies were analyzed by PCR for the presence of *masD* and the antibiotic resistance genes. Analogously to the Δ*masD* mutant, a double crossover was confirmed by the absence of *bla*, concurrent with the presence of *masD* and *kan* (data

not shown). A second homologous recombination event of the genomic DNA with the plasmid pCM184_Δ*masBCDE_aacC1* resulted in a Δ*masBCDE,* Δ*masBCDE´* mutant. The homologous recombination of the genome with this plasmid indicates that both *mas* operons need to be identical not only in the 73 bases upstream of *masA*, as shown for the Δ*masD,* Δ*masD´* mutant (Webner *et al*., unpublished results), but also in the region 1 kb upstream of *masA*. Otherwise, homologous recombination of the plasmid and the genome would have remained unsuccessful.

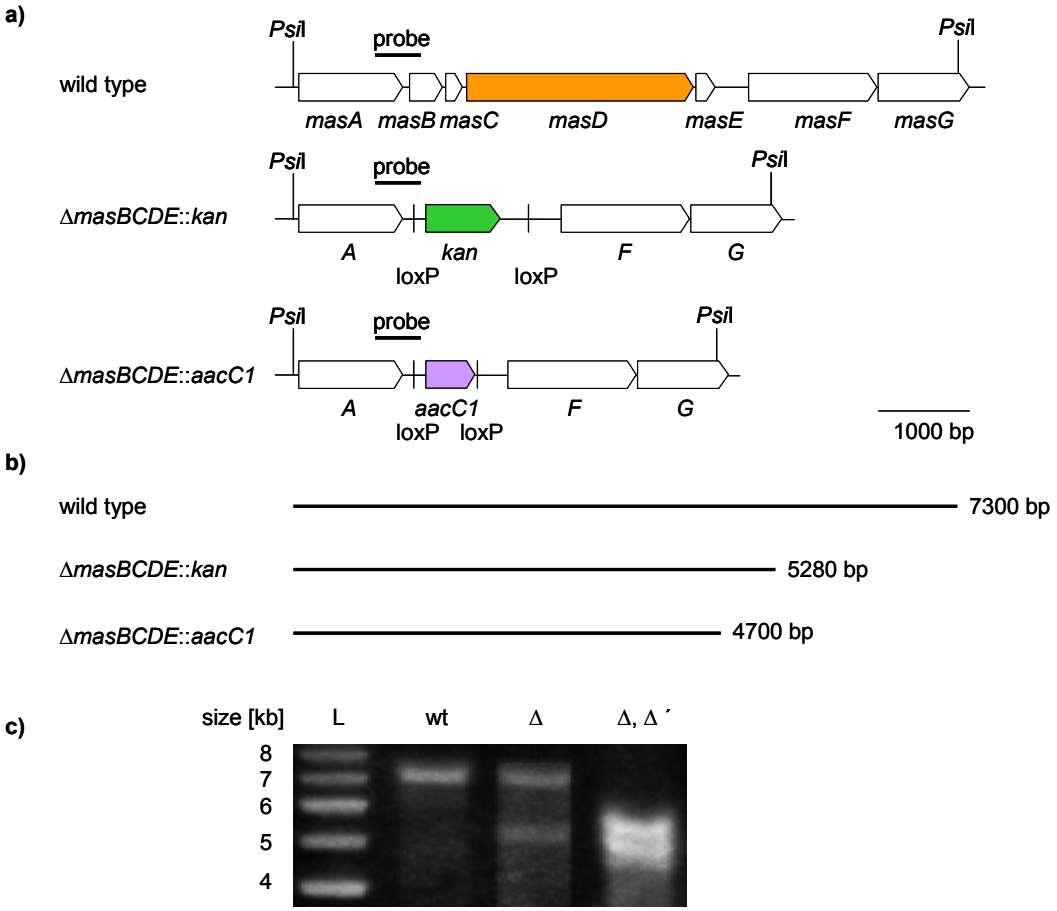

Fig. 1 Genetic characterization of strain HxN1 *masBCDE* deletion mutants by Southern blot. **a)** Schematic depiction of the chromosomal region of the wild type *mas* operon, the Δ*masBCDE::kan mas* operon and the Δ*masBCDE*::*aacC1 mas* operon. Restriction sites of *Pst*I and the hybridization site of the probe are indicated. **b)** Sizes of fragments obtained by restriction with *Pst*I in the wild type *mas* operon, the Δ*masBCDE::kan* and the Δ*masBCDE*::*aacC1 mas* operon of strain HxN1. **c)** Detection of the *Pst*I restriction fragments in the HxN1 wild type (abbreviated as wt), the Δ*masBCDE* (Δ) and the Δ*masBCDE*, Δ*masBCDE´* (Δ,Δ´) mutant by Southern blot. L: biotinylated molecular weight ladder.

The deletion of *masBCDE* was confirmed by Southern blot analysis of restriction fragments of the HxN1 wild type, the Δ*masBCDE* and the Δ*masBCDE* Δ*masBCDE´* mutant (Fig. 1). In the HxN1 wild type only one signal for the unmodified *mas* operon with a size of 7300 bp was detected. The Δ*masBCDE* mutant yielded two signals, one for the unmodified wild type *mas* operon and a second signal for the Δ*masBCDE::kan mas* operon with a size of 5280 bp. Finally, in the Δ*masBCDE* Δ*masBCDE´* mutant, a signal for the wild type *mas* operon was absent, but instead a 4780 bp fragment for the Δ*masBCDE::aacC1 mas* operon was detected additionally to the 5280 bp fragment for the Δ*masBCDE::kan mas* operon.

In trans expression of *masD* by complementation

Likewise the Δ*masD* mutants, the Δ*masBCDE* mutant grew with *n*-hexane after a prolonged lag phase, whereas the Δ*masBCDE,* Δ*masBCDE´* mutant was unable to grow with *n*-hexane anymore (data not shown). Initially, it was planned to complement the mutant with a pBBR1MCS plasmid harboring the genes *masB* to *masE* (data for generation of this plasmid are not shown). Before and behind each gene, recognition sites for restriction enzymes were inserted, which allows removal of one gene from the plasmid backbone. By introducing the plasmid lacking one of the *masBCDE* genes into the Δ*masBCDE*, Δ*masBCDE´* mutant, the effect of the missing gene on the ability of strain HxN1 to grow with *n*-hexane could have been analyzed.

However, the results of the Δ*masD* complementation pointed to polar effects (Webner *et al*., unpublished results), which probably also affect transcription of *masG*, the gene encoding the activating enzyme of (1-methylalkyl)succinate synthase. Accordingly, complementation with *masB* to *masE* was regarded to be insufficient to restore the phenotype. Therefore, the plasmid pBBR1MCS_*mas* operon, which has been used for successful complementation of Δ*masD*, was introduced into the Δ*masBCDE,* Δ*masBCDE´* mutant first. In the presence of chloramphenicol or kanamycin the complemented mutant grew with *n*-hexane again, indicating an effective reconstitution of the phenotype (data not shown). Retarded growth was also observed in the presence of gentamycin, as it has been described for the complemented Δ*masD*, Δ*masD´* mutant. Complementation with the pBBR1MCS_*masBCDE* plasmid has not been conducted so far, but is expected to remain unsuccessful.

Outlook

To analyze the function of the small subunits by deletion of their encoding genes, it is probably necessary to use the plasmid pBBR1MCS_*mas* operon, in which restriction sites need to be introduced, to allow the removal of single *mas* genes. In case of success, this plasmid can be further used for the generation of a chimeric (1-methylalkyl)succinate synthase. This could be achieved by exchange one of the *masBCDE* genes with its homologue of the related strain OcN1. Strain HxN1 and OcN1 differ by the chain length of the *n*-alkanes, which are oxidized completely. Strain HxN1 completely degrades *n*-alkanes with a chain length from C_5 to C_8, while strain OcN1 uses C_8 to C_{12} *n*-alkanes (Ehrenreich *et al.*, 2000; Schmitt *et al.*, unpublished results). The analysis of active chimeric proteins might elucidate, which one of the subunits determines the substrate range of the enzyme.

References

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

Marx, C.J. & Lidstrom, M.E. (2002) Broad-host-range *cre-lox* system for antibiotic marker recycling in gram-negative bacteria. *Biotechniques* **33**: 1062–1067.

Simon, R., Priefer, U. & Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. *Nat. Biotechnol.***1**: 784–791.

5. Manuskript

The (1-methylalkyl)succinate synthase of the *n*-alkane degrading strain HxN1 is expressed under a wide range of carbon sources

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Erstellung des Manuskriptes, Planung und Durchführung aller Versuche.

Abstract

The (1-methylalkyl)succinate synthase catalyzes the addition of *n*-alkanes to fumarate in strain HxN1 under anaerobic conditions. The encoding *mas* genes are induced in the presence of the growth substrate *n*-hexane. In the present study, we show that induction additionally occurs during cultivation with substrate mixtures of *n*-hexane and another carbon source such as a carboxylic acid. The induction of the *mas* genes under these conditions disagrees with the concept of carbon catabolite repression. Expression is even induced by *n*-alkanes, cyclic alkanes, aromatic hydrocarbons and alcohols, which are not oxidized completely or which are not a substrate for (1-methylalkyl)succinate synthase at all, probably due to a relaxed substrate specificity of the sensor mediating expression of the *mas* genes. We propose that *mas* expression induced by a wide range of carbon sources, even in the presence of other carbon sources, enables strain HxN1 to respond to utilizable *n*-alkanes immediately, once they become available and to detoxify those hydrocarbons, which are a substrate of the (1-methylalkyl)succinate synthase.

Introduction

Within the last years an increasing number of microorganisms capable to degrade petroleum derived hydrocarbons has been isolated. These isolates degrade alkanes or aromatic hydrocarbons completely to CO_2 by anaerobic respiration with nitrate, sulfate or ferric iron as electron acceptor or by phototrophy (overview in Widdel *et al.*, 2010). The most widespread mechanism to activate an inert hydrocarbon molecule under anaerobic conditions is the addition to fumarate yielding substituted succinates, a reaction which is catalyzed by a glycyl radical enzyme (Heider, 2007). In the case of toluene activation, the responsible enzyme benzylsuccinate synthase, consisting of three subunits, and its encoding *bss* genes have been identified and characterized (Leuthner *et al.*, 1998). Later, related enzymes catalyzing the addition of an *n*-alkane to fumarate were described in the denitrifying strain HxN1 (Grundmann *et al.*, 2008) and the sulfate-reducer *Desulfatibacillum alkenivorans* strain AK-01 (Callaghan *et al.*, 2008). The enzymes were named (1-methylalkyl)succinate synthase (Grundmann *et al.*, 2008), respectively alkylsuccinate synthase (Callaghan *et al.*, 2008) and the encoding genes were titled *mas*, respectively *ass*.

So far no bacterium has been isolated, which is able to grow with both alkanes and aromatic hydrocarbons. All obtained isolates use only one of these substrates for growth. Moreover, the substrate range of the isolated strains is restricted. For many strains toluene is the only growth substrate among the hydrocarbons and for the *n*-alkane degrading bacteria the utilizable *n*-alkanes lie within a certain range of chain length (Widdel *et al.*, 2010). Strain HxN1 for example grows with *n*-alkanes from C_5 to C_8, whereas the related strain OcN1 uses *n*-alkanes with a chain length from C_8 to C_{12} (Ehrenreich *et al.*, 2000, Schmitt *et al.*, unpublished results). However, strain HxN1 co-metabolizes the alkanes *n*-butane, cyclopentane and methylcyclopentane if incubated together with *n*-hexane (Wilkes *et al.*, 2003). Not only the succinated products of the activation reaction, but also metabolites of the further downstream degradation pathway, were identified. Additionally, strain HxN1 and two other alkane degrading strains, OcN1 and TD3, were shown to activate both an alkane and toluene if they were supplied together (Rabus *et al.*, 2011). For strain HxN1 metabolites of the further degradation pathway of toluene were analyzed. These metabolites proposed a pathway different to the regular toluene degradation. It still remains open, whether co-metabolism of certain hydrocarbons is incompletely to a dead-end product or whether hydrocarbons are oxidized completely but slower than the preferred ones and might contribute to a minor amount to energy gain and growth (Wilkes *et al.*, 2003). In contrast, toluene degrading bacteria were unable to co-metabolize *n*-alkanes, which was explained by the higher

bond dissociation energy of a C–H bond to overcome for an *n*-alkane at its secondary carbon atom (– 398 kJ mol^{-1}) than for the methyl group of toluene (– 368 kJ mol^{-1}) (Rabus *et al.*, 2011). Toluene-activating enzymes possibly are not able to cope with this higher bond dissociation energy, but an *n*-alkane activating enzyme should be able to overcome the weaker bond dissociation energy for toluene activation.

Another aspect that should be considered in terms of the limited substrate range is the regulation of the hydrocarbon degradation pathway. It was shown that anaerobic hydrocarbon degradation is inducible. In *Thauera aromatica*, the benzylsuccinate synthase as well as the transcript containing the *bss* genes were only detectable in cells grown on toluene and not in benzoate-grown cells (Leuthner *et al.*, 1998). The same was reported for the (1-methylalkyl)succinate synthase and its *mas* genes, which were only evident when strain HxN1 was grown with *n*-hexane and not upon growth with caproate (Grundmann *et al.*, 2008). The *bss* operon in toluene-degrading bacteria is likely regulated by a two-component regulatory system, whose encoding genes were found adjacent to the *bss* operon (Coschigano & Young, 1997; Leuthner & Heider, 1998; Achong *et al.*, 2001; Kube *et al.*, 2004).

The present study was undertaken to gain first insights into the regulation of the anaerobic *n*-alkane degradation in strain HxN1. We investigated, whether expression of the *mas* genes is inhibited in the presence of *n*-hexane and a second carbon source. Furthermore, induction of *mas* expression by organic compounds and hydrocarbons, which do not serve as growth substrates, was analyzed.

Material and Methods

Cultivation

The bacterial strain HxN1 was cultivated under denitrifying conditions in defined mineral medium as described previously (Rabus & Widdel, 1995; Ehrenreich *et al.*, 2000). Substrates with toxic potential and/or low solubility in water were supplied as 1 or 5% (v/v) dilution in 2,2,4,4,6,8,8-heptamethylnonane (HMN). Gaseous alkanes were provided as 1 bar overpressure to the headspace. Carboxylic acids, sugars and alcohols were added to a final concentration of 5 or 10 mM. Nitrate was supplied regularly in portions ≤ 5 mM.

Analysis of inhibition and induction of the *mas* expression

Strain HxN1 was grown with caproate over several passages, then harvested by centrifugation (10 000 x *g*, 10 min, 4 °C), washed and re-suspended in anoxic mineral

medium. The cell suspension served as inoculum for cultivation under different substrate conditions. Cultivation was carried out in hungate tubes containing 10 ml of anoxic mineral medium. The optical density (OD) was measured directly in the tubes at 600 nm (UV-1202, Shimadzu, Duisburg, Germany). To analyze the inhibition of *mas* expression, strain HxN1 was cultivated with caproate, acetate, succinate, fumarate, benzoate or fructose each together with *n*-hexane. Parallel control experiments were performed without *n*-hexane. For analysis of *mas* expression as response to different hydrocarbons, strain HxN1 was cultivated with caproate and one of the hydrocarbons or alcohols depicted in table 1.

Table 1 *n*-Alkanes, cyclic alkanes, aromatic hydrocarbons and alcohols used in this study.

n-alkanes	cyclic alkanes, aromatics	alcohols
methane	cyclopentane	methanol
ethane	methylcyclopentane	ethanol
propane	cyclohexane	1-propanol
n-butane	methylcyclohexane	1-butanol
n-pentane	ethylcyclohexane	1-pentanol
n-hexane	propylcyclohexane	1-hexanol
n-heptane	benzene	1,6-hexanediol
n-octane	toluene	2,5-hexanediol
n-nonane	ethylbenzene	1-heptanol
n-decane	propylbenzene	1-octanol
n-undecane		1-decanol
n-dodecane		1-dodecanol
n-tetradecane		1-tetradecanol
		cyclohexanol

A control experiment was conducted with caproate as sole carbon source. Growth was monitored at least once per day by OD measurement and by determination of nitrate and nitrite in the cultures with Merckoquant test stripes (Merck, Darmstadt, Germany). Nitrate was added in portions of 5 mM after depletion. After two or three days of incubation, samples were withdrawn, centrifuged (10 000 x *g*, 5 min, 4 °C) and re-suspended in phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) to an OD of 4 at 600 nm. These cell suspensions were run on 12 % polyacrylamide gels as described by Laemmli (1970). The PageRuler Prestained Protein

Ladder (Fermentas, St. Leon-Rot, Germany) served as molecular size marker. Proteins were stained with Coomassie R-250 (0.25% (v/v) Coomassie R250, 40% (v/v) ethanol, 10% (v/v) glacial acetic acid). Immunoblotting for detection of MasD was performed as described by Schmitt *et al*. (unpublished results). As alternative secondary antibody, horse radish peroxidase conjugated goat-anti rabbit IgG (Pierce Protein, Rockford, USA), visualized with an ECL camera system (BIS 303 PC Bioimaging, Amersham Pharmacia Biotech/GE Healthcare, Munich, Germany), was used.

Analysis of (1-methylalkyl)succinate synthase activity

Strain HxN1 was grown with *n*-hexane as described above and then transferred with an inoculation size of 10% into hungate tubes containing 10 ml of fresh anoxic mineral medium and one of the alkanes or aromatic hydrocarbons depicted above (table 1). An approach without any carbon source served as negative control. After one week of incubation samples were withdrawn to determine nitrate consumption and nitrite production with an ion chromatograph connected to an UV detector (Sykam, Fürstenfeldbruck, Germany) as described by Rabus and Widdel (1995). Data analysis was performed with the Clarity HPLC software (DataApex, Praque, Czech Republic).

Results and Discussion

MasD as a marker for *mas* expression

As reported previously, strain HxN1 consumes *n*-hexane only after a lag phase if grown on caproate before (Grundmann *et al.*, 2008), suggesting that the enzyme responsible for the activation of *n*-hexane, the (1-methylalkyl)succinate synthase, needs to be induced. In contrast, caproate was consumed immediately in *n*-hexane-grown cells, indicating a constitutive ability to degrade caproate. Correspondingly, in differential two-dimensional gel electrophoresis of *n*-hexane- versus caproate-grown cells, subunits of (1-methylalkyl)succinate synthase were only identified in the *n*-alkane-grown cells (Grundmann *et al.*, 2008). The large, catalytic α-subunit (MasD) of the enzyme is detectable in caproate-adapted cells of strain HxN1 after incubation with *n*-hexane for at least two days in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as a prominent protein band with a size of ~ 94 kDa, as well as in Western blot with immune serum against MasD (Fig. 1a, 2). The amount of MasD increases over time, which indicates continuous expression of the *mas* genes to produce high amounts of (1-methylalkyl)succinate synthase (Fig. 1b). Thus, the presence of MasD was used as marker for the expression of the *mas* genes under all tested conditions described below.

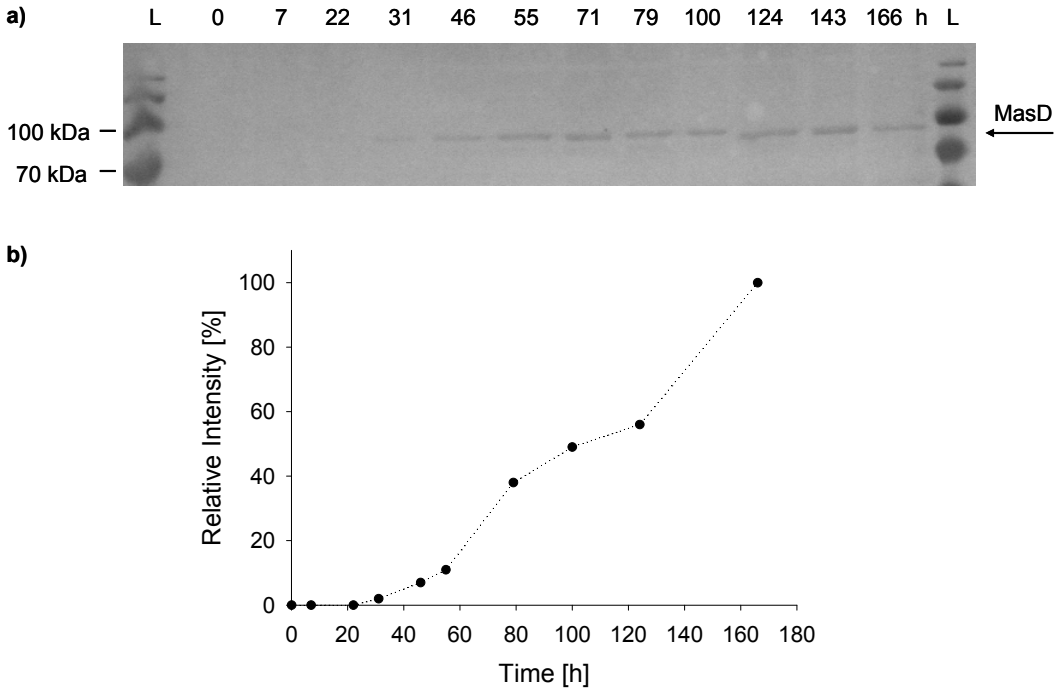

Fig. 1 Production of MasD over time in cell extracts of strain HxN1 shifted from caproate to *n*-hexane. **a)** Western blot for the detection of MasD in cell extracts of strain HxN1 sampled at different time points (0 to 166 hours). L = pre-stained ladder. **b)** Relative intensity of the MasD signal in Western blot at different time points (0 to 166 hours). The highest intensity at time point 166 h was set to 100%.

Expression of the *mas* genes during cultivation on substrate mixtures

The cultivation of strain HxN1 with two growth substrates, *n*-hexane and a carboxylic acid or sugar, showed that the induction of the *mas* expression was not inhibited in the presence of a second growth substrate (Fig 2). Apparently, strain HxN1 used the supplied caboxylic acid or fructose for growth, because growth started immediately, without showing a lag phase, in contrast to the control with *n*-hexane as sole carbon source (Fig 3). Growth with caproate and *n*-hexane was somewhat slower compared to growth with caproate alone. Similar results were obtained for carbon sources other than caproate (data not shown). Anyhow, the *mas* genes were expressed in all incubations containing *n*-hexane. The carboxylic acids and fructose were supplied in a suitable amount for complete oxidation with the provided nitrate. Both were added regularly to prevent expression of *mas* simply due to a lack of the carboxylic acids or fructose. Without *n*-hexane MasD was not detectable, which also demonstrates that the induction of the *mas* genes is dependent on the presence of *n*-alkanes (Fig 2).

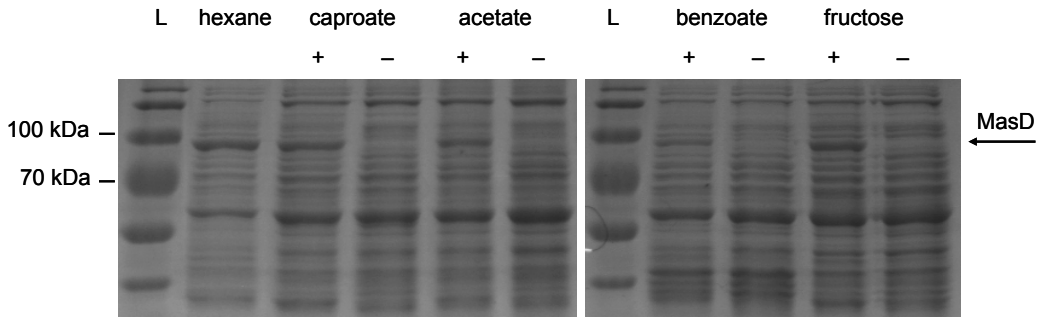

Fig. 2 Denaturing gel electrophoresis (SDS-PAGE) of cell extracts from strain HxN1. Cells were grown with caproate, acetate, benzoate or fructose either together with *n*-hexane (+) or alone (–) and in a positive control with *n*-hexane as sole carbon source for two days. MasD is apparent in all cultivations containing *n*-hexane. L: protein ladder.

For *Azoarcus* sp. strain T transcription of the *bss* operon in the presence of benzoate and toluene was reported (Achong *et al.*, 2001). These two examples (strain HxN1 and strain T) indicate that expression of the genes for hydrocarbon degradation, even in the presence of another carbon and energy source, which is more easily to degrade, might be a common feature in anaerobic hydrocarbon degrading bacteria. This is of interest, because it disagrees with the commonly established model of carbon catabolite repression, where induction of one catabolic pathway is inhibited as long as a preferred carbon source is available (Görke & Stülke, 2008). Carbon catabolite repression was for example shown in aerobic *n*-alkane degrading *Pseudomonas* strains, which preferentially used other carbon sources than *n*-alkanes (summarized in Rojo, 2010a). For anaerobic hydrocarbon degrading bacteria cultivation with a hydrocarbon and another carbon source has not been conducted so far.

It cannot be excluded that degradation of *n*-hexane takes place in parallel to the degradation of another carbon source and contributes to energy gain. However, this should be only possible after more than two days, because the same time is required for synthesis of the (1-methylalkyl)succinate synthase (Fig. 1). In addition, consumption of both substrates should result in faster growth than with one substrate, which was not observed for strain HxN1 (Fig. 3). It is also possible that the (1-methylalkyl)succinate synthase is only present after two days in its inactive, radical-free form, which requires activation by an activating enzyme of the S-adenosylmethionine radical enzyme family encoded by *masG* (Sofia *et al.*, 2001; Grundmann *et al.*, 2008). Activation might not take place until the preferred carbon source is consumed completely. From an energetic point of view, the expression of (1-methylalkyl)succinate synthase in the presence of a second carbon source is not that expensive, because the complete oxidation of carboxylic acids

or sugars provides energy. Consumption of energy for expression of the *mas* genes might be an explanation for the observed slower growth with caproate and *n*-hexane together compared to caproate alone (Fig. 3). The question is, why strain HxN1 expresses the *mas* genes in the presence of another carbon source, which is degraded immediately. With regard to the natural environment, expression might be advantageous to be prepared for changes in the availability of carbon sources. When the preferred growth substrate has been consumed completely, the cell is able to use *n*-hexane immediately by the (1-methylalkyl)succinate synthase expressed in advance. Thus, co-expression of several catabolic pathways might, in the environment, be more common than carbon catabolite repression observed in laboratory studies.

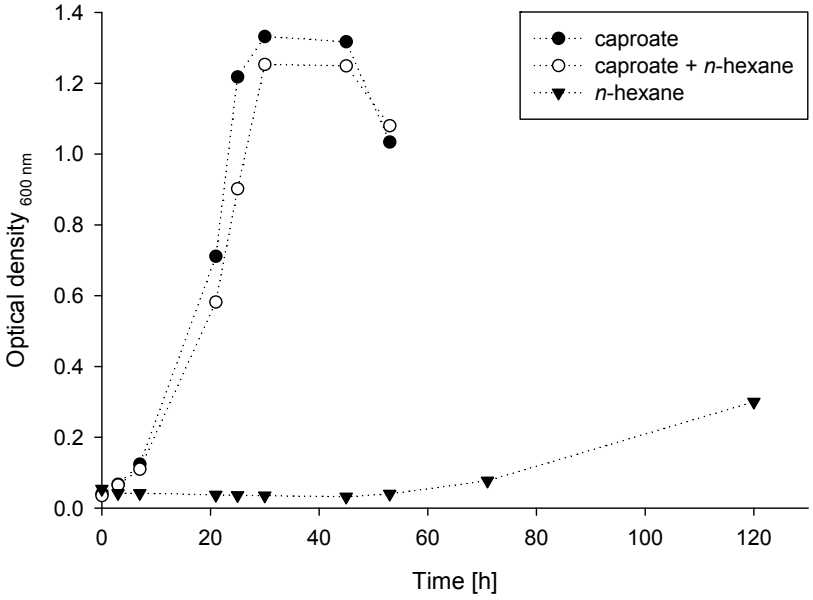

Fig. 3 Time course of growth of strain HxN1 with either caproate or *n*-hexane or both, caproate and *n*-hexane.

Another reason for the expression of (1-methylalkyl)succinate synthase, although it is not required for energy gain, might be the protection against toxic concentrations of *n*-hexane. Alkanes are supposed to diffuse freely through the cytoplasmic membrane due to their hydrophobicity and thus, the diffusion of *n*-hexane into the cell is not regulated (Sikkema *et al.*, 1995). High concentrations of hydrocarbons in the membrane change membrane structure and function by increasing fluidity. As a result, interactions between lipids and proteins are destroyed and energy conduction is affected (for an overview see Sikkema *et al.*, 1995). Expression of (1-methylalkyl)succinate synthase enables the cells to metabolize *n*-hexane immediately and consequently prevents

accumulation to toxic levels. The applied concentration of 5% *n*-hexane in HMN in this study is not toxic for strain HxN1. Anyhow, concentrations ≥ 10% inhibit growth partially and at a concentration of 75% *n*-hexane in HMN growth of strain HxN1 is inhibited completely (Behrends, 1999). In the environment, *n*-alkanes are not diluted in HMN to prevent toxicity as it is performed in the laboratory. Efflux pumps for hydrocarbons are not known for strain HxN1, but were for example identified in *Pseudomonas putida* to remove aromatic hydrocarbons (Ramos *et al.*, 2002). Conversion of a toxic substrate into a non-toxic metabolite is another protection strategy.

Induction of *mas* expression

Expression of the *mas* genes in strain HxN1 grown with *n*-hexane and caproate enabled the investigation of *mas* expression in the presence of hydrocarbons, which do not support growth. In these experiments caproate promoted growth whereas the hydrocarbon served as possible inductor of the *mas* expression. Signals for MasD in Western blot analysis were obtained upon incubation with those alkanes, which serve as growth substrate for strain HxN1: *n*-pentane, *n*-hexane, *n*-heptane and *n*-octane (Fig. 4). In addition, the hydrocarbons *n*-butane, cyclopentane, methylcyclopentane and toluene, which were shown to be activated co-metabolically (Wilkes *et al.*, 2003; Rabus *et al.*, 2011), promoted *mas* expression (Fig. 4, 5).

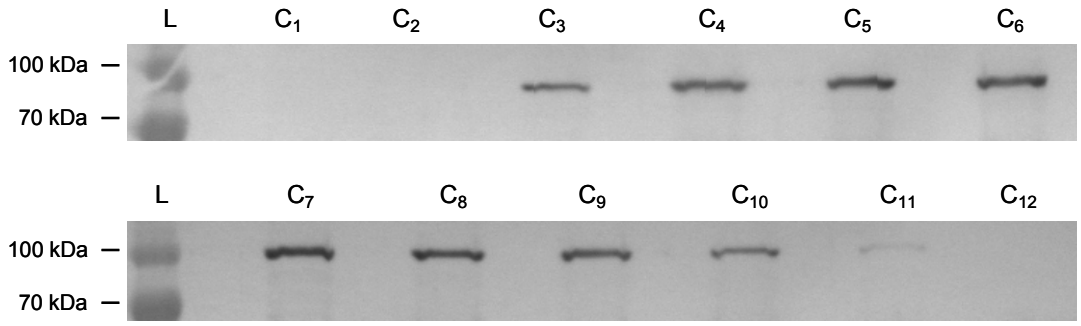

Fig. 4 Western blot for the identification of MasD in cell extracts of strain HxN1 after incubation of caproate-adapted cells for three days with *n*-alkanes of a chain length from C_1 to C_{12}. L: pre-stained ladder.

Hence, the enzyme not only converts other alkanes than those used for growth accidentally co-metabolically together with *n*-hexane, but these alkanes are also able to induce expression of the enzyme needed for their activation. According to detection of MasD in Western blot, *mas* expression is also induced by the alkanes *n*-propane, *n*-nonane, *n*-decane, *n*-undecane, cyclohexane and alkylsubstituted derivatives of it

(Fig. 4, 5), as well as alkylsubstituted derivatives of the aromatic hydrocarbon benzene (Fig. 5). None of these alkanes was shown to be activated co-metabolically before (Wilkes *et al.*, 2003), while co-metabolic activation of benzene derivatives other than toluene has not been investigated so far.

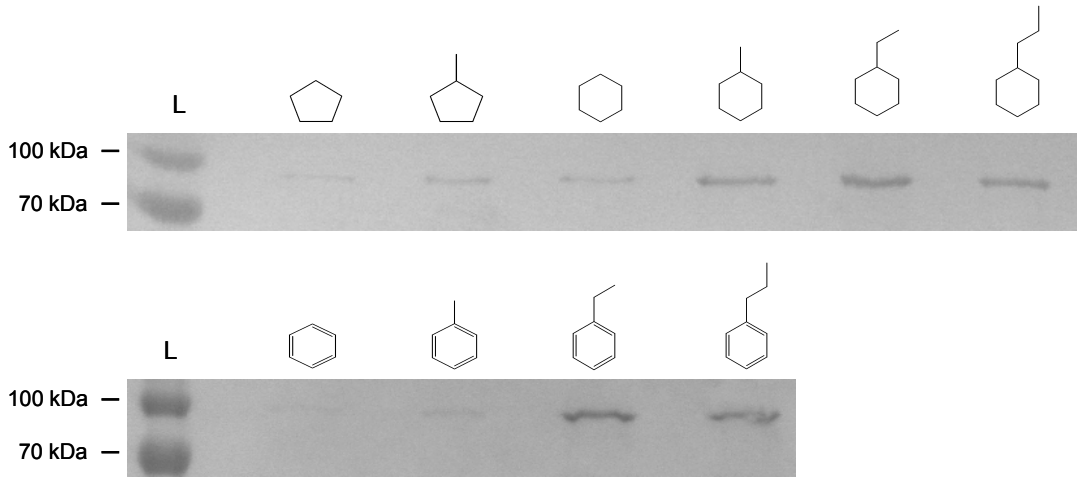

Fig. 5 Western blot for the identification of MasD in cell extracts of strain HxN1 after incubation of caproate-adapted cells for three days with cyclic alkanes (cyclopentane, methylcyclopentane, cyclohexane, methyl-, ethyl- and propylcyclohexane) and aromatic hydrocarbons (benzene, toluene, ethyl- and propylbenzene). L: pre-stained ladder.

Similar to this, in *Thauera aromatica* strain T1 the TutD protein, characterized as the catalytic subunit of the benzylsuccinate synthase, was detected after induction with *o*-xylene, which is not a growth substrate for this strain (Coschigano & Bishop, 2004). During growth of *Aromatoleum aromatica* strain EbN1 with toluene not only the *bss* operon, but also the *ebd* operon for the degradation of ethylbenzene was induced to a minor level, which was thought to be due to a relaxed specificity of the sensor for the *ebd* operon towards toluene (Kühner *et al.*, 2005).

The signal intensity for MasD was highest for the growth substrates *n*-pentane, *n*-hexane, *n*-heptane and *n*-octane and decreased with shorter and longer chain length (Fig. 4). Signals were not detectable in *n*-alkanes shorter than *n*-propane and larger than *n*-undecane. For the cyclic alkanes and the aromatic hydrocarbons the signal increased with increasing length of the alkyl moiety of the substance, only for benzene a signal was not detectable (Fig. 5). Interestingly, *mas* expression was also induced by the presence of alcohols from 1-butanol to 1-decanol, although they are not activated by the (1-methylalkyl)succinate synthase for their degradation (Fig. 6). Alcohols are channelled via aldehyde and fatty acid into the β-oxidation. Some of the tested alcohols (1-propanol,

1-butanol, 1-hexanol) were shown to allow growth of strain HxN1 (Behrends, 1999). Very light signals were obtained for the diols 1,6- and 2,5-hexanediol.

Obviously, the *mas* operon is induced by a lot of the tested substances, even though several of them are not used for growth neither are activated by (1-methylalkyl)succinate synthase. The induction by a wide range of hydrocarbons is explained by a relaxed specificity of the sensor, which regulates the *mas* operon. As concluded from the obtained results, the sensor recognizes linear and cyclic alkanes, aromatic hydrocarbons as well as alcohols. A hydrophobic character of the molecule is probably necessary for being recognized by the sensor. Alcohols with one hydroxyl group are assumed to be recognized by their hydrophobic alkyl moiety, whereas the more polar diols 1,6-hexanediol and 2,5-hexanediol induce expression only lightly.

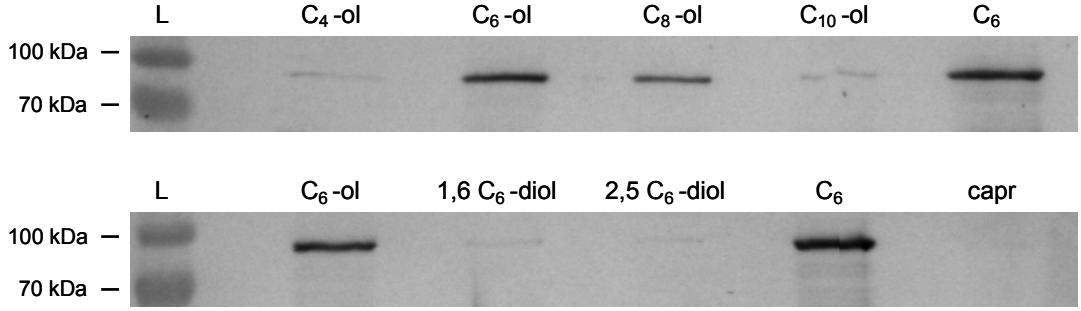

Fig. 6 Western blot for the identification of MasD in cell extracts of strain HxN1 after incubation of caproate-adapted cells for three days with the alcohols 1-butanol (C_4 -ol), 1-hexanol (C_6 -ol), 1,6-hexanediol (1,6 C_6-diol), 2,5-hexanediol (2,5 C_6-diol), 1-octanol (C_8 -ol) or 1-decanol (C_{10} -ol). Cells grown with *n*-hexane (C_6) or caproate served as positive or negative control, respectively. L: pre-stained ladder.

Although several *n*-alkanes and alcohols are recognized by the sensor, a certain length of the molecule is required. The smallest ones, methane, ethane, methanol, ethanol and propanol are probably too small to be bound by the sensor and therefore do not stimulate *mas* expression. Alcohols larger than 1-decanol and *n*-alkanes larger than *n*-undecane are likely too large for recognition by the sensor and thus, are also unable to induce *mas* expression. The largest substances, which are recognized by the sensor, 1-decanol and *n*-undecane, in fact have the same size, whereas the smallest inducers for the sensor, 1-butanol and propane do not have the same size. Simply from size, ethanol should be the smallest alcohol, which is recognized by the sensor. However, the polar hydroxyl group might prevent recognition by the sensor. Probably, a longer hydrophobic residue, as it is the case for 1-butanol, is required for an alcohol to allow

binding of the sensor. This is in accordance with the poor inducing effect of the polar 1,6- and 2,5-hexanediols. In case of the cyclic alkanes and aromatic hydrocarbons the presence of an alkyl moiety increases recognition by the sensor. Probably an aromatic ring requires an alkyl moiety to be recognized by the sensor, because no signal was obtained for benzene.

In the environment, an unspecific sensor is possibly of advantage for a strain degrading components of crude oil. Crude oils are always composed of *n*-alkanes and aromatic hydrocarbons as main components (Tissot & Welte, 1984). The amount of *n*-alkanes reaches up to 60% and the content of aromatic hydrocarbons varies from 20 to 45%. Therefore, even if cells exposed to crude oil get first into contact with aromatics due to their higher solubility, *n*-alkanes are almost certainly also present (Hildebrand solubility parameter δ for toluene: 8.9 versus 7.3 for *n*-hexane (Weast, 1990)). Expression of the *n*-alkane activating enzyme induced by aromatic hydrocarbons allows immediate use of *n*-alkanes for energy gain once they have entered the cell. In addition, a promiscuous sensor is of advantage for strain HxN1 for detoxification of aromatic hydrocarbons. The induction by alcohols can be explained by accidentally recognition of their hydrocarbon residues. Relaxed substrate specificity was reported for several regulators/sensors involved in aromatic hydrocarbon degradation (Shingler, 2003). One example is the regulating protein AlkS of the *alk* genes for aerobic degradation of *n*-alkanes in *Pseudomonas putida*. The promoter under the control of AlkS was shown to respond to branched alkanes, alkenes, haloalkanes, ethers and ketones in addition to *n*-alkanes (de Lorenzo & Perez-Martin, 1996).

Activity of (1-methylalkyl)succinate synthase from *n*-hexane adapted cells towards other alkanes and aromatic hydrocarbons

To analyze, whether the limited range of *n*-alkanes used for growth by strain HxN1 is caused by insufficient induction of the *mas* genes, we incubated strain HxN1, having the (1-methylalkyl)succinate synthase already expressed, with alkanes and aromatic hydrocarbons, which were shown to induce *mas* expression in the previous experiment. Expression of (1-methylalkyl)succinate synthase was ensured by culturing strain HxN1 on *n*-hexane before. Cultures incubated with *n*-hexane, *n*-heptane and *n*-octane had consumed the supplied 2.5 mM of nitrate after three to five days and growth became visible by OD measurement (data not shown). Besides, partial nitrate consumption had been only observed for *n*-pentane (1.4 mM), which is a growth substrate, too (Schmitt *et al.*, unpublished results), and *n*-nonane (0.1 mM). All the rest of the tested hydrocarbons did not promote consumption of any nitrate. Hence, the limited range of *n*-alkanes, which

allow growth of strain HxN1, is not explained by the ineffectiveness of hydrocarbons other than the known growth substrates to act as efficient effector for the regulating system of *mas* expression. Otherwise the hydrocarbons should have been converted by the already active (1-methylalkyl)succinate synthase. The results obtained for strain HxN1 differ from those of *Pseudomonas putida* GPo1. In this strain, it was proposed that growth is optimal for C_5 to C_{10} *n*-alkanes, because they bind efficiently as effectors to the AlkS regulator (Sticher *et al.*, 1997). AlkS initiates expression of the alkane hydroxylase encoding genes for aerobic degradation of *n*-alkanes (van Beilen *et al.*, 1994). However, slow growth with a considerable lag time was also observed for C_3 to C_4 *n*-alkanes (Johnson & Hyman, 2006). This was assumed to be due to their inability to act as efficient effector for AlkS (Rojo, 2010b).

The purified (1-methylalkyl)succinate synthase of strain HxN1 has been shown to activate cyclopentane besides *n*-pentane, -hexane, -heptane and -octane (Schmitt *et al.*, unpublished results). However, conversion of *n*-hexane was 40 times more effective than conversion of cyclopentane, which is already an indication for a restricted substrate range of (1-methylalkyl)succinate synthase. Some more hydrocarbons were activated by strain HxN1 in cultivation with substrate mixtures and it remained open, if the activated hydrocarbons are oxidized completely to CO_2 or if they are only converted to a dead end product due to a missing enzyme (Wilkes *et al.*, 2003; Rabus *et al.*, 2011). Since *n*-alkanes only differ in size, it is assumed that they are all degraded by the available enzymes, but degradation might be below the detection limit due to poor binding of these *n*-alkanes by (1-methylalkyl)succinate synthase. Cyclic alkanes and aromatic hydrocarbons possibly accumulate in a dead end product due to an enzyme needed for conversion of a ring structure, which is missing in strain HxN1. Benzoate is excluded as possible dead end product in degradation of aromatics, as proposed by Rabus *et al*. (2011), because strain HxN1 grew with benzoate as sole carbon source in the present study. Growth of strain HxN1 on benzoate had also been reported before (Behrends, 1999; Trautwein *et al.*, 2012). Conversion of substrates, which are not used for growth, into succinated products was observed in the toluene-degrading strains *Azoarcus* sp. strain T and *T. aromatica* strain K172, too (Beller & Spormann, 1997; Verfürth *et al.*, 2004). The activation product of for example toluene and fumarate, benzylsuccinate, is more hydrophilic and thus less toxic, because it does not diffuse into the membrane anymore. Therefore, even incomplete degradation of aromatic hydrocarbons might be useful in terms of detoxification for strain HxN1.

Conclusion

Our results indicate that the *mas* operon of strain HxN1 is regulated by a so far unknown protein with a relaxed substrate specificity towards various hydrocarbons and alcohols. A promiscuous sensor/regulator improves the competitiveness in the environment with regard to detoxification and energy gain by fast conversion of substrates into non-toxic intermediates, which in the case of growth substrates are oxidized completely. Further support of this theory is gained by the observation that *mas* expression is not regulated by carbon catabolite repression. The obtained results reflect effective adaptation of strain HxN1 towards fast growth with *n*-alkanes.

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References

Achong, G.R., Rodriguez, A.M. & Spormann, A.M. (2001) Benzylsuccinate synthase of *Azoarcus* sp. strain T: cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization. *J. Bacteriol.* **183**: 6763–6770.

Behrends, A. (1999) Physiologie und substratspezifische Proteinbildung denitrifizierender Bakterien mit der Fähigkeit zur anaeroben Oxidation kurzkettiger Alkane. Dissertation, Universität Bremen.

Beller, H.R. & Spormann, A.M. (1997) Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. *J. Bacteriol.* **179**: 670–676.

Callaghan, A.V., Wawrik, B., Ni Chadhain, S.M., Young, L.Y. & Zylstra, G.J. (2008) Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. *Biochem. Biophys. Res. Commun.* **366**: 142–148.

Coschigano, P.W. & Young, L.Y. (1997) Identification and sequence analysis of two regulatory genes involved in anaerobic toluene metabolism by strain T1. *Appl. Environ. Microbiol.* **63**: 652–660.

Coschigano, P.W. & Bishop, B.J. (2004) Role of benzylsuccinate in the induction of the *tutE tutFDGH* gene complex of *T. aromatica* strain T1. *FEMS Microbiol. Lett.* **231**: 261–266.

de Lorenzo, V. & Perez-Martin, J. (1996) Regulatory noise in prokaryotic promoters: how bacteria learn to respond to novel environmental signals. *Mol. Microbiol.* **19**: 1177–1184.

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

Görke, B. & Stülke, J. (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol.* **6**: 613–624.

Grundmann, O., Behrends, A., Rabus, R., Amann, J., Halder, T., Heider, J. & Widdel, F. (2008) Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN1. *Environ. Microbiol.* **10**: 376–385.

Heider, J. (2007) Adding handles to unhandy substrates: Anaerobic hydrocarbon activation mechanisms. *Curr. Opin. Chem. Biol.* **11**: 188–194.

Johnson, E.L. & Hyman, M.R. (2006) Propane and *n*-butane oxidation by *Pseudomonas putida* GPo1. *Appl. Environ. Microbiol.* **72**: 950–952.

Kube, M., Heider, J., Amann, J., Hufnagel, P., Kuhner, S., Beck, A. *et al.* (2004) Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1. *Arch. Microbiol.* **181**: 182–194.

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

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

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

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

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

Rabus, R., Jarling, R., Lahme, S., Kühner, S., Heider, J., Widdel, F. & Wilkes, H. (2011) Co-metabolic conversion of toluene in anaerobic *n*-alkane-degrading bacteria. *Environ. Microbiol.* **13**: 2576–2586.

Ramos, J.L., Duque, E., Gallegos, M.T., Godoy, P., Ramos-Gonzalez, M.I., Rojas, A. *et al.* (2002) Mechanisms of solvent tolerance in gram-negative bacteria. *Annu. Rev. Microbiol.* **56**: 743–768.

Rojo, F. (2010a) Carbon catabolite repression in *Pseudomonas* : optimizing metabolic versatility and interactions with the environment. *FEMS Microbiol. Rev.* **34**: 658–684.

Rojo, F. (2010b) Genetic features and regulation of *n*-alkane metabolism. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 1141–1154.

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

Sikkema, J., de Bont, J.A. & Poolman, B. (1995) Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59**: 201–222.

Sofia, H.J., Chen, G., Hetzler, B.G., Reyes-Spindola, J.F. & Miller, N.E. (2001) Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res.* **29**: 1097–1106.

Sticher, P., Jaspers, M.C., Stemmler, K., Harms, H., Zehnder, A.J. & van der Meer, J.R. (1997) Development and characterization of a whole-cell bioluminescent sensor for bioavailable middle-chain alkanes in contaminated groundwater samples. *Appl. Environ. Microbiol.* **63**: 4053–4060.

Tissot, B.P. & Welte, D.H. (1984) Petroleum formation and occurence. Berlin, Germany: Springer Verlag.

Trautwein, K., Grundmann, O., Wöhlbrand, L., Eberlein, C., Boll, M. & Rabus, R. (2012) Benzoate mediates repression of C4-dicarboxylate utilization in "*Aromatoleum aromaticum*" EbN1. *J. Bacteriol.* **194**: 518–528.

van Beilen, J.B., Wubbolts, M.G. & Witholt, B. (1994) Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegradation* **5**: 161–174.

Verfürth, K., Pierik, A.J., Leutwein, C., Zorn, S. & Heider, J. (2004) Substrate specificities and electron paramagnetic resonance properties of benzylsuccinate synthases in anaerobic toluene and *m*-xylene metabolism. *Arch. Microbiol.* **181**: 155–162.

Weast, R.C. (1990) CRC Handbook of Chemistry and Physics, 70th ed., 1989-1990. Boca Raton, Florida: CRC Press.

Widdel, F., Knittel, K. & Galushko, A. (2010) Anaerobic hydrocarbon-degrading microorganisms: an overview. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 1998–2021.

Wilkes, H., Kühner, S., Bolm, C., Fischer, T., Classen, A., Widdel, F. & Rabus, R. (2003) Formation of *n*-alkane and cycloalkane-derived organic acids during anaerobic growth of a denitrifying bacterium with crude oil. *Org. Geochem.* **34**: 1313–1323.

C Gesamtübergreifende Diskussion und Ausblick

1. Die Bedeutung der (1-Methylalkyl)succinat-Synthase für den anaeroben *n*-Alkanabbau

Die Aktivierung von *n*-Alkanen unter anaeroben Bedingungen durch die (1-Methylalkyl)succinat-Synthase mittels Addition an Fumarat wurde ausgehend von Metabolitstudien, der Präsenz der Mas-Proteine bei Wachstum auf *n*-Hexan, sowie Sequenzähnlichkeiten der Mas-Proteine zur Benzylsuccinat-Synthase, die die Addition von Toluol an Fumarat katalysiert, für Stamm HxN1 postuliert (Rabus *et al.*, 2001; Grundmann *et al.*, 2008).

Die im Rahmen dieser Arbeit erzeugten Deletionsmutanten erbrachten erstmalig den *in vivo* Nachweis der Notwendigkeit der (1-Methylalkyl)succinat-Synthase für den anaeroben *n*-Alkanabbau in Stamm HxN1. Bei der Herstellung der Deletionsmutanten von Stamm HxN1 wurde ein zweites, identisches *mas* Operon identifiziert. Die einfach deletierte *masD* Mutante *(*∆*masD*) zeigte, nach einer gegenüber dem Wildtyp verlängerten Lag-Phase, ebenfalls Wachstum auf *n*-Hexan, wodurch die Funktionalität beider Operone bestätigt wird. Nach Deletion beider *masD* Gene (∆*masD*, ∆*masD´*) konnte Stamm HxN1 nicht mehr mit *n*-Hexan als einziger C-Quelle wachsen. Dies beweist, dass das Genprodukt MasD für den anaeroben *n*-Alkanabbau benötigt wird. Die längere Lag-Phase im Wachstum der einfachen Mutante macht auch deutlich, dass zwei funktionale *mas* Operone eine Optimierung hinsichtlich der Verwertung von *n*-Hexan darstellen, da größere Mengen an Protein gebildet werden als mit nur einem *mas* Operon. Für eine rasche Umsetzung des Substrates ist eine große Menge an Enzym vorteilhaft, da die maximale Geschwindigkeit der Enzymreaktion schneller erreicht wird. Nach Erreichen der Maximalgeschwindigkeit ist das Wachstum limitiert durch die Substratsättigung des Enzyms. Dies zeigt sich in der identischen Verdopplungszeit von Wildtyp und ∆*masD* Mutante.

Bei Wachstum des Wildtyps von Stamm HxN1 auf *n*-Hexan ist die große Untereinheit MasD nach gelelektrophoretischer Auftrennung als dominante Bande sichtbar und daher verglichen mit anderen Zellproteinen in großer Menge vorhanden. Schätzungen zufolge macht die (1-Methylalkyl)succinat-Synthase in Stamm HxN1 bis zu 6% des löslichen Proteingehaltes aus (Rabus *et al.*, 2001). Für die Benzylsuccinat-Synthase aus *Thauera aromatica* Stamm K172 wurde ein Anteil von mindestens 2–3% an der Gesamtmenge löslichen Proteins berechnet (Leuthner *et al.*, 1998). In der ∆*masD* Mutante sollte der

Proteingehalt von MasD nach der Lag-Phase nur die Hälfte des Proteingehaltes des Wildtyps ausmachen. Dieses muss noch experimentell bestätigt werden.

Die Fähigkeit zur Aktivierung von *n*-Hexan setzt zunächst die Expression der *mas* Gene voraus. In dieser Arbeit wurde gezeigt, dass die *mas* Gene nicht durch Katabolitrepression reguliert werden und dass neben *n*-Hexan auch viele Kohlenwasserstoffe, die nicht vollständig oder gar nicht oxidiert werden, induzierend wirken. Ein Sensor mit breitem Substratspektrum und die Induktion der Expression der *mas* Gene, sobald die Zelle mit Kohlenwasserstoffen in Kontakt kommt, können in der Umwelt vorteilhaft sein für ein kohlenwasserstoffabbauendes Bakterium. Diese Art der Regulation erlaubt nicht nur den Umsatz potenziell toxischer Substanzen durch die (1-Methylalkyl)succinat-Synthase, sondern auch eine schnelle Reaktion auf Substrate, die zur Energiegewinnung genutzt werden.

2. (1-Methylalkyl)succinat-Synthasen sind heterotetramere Glycylradikalenzyme

Die ersten bekannten Glycylradikalenzyme, Pyruvat-Formiat-Lyase und anaerobe Ribonukleotid-Reduktase sind Homodimere (Conradt *et al.*, 1984; Eliasson *et al.*, 1992). Ein weiteres Glycylradikalenzym ist die 4-Hydroxyphenylacetat-Decarboxylase, die die Bildung von *p*-Cresol in *Clostridium difficile* katalysiert (Selmer & Andrei, 2001). Sie besteht neben der großen, katalytischen Untereinheit aus einer weiteren kleinen Untereinheit, die zusammen ein $\alpha_4\beta_4$-Heterooktamer bilden (Andrei *et al.*, 2004; Yu *et al.*, 2006). Die kleine Untereinheit der 4-Hydroxyphenylacetat-Decarboxylase bildet Eisen-Schwefel-Zentren aus und ist notwendig für die katalytische Aktivität der großen Untereinheit (Yu *et al.*, 2006). Die Benzylsuccinat-Synthase war das erste Glycylradikalenzym mit drei Untereinheiten (Leuthner *et al.*, 1998). Aufgrund von Ähnlichkeiten in Bezug auf die Sequenz und die katalytische Funktion wurde auch für die (1-Methylalkyl)succinat-Synthase eine Zusammensetzung aus drei Untereinheiten postuliert (Grundmann *et al.*, 2008). Bei der Reinigung der (1-Methylalkyl)succinat-Synthase aus Stamm HxN1 wurde jedoch noch eine weitere Untereinheit identifiziert (Werner, 2009). Damit ist die (1-Methylalkyl)succinat-Synthase das erste beschriebene Glycylradikalenzym, das aus vier Untereinheiten besteht. Die Existenz des die vierte Untereinheit kodierenden *masB* Gens in allen bisher bekannten Stämmen, die *n*-Alkane anaerob mittels Addition an Fumarat aktivieren, legt nahe, dass diese zusätzliche Untereinheit eine Funktion ausübt, die für die anaerobe Aktivierung von Toluol nicht benötigt wird.

Möglicherweise ist der Unterschied in der Bindungsdissoziationsenergie, die aufgewendet werden muss, um die C–H-Bindung im Toluol bzw. am sekundären C-Atom des *n*-Alkans homolytisch zu spalten, hierfür von Bedeutung. Für die Aktivierung des *n*-Alkans werden 398 kJ mol^{-1} benötigt, für die Aktivierung von Toluol nur 368 kJ mol^{-1}. Dieser Unterschied von 30 kJ mol^{-1} ist womöglich auch verantwortlich dafür, dass Toluol von Stamm HxN1 aktiviert wird, *n*-Hexan jedoch nicht von dem toluolabbauenden *Aromatoleum aromaticum* Stamm EbN1 (Rabus *et al.*, 2011). Für die (1-Methylalkyl)succinat-Synthase sollte es energetisch kein Problem darstellen eine Reaktion zu katalysieren, für die weniger Energie benötigt wird als für die üblicherweise katalysierte Reaktion, während Benzylsuccinat-Synthasen vielleicht nicht in der Lage sind die stärkere C–H-Bindung in *n*-Alkanen zu spalten (Rabus *et al.*, 2011).

Ein weiterer Unterschied zwischen der Aktivierung von *n*-Hexan und von Toluol liegt in der Stereospezifität. Bei der Aktivierung von Toluol wird zu mehr als 95% das (*R*)-(+)-Benzylsuccinat Enantiomer gebildet (Beller & Spormann, 1998; Leutwein & Heider, 1999). Von dem bei der Aktivierung von *n*-Hexan mit Fumarat entstehenden (1-Methylpentyl)succinat existieren aufgrund von zwei chiralen Zentren vier Stereoisomere (Abb. 1). Zwei dieser Stereoisomere werden von Stamm HxN1 bei der Aktivierung von *n*-Hexan in gleichen Mengen gebildet, das 2*R*,1´*R*- und das 2*S*,1´*R*-Isomer (Rabus *et al.*, 2001; Jarling *et al.*, 2012). Am C-2 des Succinatrestes treten also beide Konfigurationen auf, während am C-1´ des Produktes (= C-2 des *n*-Hexans) nur die *R*-Konfiguration gebildet wird (Abb. 1). Die Bildung zweier Diastereoisomere ist erwähnenswert, da die meisten Enzymreaktionen stereoselektiv sind (Rabus *et al.*, 2001).

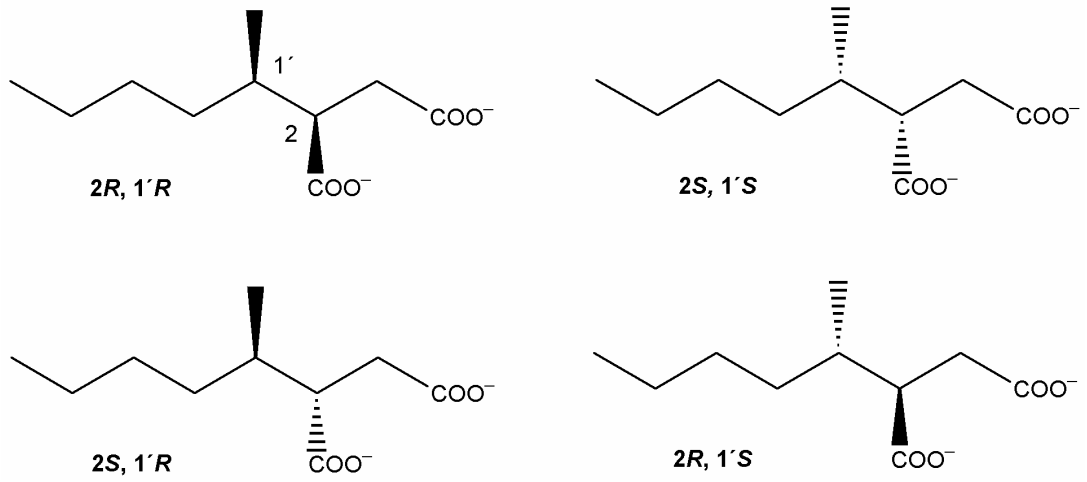

Abb. 1 Die vier Stereoisomere des (1-Methylpentyl)succinats. C-2 des Succinatrestes ist mit 2 gekennzeichnet, das ehemalige C-2 des *n*-Hexans (= C-1´) mit 1´.

Es wird spekuliert, dass in Stamm HxN1 eines der gebildeten Diastereoisomere des (1-Methylpentyl)succinats nach seiner möglichen Aktivierung zum CoA-Thioester in das andere epimerisiert wird, da nur eines der Diastereoisomere das Substrat einer möglichen Mutase ist, die die nachfolgende Reaktion zum (2-Methylhexyl)malonyl-CoA katalysiert (Wilkes *et al.*, 2002; Jarling *et al.*, 2012). Die Epimerisierung des einen CoA-Thioesters wird möglicherweise ebenfalls von der (1-Methylalkyl)succinat-Synthase katalysiert (Jarling *et al.*, 2012), wofür eine zusätzliche Untereinheit benötigt werden könnte.

Nach der Identifizierung des *mas* Operons in Stamm HxN1 wurde davon ausgegangen, dass die Genprodukte MasC und MasE homolog zu den kleinen Untereinheiten BssB und BssC der Benzylsuccinat-Synthase sind und damit die kleinen Untereinheiten der (1-Methylalkyl)succinat-Synthase repräsentieren (Grundmann *et al.*, 2008). Später wurde vermutet, dass nicht MasE sondern MasB (AssB in *Desulfatibacillum alkenivorans* Stamm AK-01) homolog zu BssB ist (Callaghan *et al.*, 2012; Hilberg *et al.*, 2012). Ursprung dieser Vermutung war ein Sequenzvergleich mehrerer BssB-Untereinheiten mit MasB und AssB, nach dem die Lage der Cysteine in MasB und AssB den BssB-Proteinen der Toluolabbauer ähnelt, auch wenn ansonsten keine Sequenzähnlichkeit besteht (Hilberg *et al.*, 2012). MasE jedoch passt von seiner Größe her besser zu den BssB-Proteinen als MasB, aber auch in diesem Fall gibt es keine Sequenzähnlichkeiten, obwohl in diesem Protein ebenfalls mehrere Cysteinreste vorkommen (Grundmann *et al.*, 2008). Die Reinigung der (1-Methylalkyl)succinat-Synthase aus Stamm HxN1 hat gezeigt, dass sowohl MasB als auch MasE Bestandteil des Enzyms sind. Daher gibt es womöglich in *n*-Alkanabbauern gar kein Homolog zu BssB der Benzylsuccinat-Synthase, weil die Aufgabe, die der BssB-Untereinheit bei der Toluolaktivierung zuteil wird, bei der Aktivierung von *n*-Alkanen von zwei Untereinheiten, MasB und MasE, durchgeführt wird. Erst eine Röntgenkristallstruktur für die (1-Methylalkyl)succinat-Synthase und die Benzylsuccinat-Synthase wird die Funktion der kleinen Untereinheiten aufklären.

3. Verbreitung kataboler Gene in Kohlenwasserstoffabbauern

Zwei funktionale *mas* Operone stellen aufgrund der größeren Menge an (1-Methylalkyl)succinat-Synthase eine Optimierung der Aktivierung von *n*-Hexan dar und sind damit für Stamm HxN1 ökonomisch sinnvoll. Die Präsenz eines für eine Transposase kodierenden Gens im *mas* Operon legt nahe, dass das *mas* Operon irgendwann im Genom verdoppelt und/oder neu ins Genom aufgenommen wurde. Transposasen inserieren DNA in andere DNA-Bereiche. Transposons bestehen aus der Sequenz, die für die Transposase kodiert, Sequenzwiederholungen, die von der

Transposase erkannt werden und aus weiteren Strukturgenen (Knippers, 2001). Das Transposon wird entweder aus der ursprünglichen Sequenz ausgeschnitten und in den Zielort wieder eingefügt oder replikativ vermehrt (Choi & Kim, 2009). Bei der replikativen Transposition erfolgt also eine Verdopplung des Transposons.

In dem *n*-Alkanabbauer *D. alkenivorans* Stamm AK-01 wurden ebenfalls zwei *ass* Operone identifiziert, die eine Alkylsuccinat-Synthase kodieren (Callaghan *et al.*, 2008). Die beiden Operone liegen auf dem 6,5 Mb großen Genom etwa 91 kb voneinander entfernt (Callaghan *et al.*, 2012). Im Gegensatz zu den *mas* Operonen in Stamm HxN1 sind die *ass* Operone jedoch nicht identisch. So beträgt die Identität der beiden katalytischen Untereinheiten AssA1 und AssA2 auf Proteinebene nur 81,6% (Callaghan *et al.*, 2008). Ob beide Operone eine aktive Alkylsuccinat-Synthase exprimieren, ist ungeklärt, da nur AssA1 in proteomischen Analysen eindeutig bei Wachstum auf *n*-Alkan nachgewiesen wurde (Callaghan *et al.*, 2008). Es wurde vermutet, dass die beiden Enzyme Ass1 und Ass2 verschiedene Substrate aktivieren oder zu unterschiedlichen Zeitpunkten des Wachstums aktiv sind (Callaghan *et al.*, 2008). Ein unterschiedliches Substratspektrum oder die differenzielle Induktion in Abhängigkeit von der Wachstumsphase der Zelle wurde für multiple, nicht identische Alkanhydroxylase Gene in mehreren aeroben *n*-Alkanabbauern nachgewiesen (Tani *et al.*, 2001; Whyte *et al.*, 2002; Marin *et al.*, 2003; van Beilen *et al.*, 2004; Liu *et al.*, 2011; Wang & Shao, 2011). Teilweise wurden Gene, die eine Transposase kodieren, in unmittelbarer Nähe der *alk* Gene identifiziert (van Beilen *et al.*, 2004). Innerhalb oder in der Nähe der *ass* Operone von Stamm AK-01 sind jedoch keine Transposasen kodiert (Callaghan *et al.*, 2012), Dies macht eine Verdopplung des Operons, so wie in Stamm HxN1 vermutet, unwahrscheinlich.

In der bekannten Sequenz vor dem *mas* Operon von Stamm HxN1 befinden sich Gene für zwei weitere Transposasen (Abb. 2a, dort annotiert als *tnpH1*, *tnpH2*). Sie beginnen in entgegengesetzter Richtung ca. 2 und 2,5 kb vor dem *mas* Operon. Auch in dem verwandten Stamm OcN1, in dessen Genom ebenfalls mindestens ein *mas* Operon kodiert ist, wurde in der bekannten Sequenz 5,3 kb hinter dem *mas* Operon ein Transposase-Gen (*tnpO1*) identifiziert (Abb. 2b). Im *mas* Operon von Stamm OcN1 selbst kodiert jedoch kein Gen eine Transposase (Werner, 2009).

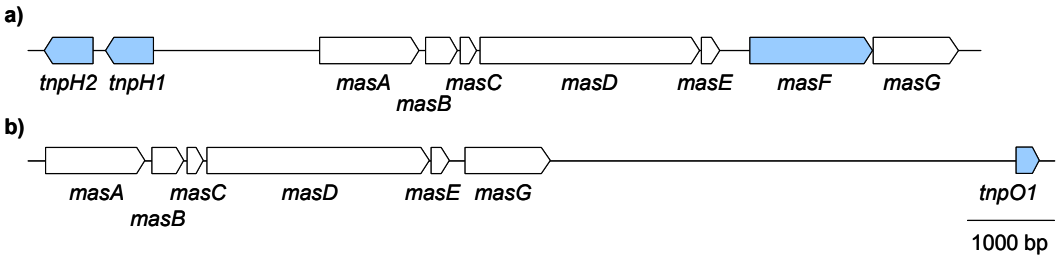

Abb. 2 Transposase-Gene in bekannten Sequenzbereichen der Stämme HxN1 (**a**) und OcN1 (**b**). Transposase-Gene sind blau markiert und wurden hier annotiert als *tnpH1* und *tnpH2* für „Transposase HxN1" bzw. *tnpO1* für „Transposase OcN1". Eine weitere Tranposase im *mas* Operon von Stamm HxN1 ist als *masF* annotiert. Weitere Gene der *mas* Operone sind weiß dargestellt. Erstes BLASTP Ergebnis für MasF mit 58% Sequenzidentität ist eine Transposase aus *Ralstonia solanacearum*, für TnpH1 mit 45% eine IS4 Transposase aus *Thauera* sp. MZ1T und für TnpH2 mit 81% Sequenzidentität bei vollständiger Abdeckung eine ISMca7 Transposase aus *Nitrococcus mobilis*. Die Transposase TnpO1 von Stamm OcN1 hat 78% Sequenzidentität zu einer Transposase aus *Acidovorax* sp. NO-1.

Von dem mit Stamm HxN1 und OcN1 verwandten Stamm EbN1 ist die Genomsequenz annotiert. Es wurden 180 Gene für Transposasen im bakteriellen Chromosom gefunden, sowie 57 weitere verteilt auf zwei Plasmide (Rabus *et al.*, 2005). Bei einer Größe des Genoms von 4,7 Mb (Chromosom: 4,3 Mb; Plasmid 1: 0,21 Mb; Plasmid 2: 0,22 Mb) kommt statistisch alle 20 000 kb ein Transposase-Gen vor. Das Genom des Sulfatreduzierers Stamm AK-01 ist ebenfalls sequenziert (Callaghan *et al.*, 2012). In diesem Genom wurden nur acht Gene für Transposasen annotiert. Dies entspricht bei einer Genomgröße von 6,5 Mb nur einem Transposase-Gen pro 812 000 kb. Die Identifizierung von drei Transposasen in einem 10,5 kb großen bekannten Bereich der DNA von Stamm HxN1 lässt vermuten, dass sich in dem größtenteils unbekannten Genom noch viele weitere Transposase-Gene, ähnlich wie in Stamm EbN1, befinden.

Die hohe Anzahl an Transposase-Genen in Stamm EbN1, teilweise in der Nähe von katabolen Genclustern, spricht für eine große Flexibilität des Genoms durch vielfachen horizontalen Gentransfer (Rabus *et al.*, 2005). Unterstützt wird diese These durch eine zum Teil hohe Sequenzidentität von katabolen Genclustern mit weiter entfernt verwandten Stämmen, wie z.B. *Thauera* sp. und das Vorkommen von paralogen Genclustern (Rabus *et al.*, 2005). Katabole Gencluster wurden womöglich durch horizontalen Gentransfer auf Stamm EbN1 übertragen, um die Adaptation des Stammes an veränderte Umweltbedingungen zu gewährleisten. Die genetische Flexibilität von Stamm AK-01 ist aufgrund der geringen Anzahl an Transposasen verglichen mit Stamm EbN1 wesentlich schwächer. Möglicherweise sind kohlenwasserstoffabbauende

Sulfatreduzierer wie Stamm AK-01 schon immer zum anaeroben Abbau von Kohlenwasserstoffen befähigt, da Kohlenwasserstoffe seit ihrer geologischen Bildung beständig aus natürlichen Erdöllagerstätten am Meeresgrund freigesetzt werden. Stamm AK-01, isoliert aus Kohlenwassterstoff-kontaminiertem Sediment, verwertet neben *n*-Alkanen auch 1-Alkene, Alkohole und Fettsäuren (So & Young, 1999), Stamm Bus5, isoliert aus dem Guaymas Basin, hingegen ausschließlich Propan und *n*-Butan (Kniemeyer *et al.*, 2007). Der Guaymas Basin im Golf von Kalifornien ist eine Hydrothermalquelle, die aufgrund tektonischer Aktivitäten beständig Alkane und aromatische Kohlenwasserstoffe freisetzt (Bazylinski *et al.*, 1989). Dort lebende Bakterien scheinen auf die Nutzung dieser Kohlenstoffquelle spezialisiert zu sein, wie das Beispiel Stamm Bus5 zeigt.

Süßwasserbakterien wie Stamm EbN1, HxN1 und OcN1 nutzen unter aeroben wie auch unter anaeroben Bedingungen eine Vielzahl an Substraten, wie z.B. Fettsäuren, Alkohole oder im Fall von Stamm HxN1 sogar Zucker als Kohlenstoffquelle (Rabus & Widdel, 1995; Ehrenreich, 1996; Behrends, 1999) und haben die Fähigkeit zum Abbau von Kohlenwasserstoffen vermutlich erst erworben, nachdem ihr Lebensraum anthropogen mit Kohlenwasserstoffen verschmutzt worden war. Stamm HxN1 und OcN1 wurden aus Grabensedimenten isoliert (Ehrenreich *et al.*, 2000), Stamm EbN1 aus einem Schlammgemisch von Gräben und der Weser (Rabus & Widdel, 1995). Die Aufnahme neuer kataboler Gene befähigte diese Stämme zum Abbau einer weiteren Kohlenstoffquelle. Dies ist unter substratlimitierenden Bedingungen von Vorteil gegenüber anderen Bakterien, die keine Kohlenwasserstoffe verwerten können. Die Enzyme für den weiteren Abbau über die β-Oxidation nach der Aktivierung des *n*-Alkans waren vermutlich schon vor der Aufnahme der neuen katabolen Gene in Stamm HxN1 vorhanden, da diese auch für den Abbau von Fettsäuren benötigt werden. Durch die Aktivierung der Kohlenwasserstoffe schützten sich die Zellen außerdem vor diesen toxischen Substanzen.

Katabole Gencluster, die eine zum Teil hohe Sequenzidentität untereinander aufweisen, wurden in vielen Bakterienstämme identifiziert, die phylogenetisch nicht miteinander verwandt sind und von geographisch unterschiedlichen Standorten isoliert wurden (Tsuda *et al.*, 1999). Das Vorkommen sehr ähnlicher Gene in verschiedenen Spezies spricht für einen gemeinsamen evolutionären Ursprung dieser Gene, die durch horizontalen Gentransfer mittels Transformation oder Konjugation zur Adaptation des Empfängers an veränderte Umweltbedingungen verbreitet wurden (Tsuda *et al.*, 1999). Daher ist es nicht überraschend, dass viele dieser Gencluster in Transposons lokalisiert sind. In dem 56 kb großen Transposon *Tn4651* befinden sich die nötigen Gene für den

aeroben Abbau von Toluol (Tsuda & Iino, 1987). Dieses Transposon ist oft Bestandteil des 117 kb großen TOL Plasmids pWWO, das in toluolabbauenden Pseudomonaden vorkommt (Burlage *et al.*, 1989; Greated *et al.*, 2002). Das Transposon *Tn4651* wird entweder über das konjugativ übertragbare Plasmid pWWO verbreitet oder es transponiert vom Plasmid ins Genom. Für die Transposition ins Genom spricht eine fast identische DNA-Sequenz des Transposons *Tn4651*, die im Genom zweier *Pseudomonas* Stämme identifiziert wurde (Sinclair *et al.*, 1986; Sinclair & Holloway, 1991). Die *alk* Gene für den aeroben Abbau von *n*-Alkanen in *Pseudomonas putida* GPo1 sind auf dem OCT-Plasmid kodiert (Chakrabarty *et al.*, 1973). Da die Gene von Insertionssequenzen flankiert sind, wird angenommen, dass die Insertionssequenzen zusammen mit den *alk* Genen ein Transposon bilden, welches in das OCT-Plasmid integriert wurde (van Beilen *et al.*, 2001). Eine ähnliche Genanordnung mit flankierenden Insertionssequenzen in *P. putida* P1 weist auf einen horizontalen Gentransfer der *alk* Gene hin (Smits *et al.*, 1999; van Beilen *et al.*, 2001). In *P. aeruginosa* PAO1 und *Alcanivorax borkumensis* AP1 befinden sich sogar je zwei nicht identische *alkB* Gene im Genom (van Beilen *et al.*, 2004). Nur eines der beiden *alkB* Gene von *A. borkumensis* ist Bestandteil eines *alk* Operons, ähnlich wie auf dem OCT-Plasmid. Das Fehlen mobiler genetischer Elemente deutet darauf hin, dass die *alk* Gene in diesem Fall vermutlich nicht über horizontalen Gentransfer von diesem Stamm erworben wurden, sondern vielmehr schon lange präsent sind. Dies ist übereinstimmend mit der Fähigkeit von *A. borkumensis* neben *n*-Alkanen nur wenige andere Kohlenstoffquellen nutzen zu können (van Beilen *et al.*, 2004). Auch für den Abbau zahlreicher anderer Kohlenwasserstoffe wurden katabole Transposons identifiziert, die auf Plasmiden oder im Genom lokalisiert sind (Nojiri *et al.*, 2004).

Katabole Gencluster werden nicht nur durch Transformation oder Konjugation, sondern auch über integrative und konjugative Elemente (ICElands) übertragen. Der Begriff ICEland umfasst mobile DNA-Elemente wie konjugative Transposons, integrative Plasmide und genomische Inseln (Burrus *et al.*, 2002). ICElands werden als zirkuläre DNA-Moleküle wie Plasmide von Zelle zu Zelle übertragen, ohne jedoch die Hilfe eines coexistierenden Plasmides zu benötigen (Tsuda *et al.*, 1999; van der Meer & Sentchilo, 2003). Als erstes wird das mobile Element aus der DNA herausgeschnitten und ligiert. Über den Mechanismus der rolling-circle Replikation wird es dann in die Empfängerzelle transferiert. Im Anschluss erfolgt die Integration ins Genom der Empfängerzelle und die Re-Integration in das Genom der Donorzelle (Tsuda *et al.*, 1999).

Die zahlreichen Beispiele horizontalen Gentransfers kataboler Gene für den Abbau von Kohlenwasserstoffen unterstützen die Hypothese, dass das *mas* Operon ebenfalls auf

einem der beschriebenen Wege in Stamm HxN1 transferiert und ins Genom integriert worden ist. Die Duplikation des *mas* Operons kann sowohl vor der Integration ins Genom als auch zu einem späteren Zeitpunkt, nachdem das Operon bereits ins Genom integriert worden war, stattgefunden haben.

4. Regulation der *mas* Operone in Stamm HxN1

Sowohl Stamm HxN1 als auch Stamm AK-01 haben in ihrem Genom zwei *mas* bzw. *ass* Operone kodiert. Die Regulation zweier Operone mit gleicher Funktion ist vermutlich komplexer als die Regulation eines einzelnen Operons. Alle bisher bekannten anaerob toluolabbauenden Bakterien haben nur ein *bss* Operon. In unmittelbarer Nähe stromaufwärts der *bss* Operone wurden Gene identifiziert, die ein Zweikomponenten-Regulationssystem kodieren (Coschigano & Young, 1997; Leuthner & Heider, 1998; Achong *et al.*, 2001; Kube *et al.*, 2004). Zweikomponentensysteme werden von Bakterien vielfach genutzt, um ihren Stoffwechsel als Antwort auf Umweltreize zu regulieren. Diese Systeme bestehen aus einer Sensorkinase und einem Transkriptionsregulator. Die membranständige Sensorkinase phosphoryliert sich als Reaktion auf die Wahrnehmung eines für sie empfänglichen Signals selbst an einem spezifischen intrazellulären Histidinrest. Durch anschließende Übertragung des Phosphorylrestes auf den Responseregulator wird dieser aktiviert. Der Responseregulator bindet an den Promotor, wodurch die Transkription der Gene aktiviert wird, die für die Verarbeitung des Umweltreizes benötigt werden.

Das *bss* Operon wird wahrscheinlich in Anwesenheit von Toluol durch das Zweikomponentensystem TdiSR (für toluene degradation induction) bzw. TutBC1 (für toluene utilization) aktiviert (Leuthner & Heider, 1998; Achong *et al.*, 2001; Kube *et al.*, 2004). In *T. aromatica* Stamm K172 wurde gezeigt, dass der Transkriptionsregulator TdiR an die 5´-DNA-Sequenz des *bss* Operons bindet (Leuthner & Heider, 1998). Auch für die Regulation des aeroben Abbaus von Toluol in *P. putida* F1 wurde ein Zweikomponentensystem (TodST; für toluene degradation) identifiziert (Lau *et al.*, 1997). Das fakultativ anaerobe Bakterium *T. aromatica* Stamm T1 oxidiert Toluol sowohl unter aeroben als auch unter anaeroben Bedigungen (Evans *et al.*, 1991). Zusätzlich zu dem TutBC1-System für die Regulation des anaeroben Abbaus ist in Stamm T1 ein weiteres Zweikomponentensystem, TutBC, vorhanden, das vermutlich für die Regulation des aeroben Abbaus zuständig ist, da es ähnlich dem TodST-System ist (Coschigano & Young, 1997; Leuthner & Heider, 1998).

Für die Regulation des Abbaus von *n*-Alkanen sind hingegen bisher keine Zweikomponentensysteme bekannt. In *P. putida* Gpo1 wird die Expression der plasmidkodierten Alkanhydroxylase-Gene von AlkS reguliert (van Beilen *et al.*, 1994). Die *n*-Alkane wirken bei dieser Regulation als Effektor, der an AlkS bindet. In Abwesenheit von *n*-Alkanen wird *alkS* in geringer Konzentration exprimiert, aber nur in Anwesenheit von *n*-Alkanen bindet AlkS an den Promotor der Alkanhydroxylase-Gene und induziert dadurch deren Expression (Rojo, 2009). Anders verhält es sich in *A. borkumensis* AP1 mit zwei für eine Alkanmonooxygenase kodierenden *alkB* Genen (van Beilen *et al.*, 2004). Die beiden *alkB* Gene sind nicht identisch, werden aber dennoch beide durch *n*-Alkane induziert. Nur vor einem der beiden *alkB* Gene ist *alkS* kodiert, dessen Expression jedoch nicht durch *n*-Alkane induziert wird. Die Induktion beider *alkB* Gene durch *n*-Alkane spricht für die Regulation durch denselben Regulator, der aber nicht AlkS ist (van Beilen *et al.*, 2004).

Die Regulation der beiden *mas* Operone in Stamm HxN1 erfolgt wahrscheinlich auch über einen gemeinsamen Regulator, der jedoch noch identifiziert werden muss. Offen ist auch, ob die Regulation über ein Zweikomponentensystem oder über einen Regulator, der direkt mit *n*-Alkanen interagiert, erfolgt. Das im Verlauf dieser Arbeit entwickelte genetische System für Stamm HxN1 kann zur Identifizierung des Regulators des anaeroben *n*-Alkanabbaus genutzt werden, sobald die Genomsequenz von Stamm HxN1 verfügbar ist. Auch der Regulator der *ass* Operone in Stamm AK-01 ist noch nicht identifiziert. Für diesen Stamm sind zwar die Genomdaten bekannt (Callaghan *et al.*, 2012), jedoch steht kein genetisches System zur Verfügung. Vorausgesetzt, dass beide *ass* Operone unter den gleichen Bedingungen aktiv sind, erfolgt vermutlich auch in diesem Fall die Regulation über denselben Regulator. In unmittelbarer Nähe der *ass* Operone wurden im Genom von AK-01 keine Gene für Regulator- oder Sensorproteine identifiziert (Callaghan *et al.*, 2012). Das nächstgelegene Gen, das einen Regulator kodiert, ist Dalk_1723, welches ca. 5 kb vor dem *ass1* Operon lokalisiert ist. Dalk_1723 ist annotiert als ein PAS-modulierter, σ54-spezifischer Transkriptionsregulator (Callaghan *et al.*, 2012). PAS-Domänen kommen in Sensoren für Sauerstoff, Licht und Redoxpotentiale vor, wurden aber auch in TodS, TutC und TdiS identifiziert (Leuthner & Heider, 1998; Taylor & Zhulin, 1999). Die Regulation des *n*-Alkanabbaus in Stamm AK-01 durch Dalk_1723 bleibt spekulativ, da im Genom noch weitere Transkriptionsregulatoren annotiert sind, denen keine Regulation zugeordnet ist (Callaghan *et al.*, 2012). Die Regulation von zwei weit voneinander entfernten Operonen setzt auch nicht voraus, dass das Gen für den Regulator in räumlicher Nähe zu einem der beiden Operone lokalisiert sein muss.

Globale Kontrollsysteme sind der spezifischen Regulation von Operonen durch einen einzelnen Regulator übergeordnet und dienen der Regulation mehrerer Stoffwechselwege. Die Katabolitrepression ist eine Kombination globaler und operonspezifischer Regulationsmechanismen, die in Anwesenheit mehrerer Wachstumssubstrate die Expression der Enzyme für deren Verstoffwechselung reguliert (Görke & Stülke, 2008). Erst nach Verbrauch des bevorzugten Substrates, welches normalerweise eine höhere Wachstumsrate ermöglicht, werden die Gene für den Abbau des anderen Substrates induziert (Harder & Dijkhuizen, 1982). Die Folge dieser sequenziellen Induktion ist diauxisches Wachstum, das durch eine kurze Lag-Phase zwischen zwei Wachstumsphasen gekennzeichnet ist (Harder & Dijkhuizen, 1982). Während der Lag-Phase werden die Proteine für den Abbau des zweiten Substrates synthetisiert. Die Katabolitrepression ist in *Enterobacteriaceae* (*Escherichia coli*) und *Firmicutes* (*Bacillus subtilis*) gut untersucht (Deutscher, 2008).

Das *lac* Operon in *E. coli* wird über einen Repressor negativ reguliert. Erst in Anwesenheit von Lactose wird das Operon induziert, indem der Induktor Allolactose an den Repressor bindet und diesen inhibiert (Görke & Stülke, 2008). Bei gleichzeitiger Anwesenheit von Glucose und Lactose im Medium wird das *lac* Operon nicht induziert, da der Transport von Lactose in die Zelle durch Inaktivierung der Lactose-Permease LacY verhindert wird (Görke & Stülke, 2008). Dieser Regulationsmechanismus wird als Induktorausschluss bezeichnet (Görke & Stülke, 2008). An der globalen Kontrolle des *lac* Operons sind der Transkriptionsaktivator CRP (für cyclic AMP receptor protein), cAMP, die Adenylatzyklase und die IIA Komponente des Glucose-spezifischen Phosphotransferasesystems ($EIIA^{Glc}$) beteiligt (Deutscher, 2008). Die effiziente Expression des *lac* Operons erfolgt nicht nur durch Inaktivierung des Repressors, sondern zusätzlich über den Aktivator CRP (Görke & Stülke, 2008). Dieser ist nur als Komplex mit cAMP aktiv, das von der Adenylatzyklase gebildet wird. Die Adenylatzyklase wiederum wird durch phosphoryliertes $EIIA^{Glc}$ aktiviert und $EIIA^{Glc}$ ist nur in Abwesenheit von Glucose phosphoryliert (Görke & Stülke, 2008). Die nicht phosphorylierte Form von $EIIA^{Glc}$ in Anwesenheit von Glucose inaktiviert die Permease LacY (Görke & Stülke, 2008). In *B. subtilis* wird die Expression alternativer kataboler Gene durch einen Repressor verhindert, der in Anwesenheit von Glucose aktiv ist (Görke & Stülke, 2008).

Die Katabolitrepression spielt auch bei der Regulation des aeroben *n*-Alkanabbaus in *P. putida* eine Rolle (Rojo, 2010b). In Anwesenheit von Succinat verhindert das globale Regulationsprotein Crc (für catabolite repression control) die Translation des Regulators AlkS durch Bindung an das 5´-Ende der kodierenden mRNA (Moreno *et al.*, 2007).

Anders als in *E. coli* und *B. subtilis* erfolgt die Regulation in *P. putida* also nicht auf Transkriptions- sondern auf Translationsebene. Der anaerobe *n*-Alkanabbau in Stamm HxN1 hingegen wird nicht durch Katabolitrepression reguliert, wie die in dieser Arbeit erhaltenen Ergebnisse zeigen. Die Expression der *mas* Gene wird bei Kultivierung von Stamm HxN1 mit *n*-Alkan und einer weiteren Kohlenstoffquelle nicht inhibiert. Eine mögliche Erklärung ist, dass nur die getesteten Kohlenstoffquellen keine Inhibierung der Expression bewirken, es aber noch andere Kohlenstoffquellen gibt, die die Expression inhibieren. Beispielsweise wird in *P. putida* die Expression der *alk* Gene zwar u.a. durch Succinat inhibiert, jedoch nicht durch Citrat (Yuste *et al.*, 1998). Auch unter substratlimitierenden Bedingungen erfolgt normalerweise keine Katabolitrepression, sondern ein simultaner Verbrauch der Substrate (Harder & Dijkhuizen, 1982). Das Vorliegen einer Substratlimitation und eines simultanen Verbrauchs bei den in dieser Arbeit durchgeführten Versuchen mit Stamm HxN1 auf *n*-Hexan und einer weiteren Kohlenstoffquelle wie z.B. Capronat ist jedoch ausgeschlossen. Solange regulatorische Proteine nicht identifiziert sind, kann über die Regulation des *mas* Operons nur spekuliert werden. Einerseits kann das *mas* Operon positiv über einen Aktivator reguliert werden, der durch Induktoren wie z.B. *n*-Hexan aktiviert wird und die Expression induziert (Abb. 3). Andererseits ist es auch möglich, dass das *mas* Operon negativ über einen Repressor reguliert wird, der die Expression in Abwesenheit eines Induktors verhindert.

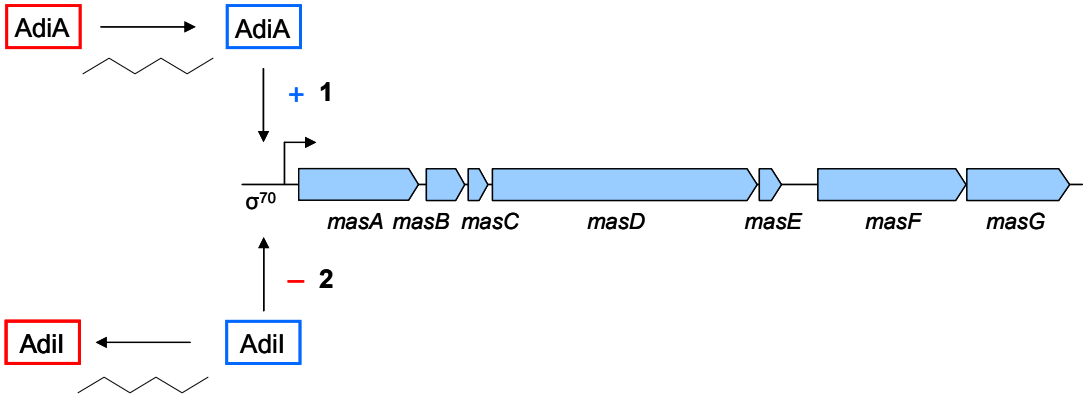

Abb. 3 Modell zur möglichen Regulation des *mas* Operons in Stamm HxN1. (**1**) Positive Regulation des *mas* Operons durch einen Aktivator AdiA (für alkane degradation induction activator), der in Anwesenheit eines Induktors wie z.B. *n*-Hexan aktiviert wird und die Expression initiiert. (**2**) Negative Regulation des *mas* Operons durch einen Repressor AdiI (für alkane degradation induction inhibitor), der in Abwesenheit eines Induktors aktiv ist und die Expression des *mas* Operons inhibiert. Blau: aktiv; rot: inaktiv; + : positive Regulation; – : negative Regulation.

Hinzuzufügen ist, dass nicht alle Bakterien ihren Stoffwechsel durch Katabolitrepression regulieren. Eine Ausnahme bilden z.B. pathogene Bakterien wie *Chlamydia trachomatis*, die aufgrund ihrer Adaptation an eine nährstoffreiche Umgebung keine Katabolit-repression benötigen (Nicholson *et al.*, 2004).

Auffällig in Stamm HxN1 ist auch die Induktion des *mas* Operons durch eine Vielzahl an Kohlenwasserstoffen, die gar nicht zur Energiegewinnung genutzt werden. Die bereits angesprochene Möglichkeit der Detoxifizierung dieser Substanzen durch ihre Aktivierung trifft nicht für alle Induktoren zu, da nicht alle positiv getesteten Induktoren nachgewiesenermaßen durch die (1-Methylalkyl)succinat-Synthase aktiviert werden (Wilkes *et al.*, 2003). Eine Erklärung liefert die „regulatory noise"-Hypothese, die besagt, dass regulatorische Gene eine gewisse Unspezifität benötigen, um auch die Regulation für die Verwertung neuer Substrate, die vorher nicht vorhanden waren, übernehmen zu können (de Lorenzo & Perez-Martin, 1996) Im Verlauf der Evolution werden neue Strukturgene, wie z.B. Gene für den Abbau von Kohlenwasserstoffen, unter die Kontrolle schon vorhandener Promotoren und ihrer Regulatoren gestellt. Die Expression dieser Strukturgene ist jedoch nur möglich, wenn das transkriptionelle Kontrollsystem einen geeigneten Induktor erkennt. Dies wiederum erfordert einen unspezifischen Regulator (de Lorenzo & Perez-Martin, 1996). Wie oben erwähnt, wurden die *mas* Gene vermutlich nachträglich von Stamm HxN1 erworben, um auf die Anwesenheit von *n*-Alkanen zu reagieren. Die große Substratspezifität des Regulators kann auf eine noch andauernde evolutionäre Optimierung hin zu dem am besten geeigneten Induktor für die Expression der *mas* Gene hindeuten.

5. Ausblick: Kohlenwasserstoffabbauende Bakterien für die biologische Sanierung

Öl wird seit Millionen von Jahren als natürlicher Prozess aus Austrittstellen am Meeresgrund in die Ozeane freigesetzt. Seit der Nutzung von Erdöl als Energieträger kommt es zusätzlich zur Freisetzung von Öl und Derivaten aus Tankern oder Förderanlagen in Gewässer. Durch derartige Unfälle werden Ökosysteme beschädigt, da anders als bei den natürlichen Ölreservoirs innerhalb kurzer Zeit große Mengen an Öl freigesetzt werden, die oftmals auch an die Küste gespült werden, so wie bei der Havarie der *Exxon Valdez* 1989 vor der Küste Alaskas (Galt *et al.*, 1991). In Ölreservoirs leben an das Öl adaptierte Mikroorganismen, die einen Beitrag zur Entfernung des Öls leisten, wohingegen in anthropogen kontaminierten Gebieten solche Mikroorganismen oft nur in geringen Mengen vorkommen (Atlas & Hazen, 2011). Daher ist es erforderlich

Maßnahmen zur Dekontamination von Ölverschmutzungen zu entwickeln. Es gibt nur zwei Möglichkeiten Öl komplett zu entfernen, entweder durch vollständige Verbrennung oder durch biologischen Abbau.

Die Dekontamination mithilfe von Bakterien, die *n*-Alkane und Aromaten des Öls abbauen, wird als biologische Sanierung (Bioremediation) bezeichnet. Das Wachstum ubiquitär vorhandener kohlenwasserstoffabbauender Bakterien zu stimulieren ist das Ziel der Biostimulation (Prince, 2010b). Normalerweise ist das Wachstum der Bakterien durch die begrenzte Verfügbarkeit von Kohlenwasserstoffen und anderer Nährstoffe wie Phosphat und Stickstoff limitiert (Prince, 2010b). Das Vorhandensein von Kohlenwasserstoffen allein ist aufgrund ihrer schlechten Löslichkeit in Wasser normalerweise nicht ausreichend für ihren mikrobiellen Abbau. Chemische Dispergenzien unterstützen die Verteilung von Öl als kleine Tröpfchen in Wasser (Prince, 2010b). Öltropfen haben ein großes Oberflächen-Volumen-Verhältnis und stehen damit einer größeren Anzahl an Bakterien zur Verfügung als ein großer Ölteppich. Die Verwendung kleiner Mineralpartikel, die mit dem Öl interagieren, bewirkt ebenfalls eine Oberflächenvergrößerung des Öls (Owens & Lee, 2003). Bakterien sind auch selbst in der Lage Emulgatoren oder Tenside wie Rhamnolipide zu sekretieren, die Micellen bilden, in deren hydrophoben Inneren das Öl eingefangen wird (van Hamme *et al.*, 2003; Perfumo *et al.*, 2010). Der Einsatz von Düngemitteln liefert für das Wachstum benötigten Stickstoff und Phosphor. In mehreren Fällen wurde eine erhöhte Rate des Abbaus von Kohlenwasserstoffen in Folge einer Düngung beobachtet (Prince, 2010b). Nach der Havarie der *Exxon Valdez* vor der Küste Alaskas wurden in drei Jahren fast 50 Tonnen biologisch verfügbarer Stickstoff eingesetzt, ohne einen sichtbaren negativen Einfluss auf die Umwelt ausgeübt zu haben (Prince, 2010b). Dennoch sind Algenblüten eine mögliche Folge zu großer Mengen an Stickstoff.

Ziel der Bioaugmentation ist es, kohlenwasserstoffabbauende Bakterien zu einem kontaminierten Gebiet hinzuzufügen, damit diese Bakterien zur Dekontamination beitragen (Prince, 2010a). Nachdem im Jahre 1981 das erste genetisch veränderte Bakterium patentiert wurde, das Kohlenwasserstoffe abbaut (US Patent 4259444), wurde davon ausgegangen, dass der Einsatz solcher Bakterien eine erfolgreiche Methode zur biologischen Sanierung mariner Ölverschmutzungen wird (Prince, 2010b). Bisher haben sich diese Erwartungen jedoch nicht erfüllt, da sich u.a. die vorherrschenden Bedingungen in der Natur stark von den optimalen (Labor-)Bedingungen der Bakterien unterscheiden. Nur sehr wenige Studien zur Dekontamination wurden mit Wildtypstämmen durchgeführt und ein klarer Nachweis des Abbaus von Kohlenwasserstoffen durch diese Bakterien konnte nicht erbracht werden

(Prince, 2010b). Im Gegensatz dazu zeigten Bioaugmentationsversuche in anaerobem, kontaminiertem Grundwasser, dass der Abbau aromatischer Kohlenwasserstoffe durch Inokulation mit kohlenwasserstoffabbauenden Anreicherungskulturen induziert wird (Weiner & Lovley, 1998; da Silva & Alvarez, 2004). Mithilfe von anaeroben Kohlenwasserstoffabbauern können also von Natur aus anaerobe Umgebungen wie z.B. Sumpfsedimente oder kontaminiertes Grundwasser saniert werden. Der Einsatz von Nitratreduzierern, wie z.B. Stamm HxN1, für die biologische Sanierung erscheint nicht nur aufgrund der größeren Energieausbeute, sondern auch wegen der hohen Wasserlöslichkeit von Nitrat (92,1 g/100 ml bei 25 °C) interessant (Mbadinga *et al.*, 2011).

Die Kenntnis der metabolischen Aktivitäten kohlenwasserstoffabbauender Bakterien ist auch für die enzymatische biologische Sanierung von Bedeutung (Sutherland *et al.*, 2004; Alcalde *et al.*, 2006; Peixoto *et al.*, 2011). Enzyme, die für die biologische Sanierung eingesetzt werden, müssen in gegebenen Umweltbedingungen funktionsfähig sein. Die Abhängigkeit vieler Enzyme von Cofaktoren limitiert, neben hohen Produktionskosten und geringen Ausbeuten bei der Enzymreinigung, ihren Einsatz. Bislang ist nur ein anwendbares, reines Enzymadditiv beschrieben. Das Produkt, Oil Spill Eater II, verringert laut Herstellerangaben unter aeroben Bedingungen die Menge an Alkanen und Aromaten nach sieben Tagen um 36,9 bzw. 33,6%, und nach 28 Tagen sogar um 89,8 bzw. 89,6% (Peixoto *et al.*, 2011). Der Einsatz von Enzymen hat jedoch den Vorteil, dass keine (gentechnisch veränderten) Mikroorganismen in die Umwelt eingebracht werden müssen und modifizierte Proteine *in vitro* produziert werden.

Mithilfe von funktionellen Markergenen wurden in kontaminierten Gebieten viele Gensequenzen identifiziert, die katabole Enzyme kodieren. Zwei bekannte Marker sind *bssA* und *assA*, die die katalytische Untereinheit der Benzylsuccinat- bzw. Alkylsuccinat-Synthase kodieren (Winderl *et al.*, 2007; Callaghan *et al.*, 2010). Solche Marker können dazu beitragen, Gene und damit Proteine von nicht-kultivierbaren Organismen zu detektieren (Peixoto *et al.*, 2011). Möglicherweise sind diese Proteine effizienter im Abbau von Kohlenwasserstoffen oder haben einen anderen Vorteil gegenüber den bisher bekannten Enzymen von kultivierbaren Organismen. Wenn die Gensequenz solcher Proteine bekannt ist, können diese Proteine *in vitro* produziert werden und dann zur enzymatischen Sanierung genutzt werden.

Nicht zuletzt ist die Kenntnis kohlenwasserstoffabbauender Bakterien auch für die Ölindustrie von Interesse, da die Qualität von Rohöl in Lagerstätten durch die mikrobiologische Aktivität vermindert wird und die Förderung hierdurch kostenintensiver und schwieriger wird (Head *et al.*, 2003). Die Erforschung von Bakterien wie Stamm

HxN1 trägt dazu bei, den Abbau von Kohlenwasserstoffen in Ölreservoirs und kontaminierten Sedimenten oder Grundwasser besser zu verstehen und bildet damit auch eine Grundlage für den Einsatz dieser Bakterien oder ihrer katabolen Proteine zur biologischen Sanierung. Stamm HxN1 eignet sich aufgrund seiner guten Kultivierbarkeit als Modellorganismus und weist darüber hinaus interessante Eigenschaften auf, die das Ergebnis der vorliegenden Arbeit sind und die für ein umfassendes Verständnis des anaeroben *n*-Alkanabbaus weiter untersucht werden sollten.

Referenzen für A und C

Achong, G.R., Rodriguez, A.M. & Spormann, A.M. (2001) Benzylsuccinate synthase of *Azoarcus* sp. strain T: cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization. *J. Bacteriol.* **183**: 6763–6770.

Aeckersberg, F., Bak, F. & Widdel, F. (1991) Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. *Arch. Microbiol.* **156**: 5–14.

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

Alcalde, M., Ferrer, M., Plou, F.J. & Ballesteros, A. (2006) Environmental biocatalysis: from remediation with enzymes to novel green processes. *Trends Biotechnol.* **24**: 281–287.

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

Anderson, R.T. & Lovley, D.R. (2000) Hexadecane decay by methanogenesis. *Nature* **404**: 722–723.

Andrei, P.I., Pierik, A.J., Zauner, S., Andrei-Selmer, L.C. & Selmer, T. (2004) Subunit composition of the glycyl radical enzyme p-hydroxyphenylacetate decarboxylase. A small subunit, HpdC, is essential for catalytic activity. *Europ. J. Biochem.* **271**: 2225–2230.

Annweiler, E., Materna, A., Safinowski, M., Kappler, A., Richnow, H.H., Michaelis, W. & Meckenstock, R.U. (2000) Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. *Appl. Environ. Microbiol.* **66**: 5329–5333.

Atlas, R.M. & Hazen, T.C. (2011) Oil biodegradation and bioremediation: a tale of the two worst spills in U.S. history. *Environ. Sci. Technol.* **45**: 6709–6715.

Baraniak, J., Moss, M.L. & Frey, P.A. (1989) Lysine 2,3-aminomutase. Support for a mechanism of hydrogen transfer involving S-adenosylmethionine. *J. Biol. Chem.* **264**: 1357–1360.

Bazylinski, D.A., Wirsen, C.O. & Jannasch, H.W. (1989) Microbial utilization of naturally occurring hydrocarbons at the guaymas basin hydrothermal vent site. *Appl. Environ. Microbiol.* **55**: 2832–2836.

Behrends, A. (1999) Physiologie und substratspezifische Proteinbildung denitrifizierender Bakterien mit der Fähigkeit zur anaeroben Oxidation kurzkettiger Alkane. Dissertation, Universität Bremen.

Bell, G.H. (1973) Solubilities of normal aliphatic acids, alcohols and alkanes in water. *Chem. Phys. Lipids* **10**: 1–10.

Beller, H.R. & Spormann, A.M. (1997a) Benzylsuccinate formation as a means of anaerobic toluene activation by sulfate-reducing strain PRTOL1. *Appl. Environ. Microbiol.* **63**: 3729–3731.

Beller, H.R. & Spormann, A.M. (1997b) Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. *J. Bacteriol.* **179**: 670–676.

Beller, H.R. & Spormann, A.M. (1998) Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. *J. Bacteriol.* **180**: 5454–5457.

Beller, H.R. & Edwards, E.A. (2000) Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. *Appl. Environ. Microbiol.* **66**: 5503–5505.

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

Boetius, A., Ravenschlag, K., Schubert, C.J., Rickert, D., Widdel, F., Gieseke, A. *et al.* (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* **407**: 623–626.

Bonin, P., Cravo-Laureau, C., Michotey, V. & Hirschler-Rea, A. (2004) The anaerobic hydrocarbon biodegrading bacteria: An overview. *Ophelia* **58**: 243–254.

Bregnard, T.P., Haner, A., Hohener, P. & Zeyer, J. (1997) Anaerobic degradation of pristane in nitrate-reducing microcosms and enrichment cultures. *Appl. Environ. Microbiol.* **63**: 2077–2081.

Buckel, W. & Golding, B.T. (2006) Radical enzymes in anaerobes. *Annu. Rev. Microbiol.* **60**: 27–49.

Burlage, R.S., Hooper, S.W. & Sayler, G.S. (1989) The TOL (pWW0) catabolic plasmid. *Appl. Environ. Microbiol.* **55**: 1323–1328.

Burrus, V., Pavlovic, G., Decaris, B. & Guédon, G. (2002) Conjugative transposons: the tip of the iceberg. *Mol. Microbiol.* **46**: 601–610.

Caldwell, M.E., Garrett, R.M., Prince, R.C. & Suflita, J.M. (1998) Anaerobic biodegradation of long-chain *n*-alkanes under sulfate-reducing conditions. *Environ. Sci. Technol.* **32**: 2191–2195.

Callaghan, A.V., Tierney, M., Phelps, C.D. & Young, L.Y. (2009) Anaerobic biodegradation of *n*-hexadecane by a nitrate-reducing consortium. *Appl. Environ. Microbiol.* **75**: 1339–1344.

Callaghan, A.V., Gieg, L.M., Kropp, K.G., Suflita, J.M. & Young, L.Y. (2006) Comparison of mechanisms of alkane metabolism under sulfate-reducing conditions among two bacterial isolates and a bacterial consortium. *Appl. Environ. Microbiol.* **72**: 4274–4282.

Callaghan, A.V., Wawrik, B., Ni Chadhain, S.M., Young, L.Y. & Zylstra, G.J. (2008) Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. *Biochem. Biophys. Res. Commun.* **366**: 142–148.

Callaghan, A.V., Davidova, I.A., Savage-Ashlock, K., Parisi, V.A., Gieg, L.M., Suflita, J.M. *et al.* (2010) Diversity of benzyl- and alkylsuccinate synthase genes in hydrocarbon-impacted environments and enrichment cultures. *Environ. Sci. Technol.* **44**: 7287–7294.

Callaghan, A.V., Morris, B.E., Pereira, I.A., McInerney, M.J., Austin, R.N., Groves, J.T. *et al.* (2012) The genome sequence of *Desulfatibacillum alkenivorans* AK-01: a blueprint for anaerobic alkane oxidation. *Environ. Microbiol.* **14**: 101–113.

Chakrabarty, A.M., Chou, G. & Gunsalus, I.C. (1973) Genetic regulation of octane dissimilation plasmid in *Pseudomonas*. *Proc. Natl. Acad. Sci. U S A* **70**: 1137–1140.

Choi, K.H. & Kim, K.J. (2009) Applications of transposon-based gene delivery system in bacteria. *J. Microbiol. Biotechnol.* **19**: 217–228.

Conradt, H., Hohmann-Berger, M., Hohmann, H.P., Blaschkowski, H.P. & Knappe, J. (1984) Pyruvate formate-lyase (inactive form) and pyruvate formate-lyase activating enzyme of *Escherichia coli*: isolation and structural properties. *Arch. Biochem. Biophys.* **228**: 133–142.

Coschigano, P.W. (2000) Transcriptional analysis of the *tutE tutFDGH* gene cluster from *Thauera aromatica* strain T1. *Appl. Environ. Microbiol.* **66**: 1147–1151.

Coschigano, P.W. & Young, L.Y. (1997) Identification and sequence analysis of two regulatory genes involved in anaerobic toluene metabolism by strain T1. *Appl. Environ. Microbiol.* **63**: 652–660.

Coschigano, P.W., Wehrman, T.S. & Young, L.Y. (1998) Identification and analysis of genes involved in anaerobic toluene metabolism by strain T1: putative role of a glycine free radical. *Appl. Environ. Microbiol.* **64**: 1650–1656.

Cravo-Laureau, C., Matheron, R., Cayol, J.L., Joulian, C. & Hirschler-Réa, A. (2004) *Desulfatibacillum aliphaticivorans* gen. nov., sp. nov., an *n*-alkane- and *n*-alkene-degrading, sulfate-reducing bacterium. *Int. J. Syst. Evol. Microbiol.* **54**: 77–83.

Cravo-Laureau, C., Grossi, V., Raphel, D., Matheron, R. & Hirschler-Réa, A. (2005) Anaerobic *n*-alkane metabolism by a sulfate-reducing bacterium, *Desulfatibacillum aliphaticivorans* strain CV2803T. *Appl. Environ. Microbiol.* **71**: 3458–3467.

da Silva, M.L.B. & Alvarez, P.J.J. (2004) Enhanced anaerobic biodegradation of benzene-toluene-ethylbenzene-xylene-ethanol mixtures in bioaugmented aquifer columns. *Appl. Environ. Microbiol.* **70**: 4720–4726.

Davidova, I.A. & Suflita, J.M. (2005) Enrichment and isolation of anaerobic hydrocarbon-degrading bacteria. *Methods Enzymol.* **397**: 17–34.

Davidova, I.A., Gieg, L.M., Nanny, M., Kropp, K.G. & Suflita, J.M. (2005) Stable isotopic studies of n-alkane metabolism by a sulfate-reducing bacterial enrichment culture. *Appl. Environ. Microbiol.* **71**: 8174–8182.

de Lorenzo, V. & Perez-Martin, J. (1996) Regulatory noise in prokaryotic promoters: how bacteria learn to respond to novel environmental signals. *Mol. Microbiol.* **19**: 1177–1184.

Deutscher, J. (2008) The mechanisms of carbon catabolite repression in bacteria. *Curr. Opin. Microbiol.* **11**: 87–93.

Ehrenreich, P. (1996) Anaerobes Wachstum neuartiger sulfatreduzierender und nitratreduzierender Bakterien auf *n*-Alkanen und Erdöl. Dissertation, Universität Bremen.

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

Eliasson, R., Pontis, E., Fontecave, M., Gerez, C., Harder, J., Jornvall, H. *et al.* (1992) Characterization of components of the anaerobic ribonucleotide reductase system from *Escherichia coli*. *J. Biol. Chem.* **267**: 25541–25547.

Ettwig, K.F., Shima, S., van de Pas-Schoonen, K.T., Kahnt, J., Medema, M.H., Op den Camp, H.J. *et al.* (2008) Denitrifying bacteria anaerobically oxidize methane in the absence of Archaea. *Environ. Microbiol.* **10**: 3164–3173.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M. *et al.* (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543–548.

Evans, P.J., Mang, D.T., Kim, K.S. & Young, L.Y. (1991) Anaerobic degradation of toluene by a denitrifying bacterium. *Appl. Environ. Microbiol.* **57**: 1139–1145.

Galt, J.A., Lehr, W.J. & Payton, D.L. (1991) Fate and transport of the Exxon Valdez oil spill, part 4. *Environ. Sci. Technol.* **25**: 202-209.

Görke, B. & Stülke, J. (2008) Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol.* **6**: 613–624.

Greated, A., Lambertsen, L., Williams, P.A. & Thomas, C.M. (2002) Complete sequence of the IncP-9 TOL plasmid pWW0 from *Pseudomonas putida*. *Environ. Microbiol.* **4**: 856–871.

Grossi, V., Cravo-Laureau, C., Guyoneaud, R., Ranchou-Peyruse, A. & Hirschler-Réa, A. (2008) Metabolism of *n*-alkanes and *n*-alkenes by bacteria: a summary. *Org. Geochem.* **39**: 1197–1203.

Grossi, V., Cravo-Laureau, C., Méou, A., Raphel, D., Garzino, F. & Hirschler-Réa, A. (2007) Anaerobic 1-alkene metabolism by the alkane- and alkene-degrading sulfate reducer *Desulfatibacillum aliphaticivorans* strain CV2803T. *Appl. Environ. Microbiol.* **73**: 7882–7890.

Grundmann, O., Behrends, A., Rabus, R., Amann, J., Halder, T., Heider, J. & Widdel, F. (2008) Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN1. *Environ. Microbiol.* **10**: 376–385.

Hanson, R.S. & Hanson, T.E. (1996) Methanotrophic bacteria. *Microbiol. Rev.* **60**: 439–471.

Harder, W. & Dijkhuizen, L. (1982) Strategies of mixed substrate utilization in microorganisms. *Philos. Trans. R. Soc. London* **297**: 459–480.

Hazen, T.C., Dubinsky, E.A., DeSantis, T.Z., Andersen, G.L., Piceno, Y.M., Singh, N. *et al.* (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. *Science* **330**: 204–208.

Head, I.M., Jones, D.M. & Larter, S.R. (2003) Biological activity in the deep subsurface and the origin of heavy oil. *Nature* **426**: 344–352.

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

Higashioka, Y., Kojima, H., Nakagawa, T., Sato, S. & Fukui, M. (2009) A novel *n*-alkane-degrading bacterium as a minor member of *p*-xylene-degrading sulfate-reducing consortium. *Biodegradation* **20**: 383–390.

Hilberg, M., Pierik, A.J., Bill, E., Friedrich, T., Lippert, M.L. & Heider, J. (2012) Identification of FeS clusters in the glycyl-radical enzyme benzylsuccinate synthase via EPR and Mossbauer spectroscopy. *J. Biol. Inorg. Chem.* **17**: 49–56.

Hinrichs, K.U., Hayes, J.M., Bach, W., Spivack, A.J., Hmelo, L.R., Holm, N.G. *et al.* (2006) Biological formation of ethane and propane in the deep marine subsurface. *Proc. Natl. Acad. Sci. U S A* **103**: 14684–14689.

Holler, T., Wegener, G., Niemann, H., Deusner, C., Ferdelman, T.G., Boetius, A. *et al.* (2011) Carbon and sulfur back flux during anaerobic microbial oxidation of methane and coupled sulfate reduction. *Proc. Natl. Acad. Sci. U S A* **108**: 1484–1490.

Jarling, R., Sadeghi, M., Drozdowska, M., Lahme, S., Buckel, W., Rabus, R. *et al.* (2012) Stereochemical investigations reveal the mechanism of the bacterial activation of *n*-alkanes without oxygen. *Angew. Chem. Int. Ed.* **51**: 1334–1338.

Jones, D.M., Head, I.M., Gray, N.D., Adams, J.J., Rowan, A.K., Aitken, C.M. *et al.* (2008) Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* **451**: 176–180.

Kane, S.R., Beller, H.R., Legler, T.C. & Anderson, R.T. (2002) Biochemical and genetic evidence of benzylsuccinate synthase in toluene-degrading, ferric iron-reducing *Geobacter metallireducens*. *Biodegradation* **13**: 149–154.

Keppler, F., Hamilton, J.T., Brass, M. & Rockmann, T. (2006) Methane emissions from terrestrial plants under aerobic conditions. *Nature* **439**: 187–191.

King, D.S. & Reichard, P. (1995) Mass spectrometric determination of the radical scission site in the anaerobic ribonucleotide reductase of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **206**: 731–735.

Knappe, J., Neugebauer, F.A., Blaschkowski, H.P. & Ganzler, M. (1984) Post-translational activation introduces a free radical into pyruvate formate-lyase. *Proc. Natl. Acad. Sci. U S A* **81**: 1332–1335.

Knappe, J., Elbert, S., Frey, M. & Wagner, A.F. (1993) Pyruvate formate-lyase mechanism involving the protein-based glycyl radical. *Biochem. Soc. Trans.* **21**: 731–734.

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

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

Kniemeyer, O., Musat, F., Sievert, S.M., Knittel, K., Wilkes, H., Blumenberg, M. *et al.* (2007) Anaerobic oxidation of short-chain hydrocarbons by marine sulphate-reducing bacteria. *Nature* **449**: 898–901.

Knippers, R. (2001) Molekulare Genetik. Stuttgart, New York: Georg Thieme Verlag.

Krieger, C.J., Beller, H.R., Reinhard, M. & Spormann, A.M. (1999) Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. *J. Bacteriol.* **181**: 6403–6410.

Krieger, C.J., Roseboom, W., Albracht, S.P. & Spormann, A.M. (2001) A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus* sp. strain T. *J. Biol. Chem.* **276**: 12924–12927.

Kropp, K.G., Davidova, I.A. & Suflita, J.M. (2000) Anaerobic oxidation of *n*-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. *Appl. Environ. Microbiol.* **66**: 5393–5398.

Krüger, M., Meyerdierks, A., Glöckner, F.O., Amann, R., Widdel, F., Kube, M. *et al.* (2003) A conspicuous nickel protein in microbial mats that oxidize methane anaerobically. *Nature* **426**: 878–881.

Kube, M., Heider, J., Amann, J., Hufnagel, P., Kuhner, S., Beck, A. *et al.* (2004) Genes involved in the anaerobic degradation of toluene in a denitrifying bacterium, strain EbN1. *Arch. Microbiol.* **181**: 182–194.

Ladygina, N., Dedyukhina, E. & Vainshtein, M. (2006) A review on microbial synthesis of hydrocarbons. *Process Biochem.* **41**: 1001–1014.

Lau, P.C., Wang, Y., Patel, A., Labbé, D., Bergeron, H., Brousseau, R. *et al.* (1997) A bacterial basic region leucine zipper histidine kinase regulating toluene degradation. *Proc. Natl. Acad. Sci. U S A* **94**: 1453–1458.

Layer, G., Heinz, D.W., Jahn, D. & Schubert, W.D. (2004) Structure and function of radical SAM enzymes. *Curr. Opin. Chem. Biol.* **8**: 468–476.

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

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

Leutwein, C. & Heider, J. (1999) Anaerobic toluene-catabolic pathway in denitrifying *Thauera aromatica*: activation and beta-oxidation of the first intermediate, (*R*)-(+)-benzylsuccinate. *Microbiol.* **145** 3265–3271.

Li, L., Patterson, D.P., Fox, C.C., Lin, B., Coschigano, P.W. & Marsh, E.N. (2009) Subunit structure of benzylsuccinate synthase. *Biochemistry* **48**: 1284–1292.

Liu, C., Wang, W., Wu, Y., Zhou, Z., Lai, Q. & Shao, Z. (2011) Multiple alkane hydroxylase systems in a marine alkane degrader, *Alcanivorax dieselolei* B-5. *Environ. Microbiol.* **13**: 1168–1178.

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

Marin, M.M., Yuste, L. & Rojo, F. (2003) Differential expression of the components of the two alkane hydroxylases from *Pseudomonas aeruginosa*. *J. Bacteriol.* **185**: 3232–3237.

Mbadinga, S.M., Wang, L.-Y., Zhou, L., Liu, J.-F., Gu, J.-D. & Mu, B.-Z. (2011) Microbial communities involved in anaerobic degradation of alkanes. *Int. Biodeter. Biodegr.* **65**: 1–13.

McInerney, M.J., Hoehler, T., Gunsalus, R.P. & Schink, B. (2010) Introduction to microbial hydrocarbon production: bioenergetics. *In* Handbook of lipid and hydrocarbon microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 321–336.

Mehboob, F., Junca, H., Schraa, G. & Stams, A.J. (2009) Growth of *Pseudomonas chloritidismutans* AW-1(T) on *n*-alkanes with chlorate as electron acceptor. *Appl. Microbiol. Biotechnol.* **83**: 739–747.

Morasch, B., Schink, B., Tebbe, C.C. & Meckenstock, R.U. (2004) Degradation of *o*-xylene and *m*-xylene by a novel sulfate-reducer belonging to the genus *Desulfotomaculum*. *Arch. Microbiol.* **181**: 407–417.

Moreno, R., Ruiz-Manzano, A., Yuste, L. & Rojo, F. (2007) The *Pseudomonas putida* Crc global regulator is an RNA binding protein that inhibits translation of the AlkS transcriptional regulator. *Mol. Microbiol.* **64**: 665–675.

Müller, J.A., Galushko, A.S., Kappler, A. & Schink, B. (1999) Anaerobic degradation of *m*-cresol by *Desulfobacterium cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate. *Arch. Microbiol.* **172**: 287–294.

Müller, J.A., Galushko, A.S., Kappler, A. & Schink, B. (2001) Initiation of anaerobic degradation of *p*-cresol by formation of 4-hydroxybenzylsuccinate in *Desulfobacterium cetonicum*. *J. Bacteriol.* **183**: 752–757.

Musat, F., Wilkes, H., Behrends, A., Woebken, D. & Widdel, F. (2010) Microbial nitrate-dependent cyclohexane degradation coupled with anaerobic ammonium oxidation. *ISME J.* **4**: 1290–1301.

Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R. *et al.* (2009) Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. *Environ. Microbiol.* **11**: 209–219.

Nauhaus, K., Boetius, A., Krüger, M. & Widdel, F. (2002) *In vitro* demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. *Environ. Microbiol.* **5**: 296–305.

Nicholson, T.L., Chiu, K. & Stephens, R.S. (2004) *Chlamydia trachomatis* lacks an adaptive response to changes in carbon source availability. *Infect. Immun.* **72**: 4286–4289.

Nojiri, H., Shintani, M. & Omori, T. (2004) Divergence of mobile genetic elements involved in the distribution of xenobiotic-catabolic capacity. *Appl. Microbiol. Biotechnol.* **64**: 154–174.

O´Brien, J.R., Raynaud, C., Croux, C., Girbal, L., Soucaille, P. & Lanzilotta, W.N. (2004) Insight into the mechanism of the B_{12}-independent glycerol dehydratase from *Clostridium butyricum*: preliminary biochemical and structural characterization. *Biochemistry* **44**: 10541–10551.

Owens, E.H. & Lee, K. (2003) Interaction of oil and mineral fines on shorelines: review and assessment. *Mar. Pollut. Bull.* **47**: 397–405.

Parales, R.E. & Ditty, J.L. (2010) Chemotaxis. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 1531–1544.

Peixoto, R.S., Vermelho, A.B. & Rosado, A.S. (2011) Petroleum-degrading enzymes: bioremediation and new prospects. *Enzyme Res.*: doi:10.4061/2011/475193.

Perfumo, A., Smyth, T.J.P., Marchant, R. & Banat, I.M. (2010) Production and roles of biosurfactants and bioemulsifiers in accessing hydrophobic substrates. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 1501–1512.

Prince, R.C. (1993) Petroleum spill bioremediation in marine environments. *Crit. Rev. Microbiol.* **19**: 217–242.

Prince, R.C. (2010a) Can we improve bioremediation? *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 3352–3355.

Prince, R.C. (2010b) Bioremediation of marine oil spills. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 2816–2830.

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

Rabus, R., Wilkes, H., Schramm, A., Harms, G., Behrends, A., Amann, R. & Widdel, F. (1999) Anaerobic utilization of alkylbenzenes and *n*-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliating with the beta-subclass of Proteobacteria. *Environ. Microbiol.* **1**: 145–157.

Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A.J. & Widdel, F. (2001) Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: Evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. *J. Bacteriol.* **183**: 1707–1715.

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

Rabus, R., Jarling, R., Lahme, S., Kühner, S., Heider, J., Widdel, F. & Wilkes, H. (2011) Co-metabolic conversion of toluene in anaerobic *n*-alkane-degrading bacteria. *Environ. Microbiol.* **13**: 2576–2586.

Ragsdale, S.W. (2007) Nickel and the carbon cycle. *J. Inorg. Biochem.* **101**: 1657–1666.

Raynaud, C., Sarcabal, P., Meynial-Salles, I., Croux, C. & Soucaille, P. (2003) Molecular characterization of the 1,3-propanediol (1,3-PD) operon of *Clostridium butyricum*. *Proc. Natl. Acad. Sci. U S A* **100**: 5010–5015.

Reinhold-Hurek, R. & Hurek, T. (2006) The genera *Azoarcus*, *Azovibrio*, *Azospira* and *Azonexus*. *In* The Prokaryotes; 3rd Edition. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. & Stackebrandt, E. (eds). Berlin, Heidelberg: Springer Verlag, pp. 873–891.

Rios-Hernandez, L.A., Gieg, L.M. & Suflita, J.M. (2003) Biodegradation of an alicyclic hydrocarbon by a sulfate-reducing enrichment from a gas condensate-contaminated aquifer. *Appl. Environ. Microbiol.* **69**: 434–443.

Rojo, F. (2009) Degradation of alkanes by bacteria. *Environ. Microbiol.* **11**: 2477–2490.

Rojo, F. (2010a) Enzymes for aerobic degradation of alkanes. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 781–798.

Rojo, F. (2010b) Genetic features and regulation of *n*-alkane metabolism. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 1141–1154.

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

Satpute, S.K., Banat, I.M., Dhakephalkar, P.K., Banpurkar, A.G. & Chopade, B.A. (2010) Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. *Biotechnol. Adv.* **28**: 436–450.

Savage, K.N., Krumholz, L.R., Gieg, L.M., Parisi, V.A., Suflita, J.M., Allen, J. *et al.* (2010) Biodegradation of low-molecular-weight alkanes under mesophilic, sulfate-reducing conditions: metabolic intermediates and community patterns. *FEMS Microbiol. Ecol.* **72**: 485–495.

Scheller, S., Goenrich, M., Boecher, R., Thauer, R.K. & Jaun, B. (2010) The key nickel enzyme of methanogenesis catalyses the anaerobic oxidation of methane. *Nature* **465**: 606–608.

Schindler, D. (2010) Tar sands need solid science. *Nature* **468**: 499–501.

Selmer, T. & Andrei, P.I. (2001) *p*-Hydroxyphenylacetate decarboxylase from *Clostridium difficile*. A novel glycyl radical enzyme catalysing the formation of *p*-cresol. *Europ. J. Biochem.* **268**: 1363–1372.

Shima, S., Krüger, M., Weinert, T., Demmer, U., Kahnt, J., Thauer, R.K. & Ermler, U. (2011) Structure of a methyl-coenzyme M reductase from Black Sea mats that oxidize methane anaerobically. *Nature* **481**: 98–101.

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

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

Sinclair, M.I. & Holloway, B.W. (1991) Chromosomal insertion of TOL transposons in *Pseudomonas aeruginosa* PAO. *J. Gen. Microbiol.* **137**: 1111–1120.

Sinclair, M.I., Maxwell, P.C., Lyon, B.R. & Holloway, B.W. (1986) Chromosomal location of TOL plasmid DNA in *Pseudomonas putida*. *J. Bacteriol.* **168**: 1302–1308.

Smits, T.H., Rothlisberger, M., Witholt, B. & van Beilen, J.B. (1999) Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environ. Microbiol.* **1**: 307–317.

So, C.M. & Young, L.Y. (1999) Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. *Appl. Environ. Microbiol.* **65**: 2969–2976.

So, C.M., Phelps, C.D. & Young, L.Y. (2003) Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, strain Hxd3. *Appl. Environ. Microbiol.* **69**: 3892–3900.

Sofia, H.J., Chen, G., Hetzler, B.G., Reyes-Spindola, J.F. & Miller, N.E. (2001) Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. *Nucleic Acids Res.* **29**: 1097–1106.

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

Sun, X., Harder, J., Krook, M., Jornvall, H., Sjoberg, B.M. & Reichard, P. (1993) A possible glycine radical in anaerobic ribonucleotide reductase from *Escherichia coli*: nucleotide sequence of the cloned *nrdD* gene. *Proc. Natl. Acad. Sci. U S A* **90**: 577–581.

Sun, X., Ollagnier, S., Schmidt, P.P., Atta, M., Mulliez, E., Lepape, L. *et al.* (1996) The free radical of the anaerobic ribonucleotide reductase from *Escherichia coli* is at glycine 681. *J. Biol. Chem.* **271**: 6827–6831.

Sutherland, T.D., Horne, I., Weir, K.M., Coppin, C.W., Williams, M.R., Selleck, M. *et al.* (2004) Enzymatic bioremediation: from enzyme discovery to applications. *Clin. Exp. Pharmacol. Physiol.* **31**: 817–821.

Tani, A., Ishige, T., Sakai, Y. & Kato, N. (2001) Gene structures and regulation of the alkane hydroxylase complex in *Acinetobacter* sp. strain M-1. *J. Bacteriol.* **183**: 1819–1823.

Taylor, B.L. & Zhulin, I.B. (1999) PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.* **63**: 479–506.

Thauer, R.K. (1998) Biochemistry of methanogenesis: a tribute to Marjory Stephenson. 1998 Marjory Stephenson Prize Lecture. *Microbiol.* **144** 2377–2406.

Thauer, R.K. (2011) Anaerobic oxidation of methane with sulfate: on the reversibility of the reactions that are catalyzed by enzymes also involved in methanogenesis from CO_2. *Curr. Opin. Biotechnol.* **14**: 292–299.

Tissot, B.P. & Welte, D.H. (1984) Petroleum formation and occurence. Berlin, Germany: Springer Verlag.

Tsuda, M. & Iino, T. (1987) Genetic analysis of a transposon carrying toluene degrading genes on a TOL plasmid pWW0. *Mol. Gen. Genet.* **210**: 270–276.

Tsuda, M., Tan, H.M., Nishi, A. & Furukawa, K. (1999) Mobile catabolic genes in bacteria. *J. Biosci. Bioeng.* **87**: 401–410.

Unkrig, V., Neugebauer, F.A. & Knappe, J. (1989) The free radical of pyruvate formate-lyase. Characterization by EPR spectroscopy and involvement in catalysis as studied with the substrate-analogue hypophosphite. *Europ. J. Biochem.* **184**: 723–728.

van Beilen, J.B. & Funhoff, E.G. (2005) Expanding the alkane oxygenase toolbox: new enzymes and applications. *Curr. Opin. Biotechnol.* **16**: 308–314.

van Beilen, J.B. & Funhoff, E.G. (2007) Alkane hydroxylases involved in microbial alkane degradation. *Appl. Microbiol. Biotechnol.* **74**: 13–21.

van Beilen, J.B., Wubbolts, M.G. & Witholt, B. (1994) Genetics of alkane oxidation by *Pseudomonas oleovorans*. *Biodegradation* **5**: 161–174.

van Beilen, J.B., Li, Z., Duetz, W.A., Smits, T.H. & Witholt, B. (2003) Diversity of alkane hydroxylase systems in the environment. *Oil Gas Sci. Technol.* **58**: 427–440.

van Beilen, J.B., Panke, S., Lucchini, S., Franchini, A.G., Rothlisberger, M. & Witholt, B. (2001) Analysis of *Pseudomonas putida* alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the *alk* genes. *Microbiol.* **147**: 1621–1630.

van Beilen, J.B., Marin, M.M., Smits, T.H., Rothlisberger, M., Franchini, A.G., Witholt, B. & Rojo, F. (2004) Characterization of two alkane hydroxylase genes from the marine hydrocarbonoclastic bacterium *Alcanivorax borkumensis*. *Environ. Microbiol.* **6**: 264–273.

van der Meer, J.R. & Sentchilo, V. (2003) Genomic islands and the evolution of catabolic pathways in bacteria. *Curr. Opin. Biotechnol.* **14**: 248–254.

van Hamme, J.D., Singh, A. & Ward, O.P. (2003) Recent advances in petroleum microbiology. *Microbiol. Mol. Biol. Rev.* **67**: 503–549.

Verfürth, K., Pierik, A.J., Leutwein, C., Zorn, S. & Heider, J. (2004) Substrate specificities and electron paramagnetic resonance properties of benzylsuccinate synthases in anaerobic toluene and *m*-xylene metabolism. *Arch. Microbiol.* **181**: 155–162.

Vollhardt, P.C. (1990) Organische Chemie. Weinheim: VCH Verlagsgesellschaft.

Wackett, L.P. (2010) Aliphatic hydrocarbon producers. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 609–614.

Wagner, A.F., Frey, M., Neugebauer, F.A., Schäfer, W. & Knappe, J. (1992) The free radical in pyruvate formate-lyase is located on glycine-734. *Proc. Natl. Acad. Sci. U S A* **89**: 996–1000.

Wang, S.C. & Frey, P.A. (2007) S-adenosylmethionine as an oxidant: the radical SAM superfamily. *Trends Biochem. Sci.* **32**: 101–110.

Wang, W. & Shao, Z. (2011) Genes involved in alkane degradation in the *Alcanivorax hongdengensis* strain A-11-3. *Appl. Microbiol. Biotechnol.*: doi: 10.1007/s00253-00011-03818-x.

Wang, Y., Rawlings, M., Gibson, D.T., Labbe, D., Bergeron, H., Brousseau, R. & Lau, P.C. (1995) Identification of a membrane protein and a truncated LysR-type regulator associated with the toluene degradation pathway in *Pseudomonas putida* F1. *Mol. Gen. Genet.* **246**: 570–579.

Washer, C.E. & Edwards, E.A. (2007) Identification and expression of benzylsuccinate synthase genes in a toluene-degrading methanogenic consortium. *Appl. Environ. Microbiol.* **73**: 1367–1369.

Weast, R.C. (1990) CRC Handbook of Chemistry and Physics, 70th ed., 1989-1990. Boca Raton, Florida: CRC Press.

Weelink, S.A., van Doesburg, W., Saia, F.T., Rijpstra, W.I., Roling, W.F., Smidt, H. & Stams, A.J. (2009) A strictly anaerobic betaproteobacterium *Georgfuchsia toluolica* gen. nov., sp. nov. degrades aromatic compounds with Fe(III), Mn(IV) or nitrate as an electron acceptor. *FEMS Microbiol. Lett.* **70**: 575–585.

Weiner, J.M. & Lovley, D.R. (1998) Anaerobic benzene degradation in petroleum-contaminated aquifer sediments after inoculation with a benzene-oxidizing enrichment. *Appl. Environ. Microbiol.* **64**: 775–778.

Werner, I. (2009) Untersuchungen zum Stoffwechsel des anaeroben Alkanabbaus. Dissertation, Universität Bremen.

Whyte, L.G., Smits, T.H., Labbe, D., Witholt, B., Greer, C.W. & van Beilen, J.B. (2002) Gene cloning and characterization of multiple alkane hydroxylase systems in *Rhodococcus* strains Q15 and NRRL B-16531. *Appl. Environ. Microbiol.* **68**: 5933–5942.

Widdel, F., Boetius, A. & Rabus, R. (2006) Anaerobic biodegradation of hydrocarbons including methane. *In* The prokaryotes; 3rd edition. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. & Stackebrandt, E. (eds). Berlin, Heidelberg: Springer Verlag, pp. 1028–1049.

Widdel, F., Knittel, K. & Galushko, A. (2010) Anaerobic hydrocarbon-degrading microorganisms: an overview. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 1998–2021.

Wilkes, H. & Schwarzbauer, J. (2010) Hydrocarbons: an introduction to structure, physico-chemical properties and natural occurence. *In* Handbook of hydrocarbon and lipid microbiology. Timmis, K.N. (ed). Berlin, Heidelberg: Springer Verlag, pp. 3–48.

Wilkes, H., Rabus, R., Fischer, T., Armstroff, A., Behrends, A. & Widdel, F. (2002) Anaerobic degradation of *n*-hexane in a denitrifying bacterium: further degradation of the initial intermediate (1-methylpentyl)succinate via C-skeleton rearrangement. *Arch. Microbiol.* **177**: 235–243.

Wilkes, H., Kühner, S., Bolm, C., Fischer, T., Classen, A., Widdel, F. & Rabus, R. (2003) Formation of *n*-alkane and cycloalkane-derived organic acids during anaerobic growth of a denitrifying bacterium with crude oil. *Org. Geochem.* **34**: 1313–1323.

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

Wöhlbrand, L. (2008) Proteomische und genetische Untersuchungen zum Aromaten-Abbau in "Aromatoleum aromaticum" Stam EbN1. Dissertation, Universität Bremen.

Yu, L., Blaser, M., Andrei, P.I., Pierik, A.J. & Selmer, T. (2006) 4-Hydroxyphenylacetate decarboxylases: properties of a novel subclass of glycyl radical enzyme systems. *Biochemistry* **45**: 9584–9592.

Yuste, L., Canosa, I. & Rojo, F. (1998) Carbon-source-dependent expression of the PalkB promoter from the *Pseudomonas oleovorans* alkane degradation pathway. *J. Bacteriol.* **180**: 5218–5226.

Zedelius, J., Rabus, R., Grundmann, O., Werner, I., Brodkorb, D., Schreiber, F. *et al.* (2011) Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. *Environ. Microbiol. Rep.* **3**: 125–135.

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

Zengler, K., Richnow, H.H., Rossello-Mora, R., Michaelis, W. & Widdel, F. (1999b) Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **401**: 266–269.

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