Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria

Florin Musat,¹ Alexander Galushko,¹ Jacob Jacob,¹ Friedrich Widdel,^{1*} Michael Kube,² Richard Reinhardt,² Heinz Wilkes,³ Bernhard Schink⁴ and Ralf Rabus^{1,5}

¹Max Planck Institute for Marine Microbiology, Celsiusstraße 1, 28359 Bremen, Germany.

²Max Planck Institute for Molecular Genetics, Ihnestr. 73, 14195 Berlin, Germany.

³Helmholtz Centre Potsdam GFZ German Research Centre for Geosciences, Telegrafenberg, 14473 Potsdam, Germany.

⁴Microbial Ecology, Department of Biology, University of Konstanz, PO Box 5560, 78457 Konstanz, Germany.

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

Summary

The anaerobic biodegradation of naphthalene, an aromatic hydrocarbon in tar and petroleum, has been repeatedly observed in environments but scarcely in pure cultures. To further explore the relationships and physiology of anaerobic naphthalene-degrading microorganisms, sulfate-reducing bacteria (SRB) were enriched from a Mediterranean sediment with added naphthalene. Two strains (NaphS3, NaphS6) with oval cells were isolated which showed naphthalene-dependent sulfate reduction. According to 16S rRNA gene sequences, both strains were *Deltaproteobacteria* and closely related to each other and to a previously described naphthalene-degrading sulfate-reducing strain (NaphS2) from a North Sea habitat. Other close relatives were SRB able to degrade alkylbenzenes, and phylotypes enriched anaerobically with benzene. If in adaptation experiments the three naphthalene-grown strains were exposed to 2-methylnaphthalene, this compound was utilized after a pronounced lag phase, indicating

that naphthalene did not induce the capacity for 2-methylnaphthalene degradation. Comparative denaturing gel electrophoresis of cells grown with naphthalene or 2-methylnaphthalene revealed a striking protein band which was only present upon growth with the latter substrate. Peptide sequences from this band perfectly matched those of a protein predicted from genomic libraries of the strains. Sequence similarity (50% identity) of the predicted protein to the large subunit of the toluene-activating enzyme (benzylsuccinate synthase) from other anaerobic bacteria indicated that the detected protein is part of an analogous 2-methylnaphthalene-activating enzyme. The absence of this protein in naphthalene-grown cells together with the adaptation experiments as well as isotopic metabolite differentiation upon growth with a mixture of *d*₈-naphthalene and unlabelled 2-methylnaphthalene suggest that the marine strains do not metabolize naphthalene by initial methylation via 2-methylnaphthalene, a previously suggested mechanism. The inability to utilize 1-naphthol or 2-naphthol also excludes these compounds as free intermediates. Results leave open the possibility of naphthalene carboxylation, another previously suggested activation mechanism.

Introduction

Naphthalene [C₁₀H₈; melting point, 80.2°C; solubility in H₂O at 25°C, 0.24 mM; $\Delta G_f^\circ = 201 \text{ kJ mol}^{-1}$ (Dean, 1992; Eastcott *et al.*, 1988)], a hydrocarbon with two fused aromatic rings, is an abundant component of coal tar (first description by Brande, 1820; Garden, 1820; Kidd, 1821). Also petroleum commonly contains naphthalene (Tissot and Welte, 1984). Furthermore, naphthalene may originate from some biological sources such as endophytic fungi (Daisy *et al.*, 2002; Ezra *et al.*, 2004), *Magnolia* flowers (Azuma *et al.*, 1996), deer hair (Gassett *et al.*, 1997) and termite nests (Chen *et al.*, 1998a,b) or the plants and natural soil near termite nests (Krauss *et al.*, 2005). Naphthalene is a raw chemical for industrial syntheses, in particular of phthalic anhydride (Franck and Stadelhofer, 1987; Preuss *et al.*, 2003). Human exposure to naphthalene was formerly not uncommon due to its

Received 15 February 2008; accepted 31 July, 2008. *For correspondence. E-mail fwiddel@mpi-bremen.de; Tel. (+49) 421 20287 02; Fax (+49) 421 2028 790.

(now obsolete) use as a moth repellent. Naphthalene at high concentration may cause haemolytic anaemia and some other conditions, whereas the tumorigenic potential is presently considered low (Umweltbundesamt, 2007; Bogen *et al.*, 2008).

Being a potential environmental contaminant from tar or petroleum and representing the 'archetype' of polycyclic aromatic hydrocarbons, naphthalene has early become of interest as a study substrate in biodegradation research (overview by Gibson and Subramanian, 1984). Accordingly, aerobic naphthalene degradation has been known since several decades and has been studied in much detail on the molecular level (Habe and Omori, 2003; Karlsson *et al.*, 2003).

In contrast, the capacity for anaerobic biodegradation of naphthalene has become evident relatively recently, in most cases from observations in anoxic habitats and microcosms under conditions of nitrate reduction, sulfate reduction or methanogenesis (Thierrin *et al.*, 1993; Coates *et al.*, 1996; 1997; Langenhoff *et al.*, 1996; Bedessem *et al.*, 1997; Sharak Genthner *et al.*, 1997; Zhang and Young, 1997; Hayes *et al.*, 1999; Hayes and Lovley, 2002; Rothermich *et al.*, 2002). Reports on the isolation and physiology of anaerobic naphthalene degraders are scarce. *Pseudomonas*- and *Vibrio*-like strains isolated under oxic conditions from tar-contaminated marine sediment were reported to mineralize naphthalene partially under conditions of denitrification (Rockne and Strand, 1998, 2001; Rockne *et al.*, 2000). A marine sulfate-reducing bacterium (SRB), strain NaphS2, was isolated that grew with naphthalene under strictly anoxic conditions (Galushko *et al.*, 1999). In addition, a highly enriched sulfate-reducing freshwater culture has been studied which utilized naphthalene anaerobically (Meckenstock *et al.*, 2000; Annweiler *et al.*, 2002; Safinowski and Meckenstock, 2006).

The initial biochemical steps in anaerobic naphthalene degradation are insufficiently understood. Naphthalene is rather unreactive, due to the stabilizing aromatic 10 π -electron system and lack of alkyl or polar groups. Still, it reacts more readily than benzene, for instance, with electrophilic and hydrogenating agents. According to present knowledge, naphthalene hydroxylation by use of a reactive oxygen species as in aerobic bacteria (Karlsson *et al.*, 2003) is excluded or not expected in an anaerobic metabolism with sulfate or nitrate as electron acceptors. After the suggestion of anaerobic hydroxylation through dehydrogenation to a naphthol (Bedessem *et al.*, 1997) or carboxylation to 2-naphthoate (Zhang and Young, 1997; Annweiler *et al.*, 2002), methylation of naphthalene has become an attractive hypothesis (Safinowski and Meckenstock, 2006; Safinowski *et al.*, 2006). The formed 2-methylnaphthalene would subsequently react with fumarate to yield (2-naphthylmethyl)succinate (Annweiler

et al., 2000), analogous to the anaerobic reaction of toluene yielding benzylsuccinate (Heider, 2007).

Considering the scarcity of pure cultures that degrade naphthalene anaerobically, the present study was undertaken to enrich and isolate further strains with such capacity so as to gain insights into their phylogenetic relationships and degradative capacities; this task also included an examination of the discussed possibilities for naphthalene activation. Since capacities for anaerobic biodegradation of various hydrocarbons are apparently widespread among marine SRB (Widdel *et al.*, 2007), the present study employed marine sediment as starting material and sulfate as electron acceptor.

Results and discussion

Enrichment, isolation and characterization

Anaerobically incubated medium containing sediment from a Mediterranean lagoon, sulfate and naphthalene (in an inert liquid carrier phase as a non-toxic reservoir) exhibited significant sulfide formation in comparison with a naphthalene-free control (up to 18 versus 9 mM sulfide) within 8 months. Subcultures (10% inoculum, v/v) reached 18 mM sulfide within 6–7 weeks. Microscopy of sediment-free subcultures revealed oval to rod-shaped cells, several of which contained light-refracting vesicles. Anoxic agar dilution series essentially yielded two distinct types of colonies. The beige colonies were either slightly translucent and consisted of vesicle-free cells, or they were opaque ('chalky') and consisted of cells with vesicles. One strain of each cell type was isolated (Fig. 1). The isolates are referred to as strains NaphS3 (without vesicles) and NaphS6 (with vesicles).

In purity tests with strain NaphS3, cell types other than in the inoculum were not detectable. However, the purity tests with strain NaphS6 employing pyruvate, lactate or fumarate revealed the presence of an accompanying spirilloid SRB with much smaller and curved cells. In cultures grown with naphthalene or other aromatic substrates (see below), these cells occurred at low numbers (approximately 3% of total); considering their size, their estimated biovolume contribution (< 0.3%) during growth of strain NaphS6 with aromatic growth substrates is therefore negligible.

Sequence comparison of 16S rRNA genes of strains NaphS3, NaphS6 and the formerly isolated naphthalene degrader, strain NaphS2 from North Sea sediment (Galushko *et al.*, 1999), revealed an affiliation with the *Desulfobacteraceae* (*Deltaproteobacteria*) and significant sequence similarity among each other (NaphS3–NaphS6, 97.5%; NaphS3–NaphS2, 99.8%; NaphS6–NaphS2, 97.4%; Fig. 2). Other close relatives were marine SRB previously isolated with *m*-xylene (Harms *et al.*, 1999)

Fig. 1. Phase-contrast images of viable cells of sulfate-reducing bacteria isolated and grown anaerobically with naphthalene. Bars, 10 µm.

A. Strain NaphS3.

B. Strain NaphS6, with characteristic gas vesicles. This strain tended to aggregate loosely around small flocs of FeS (from trace element solution).

and ethylbenzene (Kniemeyer *et al.*, 2003), as well as sequence clones retrieved from enrichment cultures with benzene (Phelps *et al.*, 1998; Musat and Widdel, 2008).

If cells of strain NaphS6 were exposed to pressure by centrifugation or inside a 1 ml syringe with application of a jerky push, the light-refracting vesicles disappeared (collapsed), which is characteristic of gas vesicles (Walsby, 1994). Gas vesicles have been observed in a few SRB (Widdel, 1988) including the closely related ethylbenzene-degrading strain EbS7 (Kniemeyer *et al.*, 2003). The role of gas vesicles in SRB is unknown.

In addition to naphthalene, strains NaphS2, NaphS3 and NaphS6 also utilized 2-methylnaphthalene (1% in carrier phase) and 2-naphthoate (1 mM), but not 1-methylnaphthalene or 1-naphthoate at the same concentrations. Benzoate (2 mM) was utilized by strains

NaphS2 and NaphS3, but not so far by strain NaphS6. In substrate tests with 1-naphthol and 2-naphthol, possible toxic effects of these compounds (as two-ring analogues of phenol) had to be excluded. If they were added at concentrations of 0.2 mM to naphthalene cultures, sulfide production was completely inhibited. Only concentrations of ≤ 0.1 mM were non-toxic. However, even at low, non-toxic concentrations, consumption of naphthols was insignificant (experiment with 1-naphthol depicted in Fig. S1).

Naphthalene consumption and sulfide formation were quantified in growth experiments. Per mol naphthalene consumed in the cultures of strains NaphS3 and NaphS6, the measured sulfide formation was 6.6 and 4.9 mol respectively. This indicates the capacity for complete oxidation, according to $C_{10}H_8 + 6SO_4^{2-} + 2H^+ + 6H_2O \rightarrow 10HCO_3^- + 6H_2S$. The apparent surplus of sulfide in one experiment is explained by a transfer of carrier phase droplets from the naphthalene-containing carrier phase in the pre-culture. The capacity for complete naphthalene oxidation was previously demonstrated with strain NaphS2 (Galushko *et al.*, 1999).

Test of 2-methylnaphthalene as a possible intermediate of naphthalene degradation

The observed failure of strains NaphS2, NaphS3 and NaphS6 (pre-grown with naphthalene) in the substrate tests to utilize naphthols (see above and Fig. S1) excludes them as free intermediates during naphthalene degradation, assuming that their hydrophobicity allows diffusive diffusion into the cells (see analogous discussion for phenol; Musat and Widdel, 2008).

In contrast, the utilization of 2-methylnaphthalene in the growth tests leaves open the possibility that this compound is an intermediate during naphthalene degradation. Via the suggested methylation (Safinowski and Meckenstock, 2006), naphthalene would be channelled into the pathway of 2-methylnaphthalene by a single and 'elegant' step. Naphthalene is indeed susceptible to electrophilic substitution so that *S*-adenosylmethionine or methyl-coenzyme B₁₂ could, in principle, serve as co-substrates (CH₃⁺ donors). 2-Methylnaphthalene as a hydrophobic compound is expected to diffuse freely into the cells, like naphthalene. If naphthalene degradation in the marine SRB occurs via 2-methylnaphthalene, naphthalene-grown cells should utilize 2-methylnaphthalene without an induction phase. Whereas such a response is a requisite albeit not a sufficient proof of the role of 2-methylnaphthalene as an intermediate (there might be an independent constitutive utilization), the lack of a response would rule out this compound as an intermediate as long as the added concentration is not toxic. The non-toxic concentration ($\leq 1\%$ in the carrier

Fig. 2. Phylogenetic 16S rRNA-based affiliation of the presently isolated sulfate-reducing strains NaphS3 and NaphS6 (from Mediterranean lagoon) and the previously isolated strain NaphS2 (from a North Sea harbour; Galushko *et al.*, 1999). Sulfate-reducing isolates able to degrade aromatic hydrocarbons are indicated in bold, their substrates being as follows: EbS7, ethylbenzene (Kniemeyer *et al.*, 2003); mXyS1, toluene and *m*-xylene (Harms *et al.*, 1999); oXyS1, toluene and *o*-xylene (Harms *et al.*, 1999); *Desulfosarcina cetonica*, *Desulfobacula toluolica* and PRTOL1, toluene. Clones SB-21, SB-30 (Phelps *et al.*, 1998) and BznS295 (Musat and Widdel, 2008) were retrieved from sulfate-reducing enrichments with benzene. The scale bar represents 5% estimated sequence divergence.

phase) was verified during the substrate tests (see above). The tests always included parallel controls with naphthalene alone to verify that all used cell batches were in an active state. If naphthalene-grown cells were exposed to 2-methylnaphthalene, sulfide formation began only after a pronounced lag phase (Fig. 3). According to this result, 2-methylnaphthalene is unlikely to be an intermediate during naphthalene degradation by the three marine strains. If in an additional control the three strains were grown with 2-methylnaphthalene, they immediately began to consume this compound again upon its addition.

The present results are analogous to those obtained with a benzene-grown sulfate-reducing marine enrichment culture which was unlikely to involve free phenol or toluene as an intermediate (Musat and Widdel, 2008).

Electrophoretic display of major proteins formed with different substrates

If extracts from cells of the three strains grown with naphthalene or 2-methylnaphthalene were subjected to denaturing gel electrophoresis, the most obvious difference between the protein patterns were 2-methylnaphthalene-specific co-migrating bands of high molecular mass (Fig. 4). Their peptide sequences perfectly matched those of gene products predicted from genomic shotgun librar-

ies of the three strains (Fig. S2). These gene products showed sequence similarity to the catalytic (large) subunit (BssA) of benzylsuccinate synthase, the enzyme for the anaerobic activation of toluene (Fig. 5). Based on these similarities, we assume that the 2-methylnaphthalene-specific band in strains NaphS2, NaphS3 and NaphS6 represents the catalytic subunit of the 2-methylnaphthalene-activating enzyme (2-naphthylmethyl)succinate synthase, which may be termed Nms. This enzyme has not been purified and characterized thus far, but high similarity to benzylsuccinate synthase is expected, due to the activation of a methyl group next to an aromatic ring, and addition to fumarate. The predicted NmsA protein also reveals the possible radical-bearing glycine (Fig. S2; Heider, 2007). The apparent absence of the striking protein band in naphthalene-grown cells again indicates that naphthalene degradation in the marine strains is unlikely to occur via 2-methylnaphthalene.

Other proteins related to the apparent (2-naphthylmethyl)succinate synthase subunit were predicted enzymes for the anaerobic activation of alkanes (Callaghan *et al.*, 2008; Grundmann *et al.*, 2008; Fig. 5). Hence, the presently known or assumed fumarate-dependent hydrocarbon-activating enzymes constitute three phylogenetic sub-branches, in accordance with their different substrates (toluene, 2-methylnaphthalene, *n*-alkanes).

Fig. 3. Incubation of naphthalene-grown cells of strains NaphS2 (A), NaphS3 (B) and NaphS6 (C and D) with 2-methylnaphthalene and naphthalene. Comparison of sulfide formation with 2-methylnaphthalene (▲), with naphthalene (●, positive control) and without any addition (□, negative control) shows that there was no initial activity towards 2-methylnaphthalene. The capacity was induced after more than 20 days, as depicted for strain NaphS6 (D).

Chemical analysis of metabolites

In accordance with other findings (Zhang and Young, 1997; Annweiler *et al.*, 2000; 2002; Galushko *et al.*, 2003; Safinowski and Meckenstock, 2006), the polar compound 2-naphthoate was detected (as methyl ester upon derivatization) in both, naphthalene- and 2-methylnaphthalene-grown cultures. 2-Naphthoate or naphthoyl-CoA is an expected central intermediate in the degradation of naphthalene and 2-methylnaphthalene (Fig. S4), as benzoyl-CoA is a central intermediate in the anaerobic degradation of many monoaromatic compounds (Boll *et al.*, 2002). Agreeing with an earlier report (Zhang and Young, 1997), cells grown with naphthalene in the presence of ^{13}C -bicarbonate incorporated the label into the carboxyl function of 2-naphthoate, according to mass spectrometric fragmentation. Even though this result cannot exclude naphthalene methylation (a methyl group may be derived from CO_2), such a reaction is unlikely in view of the other results obtained with the marine strains.

The most obvious metabolite in 2-methylnaphthalene-grown cultures was (2-naphthylmethyl)succinate, the amount being 20-fold higher than that of 2-naphthoate. Surprisingly (2-naphthylmethyl)succinate was also detected in some (but not all) naphthalene-grown cultures of the three strains; however, the amount of (2-naphthylmethyl)succinate was c. 100-fold lower than that of 2-naphthoate. 2-Methylnaphthalene was detected neither (as a possible impurity) in the added naphthalene nor (as a possible metabolite) in the naphthalene-grown cultures. Because the other experiments with the marine strains did not support naphthalene methylation and subsequent conversion to (2-naphthylmethyl)succinate, one present explanation for the detection of traces of this compound in some naphthalene cultures is its formation from the postulated intermediate, 2-naphthoyl-CoA via side-reactions. With exception of the special, possibly highly synthesis-controlled (2-naphthylmethyl)succinate synthase, the more 'usual' enzymes for the assumed subsequent β -oxidation-like conversion of (2-naphthylmethyl)succinate to 2-naphthoyl-CoA and succinyl-CoA (Annweiler *et al.*, 2000) may be present at a certain level (or constitutive) during growth with naphthalene. Since this conversion is reversible from a biochemical point of view, a reverse (reductive) reaction sequence

Fig. 4. Denaturing gel electrophoresis (SDS-PAGE) of cell extracts from strains NaphS2, NaphS3 and NaphS6 grown with benzoate (Bzt), naphthalene (Nph) or 2-methylnaphthalene (2MNph). Mass spectrometry of peptides from the 2-methylnaphthalene-specific band (arrowhead) allowed identification of a genome-deduced protein from each strain (see Fig. S2) with sequence similarity (see Fig. 5) to the catalytic subunit of benzylsuccinate synthase (BssA), which activates toluene anaerobically.

starting with naphthoyl-CoA (formed from naphthalene via still unexplored reactions) and succinyl-CoA (a ubiquitous metabolite) could lead to minor amounts of (2-naphthylmethyl)succinate during growth with naphthalene (Fig. S4).

To further substantiate that naphthalene in the marine strains is not metabolized via 2-methylnaphthalene, a naphthalene-grown culture of strain NaphS2 was cultivated with a mixture of deuterated naphthalene (d_8 -naphthalene) and unlabelled 2-methylnaphthalene. Mass spectrometric analysis of metabolite labelling revealed that the major proportion (86%) of the common intermediate, 2-naphthoate, was deuterated and hence derived from naphthalene. Naphthalene in this experiment was obviously metabolized to a greater extent than 2-methylnaphthalene. Nevertheless, almost all (> 99.5%) of the detected (2-naphthylmethyl)succinate was unlabelled and thus derived from 2-methylnaphthalene. This

result cannot be explained by naphthalene metabolism via 2-methylnaphthalene (Fig. S5).

As observed elsewhere (Sullivan *et al.*, 2001), 2-methylnaphthalene-grown cultures also formed isomers of methylnaphthoate, $H_3C-(C_{10}H_6)-COO^-$ (Fig. S6). These metabolites were not detected in naphthalene-grown cultures. 2-Methylnaphthalene apparently not only underwent fumarate addition yielding (2-naphthylmethyl)succinate (presumably the main reaction) but also an alternative addition of a carboxyl group as occurring with naphthalene. If cultures were grown with naphthalene in the presence of 1-methylnaphthalene, which is not a growth substrate, other isomers of methylnaphthoates were detected. These were probably dead-end metabolites from a co-activation of 1-methylnaphthalene by the naphthalene-activating enzyme, similar as reported previously (Safinowski *et al.*, 2006). There was no evidence that the carboxyl function of the methylnaphthoates origi-

Fig. 5. Sequence similarity of the proteins specifically formed with 2-methylnaphthalene (Fig. 4), the assumed large subunit of (2-naphthylmethyl)succinate synthase, NmsA, with other glycol radical proteins. For sequence alignment of the 2-methylnaphthalene-specific proteins see Fig. S3.

nated from a preceding methylation and subsequent metabolism via fumarate addition, because [(methyl-2-naphthyl)methyl]succinates were not detectable.

Concluding remarks

In view of the substrate adaptation experiments, the 2-methylnaphthalene-specific proteins and the isotopic differentiation of metabolites, an activation of naphthalene through methylation is unlikely in the presently studied marine SRB. However, we cannot exclude that this reaction is used, as suggested, for naphthalene activation in a different, terrestrial sulfate-reducing culture (Safinowski and Meckenstock, 2006). The present experiments leave open the possibility for naphthalene activation by carboxylation (Zhang and Young, 1997; Annweiler *et al.*, 2002), a mechanism also suggested for the other unsubstituted aromatic hydrocarbons, benzene (Caldwell and Suffita, 2000) and phenanthrene (Zhang and Young, 1997; Davidova *et al.*, 2007). An activation by hydrogen atom abstraction and addition to fumarate is presently regarded as unlikely. The resulting product, naphthylsuccinate, has never been observed. Also, such hydrogen abstraction would have to overcome a very high C–H bond strength (469 kJ mol^{-1} ; Reed and Kass, 2000) in comparison with that of the methyl group of toluene or at the secondary carbon atom of alkanes (368 and 398 kJ mol^{-1} respectively; McMillen and Golden, 1982).

Experimental procedures

Source of bacteria and cultivation

Black (anoxic) sediment for enrichment cultures was collected from a Mediterranean lagoon, Étang de Berre (France), and stored under nitrogen at 12°C . Strain NaphS2 has been maintained since its isolation (Galushko *et al.*, 1999) in the laboratory.

Techniques used for preparation of media and anaerobic cultivation were as described elsewhere (Widdel and Bak, 1992; Galushko *et al.*, 1999; Widdel *et al.*, 2004). Enrichments and subcultures were grown in flat 200 ml bottles; they were provided with 110 ml of $\text{HCO}_3^-/\text{CO}_2$ -buffered sulfide-reduced artificial seawater medium, 10 ml of inoculum, 5–7 ml of 2,2,4,4,6,6,8,8-heptamethylnonane as an inert, insoluble carrier phase containing naphthalene (20 mg ml^{-1}), a head space of an $\text{N}_2\text{--CO}_2$ mixture (9:1, v/v), and black stoppers (Wheaton). Culture bottles were incubated nearly horizontally with the stoppers below the medium surface so as to avoid their contact with the hydrocarbon phase (Widdel *et al.*, 2004). Cultures containing sediment were only briefly (5 s) shaken every 2 days while consecutive sediment-free cultures were incubated with slow horizontal shaking (50 r.p.m.) or magnetic stirring (100 r.p.m.). All cultures were incubated at 28°C .

Bacterial strains were isolated under anoxic conditions via serial agar dilutions (Widdel and Bak, 1992). The agar in the

tubes was overlaid with naphthalene in heptamethylnonane (Galushko *et al.*, 1999). For purity tests, inocula were transferred to media containing yeast extract (0.5 g l^{-1}) and pyruvate, lactate, fumarate or glucose (always 1 mM). Cells from purity and substrate tests were regularly examined microscopically.

In substrate tests, methylnaphthalenes were provided like naphthalene in heptamethylnonane, the concentration being 10 mg ml^{-1} . Naphthoates and benzoate were added from aqueous 0.2 M stock solutions prepared from the acids and NaOH. Naphthols were added from 2 mM stock solutions in seawater medium; the poorly soluble, solid compounds were dissolved by shaking for 1 day.

Adaptation experiments

To prepare metabolically active concentrated cell suspensions, a total volume of 1 l combined from naphthalene-grown cultures was separated inside an anoxic chamber from the liquid carrier phase by means of a separatory funnel and centrifuged under an $\text{N}_2\text{--CO}_2$ mixture (9:1, v/v). Cells were re-suspended in 300 ml of anoxic medium. Aliquots of 100 ml for individual experiments were distributed into anoxic 130 ml bottles and provided with 3.7 ml of heptamethylnonane containing naphthalene (20 mg ml^{-1}) or 2-methylnaphthalene (10 mg ml^{-1}). Consumption of naphthols or formation of sulfide (in case of naphthalene or 2-methylnaphthalene) was followed by chemical analysis of samples that were withdrawn with N_2 -flushed syringes.

Chemical analyses

Sulfide was quantified photometrically as fresh colloidal CuS (Cord-Ruwisch, 1985).

Naphthalene in the carrier phase was measured with an AutoSystem gas chromatograph (Perkin Elmer) equipped with a fused silica capillary column (OPTIMA 5, Macherey-Nagel; length, 50 m; inner diameter, 0.32 mm ; film thickness, $0.25 \mu\text{m}$) and a flame ionization detector. H_2 was used as carrier gas. The oven temperature was programmed from 40°C (5 min isothermal) to 200°C at a rate of 5°C min^{-1} and further to 320°C (5 min isothermal) at a rate of $20^\circ\text{C min}^{-1}$.

Naphthols were measured by using a high-performance liquid chromatograph (Sykam) with an IBJ A3 column ($4 \times 60 \text{ mm}$). Elution was by 20 mM NaCl in 45% (v/v) aqueous ethanol at a flow rate of 1 ml min^{-1} at 50°C . Naphthols were detected at 220 nm with an S 3200 UV detector (Sykam).

To analyse metabolites of naphthalene and 2-methylnaphthalene degradation, cultures grown with these substrates were acidified and extracted essentially as described (Rabus *et al.*, 2001) using dichloromethane as solvent. Methylated extracts were analysed by gas chromatography-mass spectrometry either with a type 6890 gas chromatograph (Hewlett Packard) connected to a type 95XL mass spectrometer, or with a Trace GCMS (Thermoelectron, Dreieich, Germany) as described (Wilkes *et al.*, 2000; Rabus *et al.*, 2001; Wöhlbrand *et al.*, 2008). Separation of methyl-2-naphthoic acid methyl esters was achieved on a fused silica capillary column (SGE BP21;

length, 25 m; inner diameter, 0.22 mm; film thickness, 0.25 µm). The oven temperature was programmed from 50°C (1 min isothermal) to 240°C (30 min isothermal) at a rate of 3°C min⁻¹.

16S rRNA-based analyses

Extracted DNA (Zhou *et al.*, 1996) was used for almost full-length 16S rRNA gene amplification by polymerase chain reaction (PCR) using the *Bacteria*-specific primers 8f (Hicks *et al.*, 1992) and 1492r (Kane *et al.*, 1993). Products were purified with the QIAquick Purification Kit (Qiagen) and cloned into the pCR4 vector (Invitrogen) that were used to transform *Escherichia coli* Top10 (Invitrogen). Positive clones were sequenced using the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyser (Applied Biosystems). The procedure for strain NaphS3 included 25 PCR cycles. In case of strain NaphS6, amplification of the sequence from the contaminant which occurred at low number was avoided by using only 20 PCR cycles. Subsequent direct sequencing, which did not show a contaminating sequence, and sequencing after cloning always yielded identical sequences, indicating their origin from the abundant cell type.

Sequences were assembled using the DNA Baser software (<http://www.dnabaser.com>). The sequences were aligned with those from the SILVA database (<http://www.arb-silva.de>). Phylogenetic trees were constructed by tools of the ARB software package (Ludwig *et al.*, 2004) using maximum likelihood analysis and applying different sets of filters. Only almost full-length sequences (> 1400 bp) were used for tree construction.

Protein analysis and shotgun sequencing of genomic DNA

Preparation of cell lysates of naphthalene- or 2-methylnaphthalene-grown cells, sodium dodecylsulfate gel electrophoresis and Coomassie blue staining were performed as described (Rabus and Heider, 1998). The 2-methylnaphthalene-specific bands were excised for analysis by mass spectrometry (MS). Peptide masses (via MS) and sequences (via MS/MS) were determined as described (Hufnagel and Rabus, 2007). Peptide masses and peptide fragment fingerprints were mapped to the *in silico* digests of the predicted proteins by using the MS-Digest program (Clauser *et al.*, 1999).

Isolation of genomic DNA and generation of plasmid libraries (average insert size of 1.8 and 3.5 kb) were carried out as described (Rabus *et al.*, 2002). Whole-genome shotgun sequencing was performed via dye terminator sequencing using ABI3730XL capillary sequencers. Shotgun sequences were assembled using PHRAP (<http://www.phrap.org>). The assembly was edited and verified using Consed (Gordon, 2004) for selected sequences of the draft.

Potentially coding sequences were predicted by Glimmer3 and manually curated in Artemis, as described (Rabus *et al.*, 2002). Gene sequences encoding the assumed large subunit of (2-naphthylmethyl)succinate synthase (NmsA) were identified by searching with peptide masses and sequences.

Sequence quality of the identified *nmsA* genes was manually inspected.

The MEGA 3.1 program was used for alignment and tree construction (Kumar *et al.*, 2004) of glycol radical enzymes. Full-length amino acid sequences were aligned with CLUSTALW using the Gonnet matrix. A consensus neighbour-joining tree was constructed. Inferred phylogeny was tested by bootstrap analysis (1000 replications).

Data deposition

The 16S rRNA gene sequences of the presently obtained isolates, strains NaphS3 and NaphS6, have been submitted to GenBank under Accession Nos EU908726 and EU908727 respectively. The previously determined 16S rRNA sequence from strain NaphS2 is available under GenBank Accession No. AJ132804. Nucleotide sequences of the *nmsA* genes that encode the assumed 2-methylnaphthalene-activating enzyme, (2-naphthylmethyl)succinate synthase, in strains NaphS2, NaphS3 and NaphS6, have been submitted to EMBL/GenBank/DBJ under Accession Nos CU466266, CU466267 and CU466269, respectively.

Acknowledgements

We thank Ramona Appel (Bremen) and Ines Müller (Berlin) for technical assistance. This work was supported by the Max-Planck-Gesellschaft, the Deutsche Forschungsgemeinschaft (grant to B.S.) and the European Community project MATBIOPOL (EVK3-CT1999-00010; grant to F.W.).

References

- Anneweiler, E., Materna, A., Safinowski, M., Kappler, A., Richnow, H.H., Michaelis, W., and Meckenstock, R.U. (2000) Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. *Appl Environ Microbiol* **66**: 5329–5333.
- Anneweiler, E., Michaelis, W., and Meckenstock, R.U. (2002) Identical ring cleavage products during anaerobic degradation of naphthalene, 2-methylnaphthalene, and tetralin indicate a new metabolic pathway. *Appl Environ Microbiol* **68**: 852–858.
- Azuma, H., Toyota, M., Asakawa, Y., and Kawano, S. (1996) Naphthalene – a constituent of *Magnolia* flowers. *Phytochemistry* **42**: 999–1004.
- Bedessem, M.E., Swoboda-Colberg, N.G., and Colberg, P.J.S. (1997) Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. *FEMS Microbiol Lett* **152**: 213–218.
- Bogen, K.T., Beson, J.M., Yost, G.S., Morris, J.B., Dahl, A.R., Clewell, H.J., *et al.* (2008) Naphthalene metabolism in relation to target tissue anatomy, physiology, cytotoxicity and tumorigenic mechanism of action. *Regul Toxicol Pharmacol* **51**: 27–36.
- Boll, M., Fuchs, G., and Heider, J. (2002) Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr Opin Chem Biol* **6**: 604–611.
- Brandt, W.T. (1820) On a substance produced during the distillation of coal tar. *Q J Sci* **8**: 287–290.

- Caldwell, M.E., and Suflita, J.M. (2000) Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. *Environ Sci Technol* **34**: 1216–1220.
- Callaghan, A.V., Wawrik, B., Ní Chadhain, S.M., Young, L.Y., and Zylstra, G.J. (2008) Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. *Biochem Biophys Res Commun* **366**: 142–148.
- Chen, J., Henderson, G., Grimm, C.C., Lloyd, S.W., and Laine, R.A. (1998a) Termites fumigate their nests with naphthalene. *Nature* **392**: 558–559.
- Chen, J., Henderson, G., Grimm, C.C., Lloyd, S.W., and Laine, R.A. (1998b) Naphthalene in Formosan subterranean termite carton nests. *J Agric Food Chem* **46**: 2337–2339.
- Clauser, K.R., Baker, P., and Burlingame, A.L. (1999) Role of accurate mass measurement (± 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. *Anal Chem* **71**: 2871–2882.
- Coates, J., Anderson, R., and Lovley, D. (1996) Oxidation of polycyclic aromatic hydrocarbons under sulfate-reducing conditions. *Appl Environ Microbiol* **62**: 1099–1101.
- Coates, J., Woodward, J., Allen, J., Philp, P., and Lovley, D.R. (1997) Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. *Appl Environ Microbiol* **63**: 3589–3593.
- Cord-Ruwisch, R. (1985) A quick method for the determination of dissolved and precipitated sulfides in cultures of sulfate-reducing bacteria. *J Microbiol Methods* **4**: 33–36.
- Daisy, B.H., Strobel, G.A., Castillo, U., Ezra, D., Sears, J., Weaver, D.K., and Runyon, J.B. (2002) Naphthalene, an insect repellent, is produced by *Muscodora vitigenus*, a novel endophytic fungus. *Microbiology* **148**: 3737–3741.
- Davidova, I.A., Gieg, L.M., Duncan, K.E., and Suflita, J.M. (2007) Anaerobic phenanthrene mineralization by a carboxylating sulfate-reducing bacterial enrichment. *ISME J* **1**: 436–442.
- Dean, J.A. (1992) *Lange's Handbook of Chemistry*. New York, USA: McGraw-Hill.
- Eastcott, L., Shiu, W.Y., and Mackay, D. (1988) Environmentally relevant physical-chemical properties of hydrocarbons: a review of data and development of simple correlations. *Oil Chem Poll* **4**: 191–216.
- Ezra, D., Hess, W.M., and Strobel, G.A. (2004) New endophytic isolates of *Muscodora albus*, a volatile-antibiotic-producing fungus. *Microbiology* **150**: 4023–4031.
- Franck, H.-G., and Stadelhofer, J.W. (1987) *Industrielle Aromatenchemie*. Berlin, Germany: Springer-Verlag.
- Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999) Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ Microbiol* **1**: 415–420.
- Galushko, A.S., Kieseke-Lang, U., and Kappler, A. (2003) Degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture of mesophilic freshwater bacteria. *Polycyclic Aromatic Compounds* **23**: 207–218.
- Garden, A. (1820) Singular substance found in a coal tar apparatus. *Ann Phil* **15**: 74–75.
- Gassett, J.W., Wiesler, D.P., Baker, A.G., Osborn, D.A., Miller, K.V., Marchinton, R.L., and Novotny, M. (1997) Volatile compounds from the forehead region of male white-tailed deer (*Odocoileus virginianus*). *J Chem Ecol* **23**: 569–578.
- Gibson, D.T., and Subramanian, V. (1984) Microbial degradation of aromatic compounds. In *Microbial Degradation of Organic Compounds*. Gibson, D.T. (ed.). New York, NY, USA: Marcel Dekker, pp. 181–252.
- Gordon, D. (2004) Viewing and editing assembled sequences using Consed. In *Current Protocols in Bioinformatics*. Baxevanis, A.D., and Davison, D.B. (eds). New York, NY, USA: John Wiley, pp. 11.2.1–11.2.43.
- Grundmann, O., Behrends, A., Rabus, R., Amann, J., Halder, T., Heider, J., and 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.
- Habe, H., and Omori, T. (2003) Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. *Biosci Biotechnol Biochem* **67**: 225–243.
- Harms, G., Zengler, K., Aeckersberg, F., Minz, D., Rabus, R., and Widdel, F. (1999) Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. *Appl Environ Microbiol* **65**: 999–1004.
- Hayes, L.A., and Lovley, D.R. (2002) Specific 16S rDNA sequences associated with naphthalene degradation under sulfate-reducing conditions in harbor sediments. *Microb Ecol* **43**: 134–145.
- Hayes, L.A., Nevin, K.P., and Lovley, D.R. (1999) Role of prior exposure on anaerobic degradation of naphthalene and phenanthrene in marine harbor sediments. *Org Geochem* **30**: 937–945.
- Heider, J. (2007) Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. *Curr Opin Chem Biol* **11**: 188–194.
- Hicks, R.E., Amann, R.L., and Stahl, D.A. (1992) Dual staining of natural bacterioplankton with 4',6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. *Appl Environ Microbiol* **58**: 2158–2163.
- Hufnagel, P., and Rabus, R. (2007) Mass spectrometric identification of proteins in complex post-genomic projects. Soluble proteins of the metabolic versatile, denitrifying 'Aromatoleum' sp. strain EbN1. *J Mol Microbiol Biotechnol* **11**: 53–81.
- Kane, M.D., Poulsen, L.K., and Stahl, D.A. (1993) Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. *Appl Environ Microbiol* **59**: 682–686.
- Karlsson, A., Parales, J.V., Parales, R.E., Gibson, D.T., Eklund, H., and Ramaswamy, S. (2003) Crystal structure of naphthalene dioxygenase: side-on binding of dioxygen to iron. *Science* **299**: 1039–1042.
- Kidd, J. (1821) Observations on naphthaline, a peculiar substance resembling a concrete essential oil, which is apparently produced during the decomposition of coal tar, by exposure to a red heat. *Philos Trans R Soc Lond* **111**: 209–221.
- Kniemeyer, O., Fischer, T., Wilkes, H., Glöckner, F.O., and Widdel, F. (2003) Anaerobic degradation of ethylbenzene

- by a new type of marine sulfate-reducing bacterium. *Appl Environ Microbiol* **69**: 760–768.
- Krauss, M., Wilcke, W., Martius, C., Bandeira, A.G., Garcia, M.V.B., and Amelung, W. (2005) Atmospheric versus biological sources of polycyclic aromatic hydrocarbons (PAHs) in a tropical rain forest environment. *Environ Pollut* **135**: 143–154.
- Kumar, S., Tamura, K., and Nei, M. (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**: 150–163.
- Langenhoff, A.A.M., Zehnder, A.J.B., and Schraa, G. (1996) Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. *Biodegradation* **7**: 267–274.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.
- McMillen, D.F., and Golden, D.M. (1982) Hydrocarbon bond dissociation energies. *Ann Rev Phys Chem* **33**: 493–532.
- Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B. (2000) Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl Environ Microbiol* **66**: 2743–2747.
- Musat, F., and Widdel, F. (2008) Anaerobic degradation of benzene by a marine sulfate-reducing enrichment culture, and cell hybridization of the dominant phylotype. *Environ Microbiol* **10**: 10–19.
- Phelps, C.D., Kerkhof, L.J., and Young, L.Y. (1998) Molecular characterization of a sulfate-reducing consortium which mineralizes benzene. *FEMS Microbiol Ecol* **27**: 269–279.
- Preuss, R., Angerer, J.R., and Drexler, H. (2003) Naphthalene – an environmental and occupational toxicant. *Int Arch Occup Environ Health* **76**: 556–576.
- Rabus, R., and Heider, H. (1998) Initial reactions of anaerobic metabolism of alkylbenzenes in denitrifying and sulfate-reducing bacteria. *Arch Microbiol* **170**: 377–384.
- Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A.J., and 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., Beck, A., Widdel, F., and Reinhardt, R. (2002) Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EbN1. *Arch Microbiol* **178**: 506–516.
- Reed, D.R., and Kass, S.R. (2000) Experimental determination of the α and β C–H bond dissociation energies in naphthalene. *J Mass Spectrom* **35**: 534–539.
- Rockne, K.J., and Strand, S.E. (1998) Biodegradation of bicyclic and polycyclic aromatic hydrocarbons in anaerobic enrichments. *Environ Sci Technol* **32**: 3962–3967.
- Rockne, K.J., and Strand, S.E. (2001) Anaerobic biodegradation of naphthalene, phenanthrene, and biphenyl by a denitrifying enrichment culture. *Water Res* **35**: 291–299.
- Rockne, K.J., Chee-Sanford, J.C., Sanford, R.A., Hedlund, B.P., Staley, J.T., and Strand, S.E. (2000) Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Appl Environ Microbiol* **66**: 1595–1601.
- Rothermich, M.M., Hayes, L.A., and Lovley, D.R. (2002) Anaerobic, sulfate-dependent degradation of polycyclic aromatic hydrocarbons in petroleum-contaminated harbor sediment. *Environ Sci Technol* **36**: 4811–4817.
- Safinowski, M., and Meckenstock, R.U. (2006) Methylation is the initial reaction in anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Environ Microbiol* **8**: 347–352.
- Safinowski, M., Griebler, C., and Meckenstock, R.U. (2006) Anaerobic cometabolic transformation of polycyclic and heterocyclic aromatic hydrocarbons: evidence from laboratory and field studies. *Environ Sci Technol* **40**: 4165–4173.
- Sharak Genthner, B.R., Townsend, G.T., Lantz, S.E., and Mueller, J.G. (1997) Persistence of polycyclic aromatic hydrocarbon components of creosote under anaerobic enrichment conditions. *Arch Environ Contam Toxicol* **32**: 99–105.
- Sullivan, E.R., Zhang, X., Phelps, C., and Young, L.Y. (2001) Anaerobic mineralization of stable isotope-labeled 2-methylnaphthalene. *Appl Environ Microbiol* **67**: 4353–4357.
- Thierrin, J., Davis, G.B., Barber, C., Patterson, B.M., Pribac, F., Power, T.R., and Lambert, M. (1993) Natural degradation rates of BTEX compounds and naphthalene in a sulfate-reducing groundwater environment. *Hydrol Sci* **38**: 309–322.
- Tissot, B.P., and Welte, D.H. (1984) *Petroleum Formation and Occurrence*, 2nd edn. New York, NY, USA: Springer-Verlag.
- Umweltbundesamt (2007) Naphthalin/Naphthole und Human-Biomonitoring. *Bundesgesundheitsbl Gesundheitsforsch Gesundheitsschutz* **50**: 1357–1364. doi:10.1007/s00103-007-0342-3.
- Walsby, A.E. (1994) Gas vesicles. *Microbiol Rev* **58**: 94–144.
- Widdel, F. (1988) Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In *Biology of Anaerobic Microorganisms*. Zehnder, A.J.B. (ed.). New York, NY, USA: Wiley & Sons, pp. 469–585.
- Widdel, F., and Bak, F. (1992) Gram-negative mesophilic sulfate-reducing bacteria. In *The Prokaryotes*, 2nd edn. Balows, A., Trüper, H.G., Dworking, M., Harder, W., and Schleifer, K.-H. (eds). New York, NY, USA: Springer, pp. 3352–3378.
- Widdel, F., Boetius, A., and Rabus, R. (2004) Anaerobic biodegradation of hydrocarbons including methane. In *The Prokaryotes*, 3rd edn, Vol. 2. Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds). New York, NY, USA: Springer-Verlag, pp. 1028–1049.
- Widdel, F., Musat, F., Knittel, K., and Galushko, A. (2007) Anaerobic degradation of hydrocarbons with sulphate as electron acceptor. In *Sulphate-Reducing Bacteria: Environmental and Engineered Systems*. Barton, L.L., and Hamilton, W.A. (eds). Cambridge, UK: Cambridge University Press, pp. 265–303.
- Wilkes, H., Boreham, C., Harms, G., Zengler, K., and Rabus, R. (2000) Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. *Org Geochem* **31**: 101–115.
- Wöhlbrand, L., Wilkes, H., Halder, T., and Rabus, R. (2008)

Anaerobic degradation of *p*-ethylphenol by 'Aromatoleum aromaticum' strain EbN1: pathway, regulation, and involved proteins. *J Bacteriol* **190**: 5699–5709.

Zhang, X., and Young, L.Y. (1997) Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microbiol* **63**: 4759–4764.

Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62**: 316–322.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Incubation experiments of strain NaphS2 with non-toxic concentrations of 1-naphthol indicating that it did not serve as a substrate.

Fig. S2. Mapping of peptides (identified by mass spectrometry) from 2-methylnaphthalene-specific proteins (Fig. 4)

against the gene-deduced assumed (2-naphthylmethyl)-succinate synthases in strains NaphS2, NaphS3 and NaphS6.

Fig. S3. CLUSTALW alignment of protein sequences used for phylogenetic tree construction (Fig. 5).

Fig. S4. Assumed separate degradation of naphthalene and 2-methylnaphthalene to the common intermediate, 2-naphthoyl-CoA, in marine sulfate-reducing bacteria.

Fig. S5. Isotope-based differentiation between the pathways of naphthalene and 2-methylnaphthalene in marine sulfate-reducing bacteria.

Fig. S6. Partial ion chromatograms revealing naphthalene-2-carboxylic acid methyl ester and methylnaphthalene-2-carboxylic acid methyl esters obtained after growth with 2-methylnaphthalene or a mixture of naphthalene and 1-methylnaphthalene respectively.

Please note: Blackwell Publishing are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.