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Effect of energy deprivation on metabolite release by anaerobic marine naphthalene-degrading sulfate-reducing bacteria

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Summary

The aromatic hydrocarbon naphthalene, which occurs in coal and oil, can be degraded by aerobic or anaerobic microorganisms. A wide-spread electron acceptor for the latter is sulfate. Evidence for in situ naphthalene degradation stems in particular from the detection of 2-naphthoate and [5,6,7,8]-tetrahydro-2-naphthoate in oil field samples. Because such intermediates are usually not detected in laboratory cultures with high sulfate concentrations, one may suppose that conditions in reservoirs, such as sulfate limitation, trigger metabolite release. Indeed, if naphthalene-grown cells of marine sulfate-reducing Deltaproteobacteria (strains NaphS2, NaphS3 and NaphS6) were transferred to sulfate-free medium, they released 2-naphthoate and [5,6,7,8]-tetrahydro-2-naphthoate while still consuming naphthalene. With 2-naphthoate as initial substrate, cells produced [5,6,7,8]-tetrahydro-2-naphthoate and the hydrocarbon, naphthalene, indicating reversibility of the initial naphthalene-metabolizing reaction. The reactions in the absence of sulfate were not coupled to observable growth. Excretion of naphthalene-derived metabolites was also achieved in sulfate-rich medium upon addition of the protonophore carbonyl cyanide4-(trifluoromethoxy)phenylhydrazone or the ATPase inhibitor *N,N′*-dicyclohexylcarbodiimide. In conclusion, obstruction of electron flow and energy gain by sulfate limitation offers an explanation for the occurrence of naphthalene-derived metabolites in oil reservoirs, and provides a simple experimental tool for gaining insights into the anaerobic naphthalene oxidation pathway from an energetic perspective.

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Introduction

Naphthalene, the simplest representative of unsubstituted polycyclic aromatic hydrocarbons (PAH), is a major constituent of coal tar, amounting to up to 10% by mass (Wise *et al*., 1988). To a lesser extent naphthalene is found in crude oils (Tissot and Welte, 1984). In addition to geochemical sources, naphthalene can be produced by living organisms such as fungi (Daisy *et al*., 2002), plants (Azuma *et al*., 1996), and insects (Chen *et al*., 1998).

Whereas biodegradation of naphthalene with oxygen has been studied in depth over several decades (Boronin and Kosheleva, 2010), degradation in the absence of oxygen is a more recently established topic. Besides enriched microcosms (e.g. Mihelcic and Luthy, 1988; Bedessem *et al*., 1997; Coates *et al*., 1997; Chang *et al*., 2006), relatively few pure cultures of anaerobic naphthalene degraders have been obtained; electron acceptors were sulfate (Galushko *et al*., 1999; Meckenstock *et al*., 2000; Musat *et al*., 2009), ferric iron (Kleemann and Meckenstock, 2011), or nitrate (Rockne *et al*., 2000). Because functionalization of the apolar hydrocarbon by introduction of hydroxyl groups via dioxygenases is excluded in anaerobes (with possible exception of denitrifiers employing a nitric oxide dismutase; Ettwig *et al*., 2010), the initial reaction of naphthalene in anaerobes must basically differ from that in aerobes. The presently favoured one is a carboxylation-like reaction leading to 2-naphthoate (Zhang and Young, 1997; Mouttaki *et al*., 2012). Based on genomic and proteomic analyses of the sulfate-reducing marine strain NaphS2 (DiDonato *et al*., 2010) and the fresh-water strain N47 (Bergmann *et al*., 2011), enzymes related to phenylphosphate carboxylase of the anaerobic phenol degradation were proposed to be involved in naphthalene functionalization. Subsequent processing, which has been studied in a highly enriched freshwater sulfate-reducing bacterium (strain N47) occurs

via thioesterification to 2-naphthoyl-CoA, reduction of the distal ring to [5,6,7,8]-tetrahydro-2-naphthoyl-CoA (Eberlein *et al*., 2013a; Eberlein *et al*., 2013b; Estelmann *et al*., 2015), and further reduction to hexahydro-2-naphthoyl-CoA (Eberlein *et al*., 2013b). Ring opening and beta-oxidation converts the latter via pimeloyl-CoA and glutaconyl-CoA to acetyl-CoA (Weyrauch *et al*., 2017) that is oxidized to CO_2 via the CO-dehydrogenase (oxidative Wood-Ljungdahl, C_1 pathway Galushko *et al*., 1999; Musat *et al*., 2009).

Free metabolites of the anaerobic degradation pathways of aromatic and aliphatic compounds, mostly carboxylic acids, in aqueous phases of deep anoxic strata may serve as indicators (biomarkers) of *in situ* microbial activity towards oil, gas or coal hydrocarbons (Griebler *et al*., 2004; Gruner *et al*., 2017). Anaerobic utilization of naphthalene in oil reservoirs was inferred from the presence of 2-naphthoate and [5,6,7,8]-tetrahydro-2-naphthoate (Aitken *et al*., 2004). However, laboratory cultures of naphthalene-degrading sulfate-reducing bacteria usually do not release detectable concentrations of such metabolites into the medium. For their identification, entire cultures are extracted which liberates cellular pools (e.g., Rabus *et al*., 2001; Musat *et al*., 2009). One may speculate that the availability of sulfate as electron acceptor for respiratory energy conservation is one of the factors that influence the containment of metabolites inside the cell. Whereas laboratory cultures have been grown with optimal supply of the electron acceptor, sulfate in oil reservoirs is frequently depleted or strongly limiting (Nilsen *et al*., 1996; Orphan *et al*., 2000). Here we investigated the effect of sulfate depletion on naphthalene-metabolizing cells of three strains of marine sulfate-reducing bacteria. Sulfate depletion indeed led to detectable concentrations of naphthalene-derived carboxylates in the medium. Such excretion was also triggered by impediment of energy conservation by agents dissipating the transmembrane proton gradient, or blocking ATP synthesis. Moreover, sulfate-depleted non-growing yet metabolically intact cells catalysed net conversion of 2-naphthoate to the hydrocarbon naphthalene (besides [5,6,7,8]-tetrahydro-2-naphthoate). Impediment of the energy-conserving electron flow to the external electron acceptor of the investigated sulfate-reducing bacteria thus not only sheds light on metabolite excretion in situ, but also on energetic aspects such as reversibility of the initial ('upper') reactions of naphthalene.

Results

Use of a carrier phase for naphthalene versus direct addition

The marine sulfate-reducing strains NaphS2, NaphS3 and NaphS6 (hereafter NaphS strains) were hitherto grown with naphthalene provided from an inert hydrophobic carrier phase (2,2,4,4,6,8,8-heptamethylnonane) in the presence of high sulfate concentrations (28 mM). The carrier phase serves as a reservoir while simultaneously diluting the pure naphthalene and diminishing its activity (chemical potential) and possible toxic effects. However, this cultivation method has its drawbacks if time courses of naphthalene consumption and product formation are to be studied. The carrier phase constantly resupplies naphthalene to the aqueous phase and thus interferes with a quantitative (e.g. stoichiometric) analysis. Also, some adsorption of metabolites to the carrier phase cannot be excluded.

Quantitative measurement of time courses is therefore more reliable without a carrier phase. However, naphthalene dissolves poorly (24 mg l^{-1} or 0.19 mM, at 20°C) and slowly in water, and undissolved crystals would also act as an interfering reservoir. Defined amounts of naphthalene were therefore added from a solution in methanol. To test for potential interferences of methanol with the energy metabolism, NaphS strains growing with naphthalene were provided with concentrations of up to 2.5 mM methanol. No effects on the naphthalene-dependent reduction of sulfate to sulfide were observed (Fig. S3). A certain minor problem of this method is that a noticeable proportion of the relatively small amount of total naphthalene can be lost by adsorption to the stoppers of cultivation flasks.

Utilization of naphthalene and 2-naphthoate in the absence of sulfate

Naphthalene applied without a carrier phase directly to medium with sufficient sulfate (starting concentration, 28 mM) did not yield detectable concentrations of UV-adsorbing metabolites (limit of detection, < 0.1 μM; Fig. S1), not even in assays with 80-fold concentrated cell suspensions ($OD_{600} \approx 8$). Similar findings were observed in the presence of a carrier phase. To investigate whether a hampered energy metabolism, as it may occur in oil reservoirs, can trigger metabolite release, cell suspensions were incubated with naphthalene in sulfate-free medium. Indeed, transient formation of 2-naphthoate (2-Npht) and accumulation of [5,6,7,8]-tetrahydro-2-naphthoate (H_4-2-Npht) were observed. Despite the absence of sulfate, these cell suspensions consumed all added naphthalene, albeit more slowly than cells with sulfate (Figs. 1A and S1). Formation of 2-Npht and H_4-2-Npht was strictly dependent on the addition of naphthalene. Naphthalene-dependent formation of acetate at concentrations of about 800 μM was also observed (Fig. 1A, inset). Cell suspensions in control assays not supplied with naphthalene produced about 400 μM acetate (Fig. 1A, inset), which was attributed to the

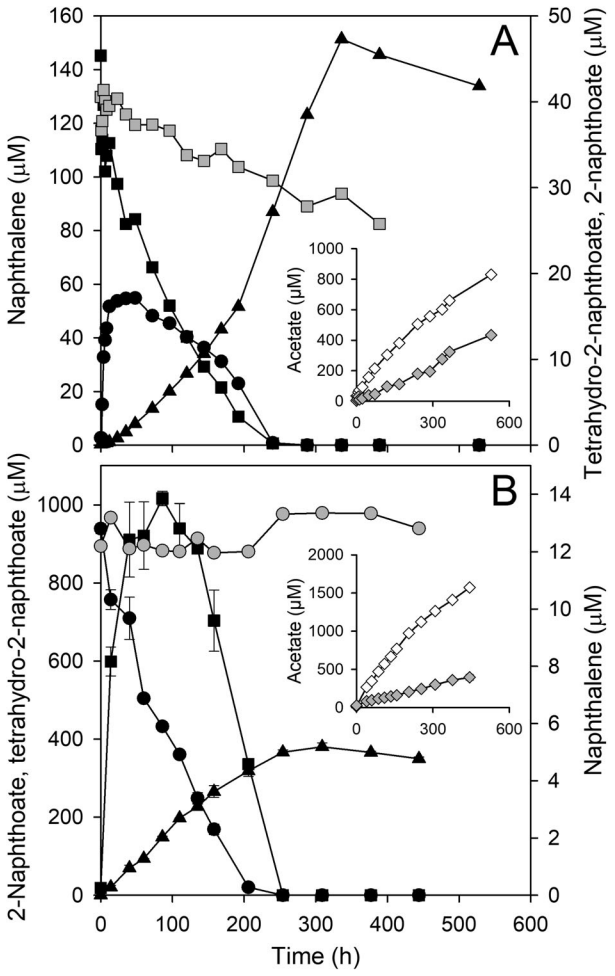

Fig 1. Strain NaphS2 metabolized naphthalene (A) and 2-naphthoate (B) in the absence of sulfate.
A. Consumption of naphthalene (■) was accompanied by the transient formation of 2-naphthoate (●), and accumulation of tetrahydro-2-napthoate (▲). Naphthalene concentrations decreased slightly in sterile controls (□)
B. Consumption of 2-Npht (●) was accompanied by the transient formation of small amounts of naphthalene (■), and by the accumulation of tetrahydro-2-napthoate (▲).
2-Npht concentrations were stable in sterile control incubations (○). Acetate (insets of panels A and B) was detected in both cell suspensions incubated with (◇) and without (◈) added substrate (naphthalene or 2-Npht respectively). Error bars represent standard deviation based on replicate incubations.

transformation of intracellularly stored intermediates. Other intermediates downstream of H_4-2-Npht were not quantified. The formed H_4-2-Npht accounted for approximately 33% of the added naphthalene.

Because naphthalene-derived 2-Npht appeared only transiently in the sulfate-free incubations and was consumed again, cells were expected to metabolize also exogenously added 2-Npht without an electron acceptor. Indeed, naphthalene-grown cells resuspended in sulfate-free medium consumed all added 2-Npht within a time frame comparable to that of naphthalene degradation (i.e. ca. 240 h; Fig. 1B). Like naphthalene without sulfate, also 2-Npht led to an accumulation of H_4-2-Npht and acetate (Fig. 1B and inset). Moreover and remarkably, 2-Npht was in addition converted to the hydrocarbon, naphthalene at concentrations up to 14 μM, which is ca. 1.5% of the added 2-Npht (Fig. 1B). The formed naphthalene was subsequently consumed, similar as in the incubations receiving naphthalene as the starting substrate. These results demonstrate that external conditions can enforce net reversion of the reaction leading from naphthalene to 2-Npht, that is, of the functionalization (activation) of the hydrocarbon. Before, reversibility was shown by an isotope exchange between labelled bicarbonate and 2-Npht in assays with cell extracts of the naphthalene-grown culture N47 (Mouttaki *et al*., 2012). A similar isotope exchange was observed in whole cell assays of the NaphS strains (Fig. S2).

Naphthalene metabolism in cells treated with energy-depriving agents

The obvious triggering of metabolite release by sulfate limitation, that is, by an impeded energy metabolism and flow of reducing equivalents, suggested that artificial energy-depriving agents may have similar effects. Analyses of catabolically affected cell suspensions were therefore also conducted upon treatment with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and *N*,*N*′-dicyclohexylcarbodiimide (DCCD). FCCP is a weakly proton-binding, membrane permeable compound (protonophore) that dissipates the transmembrane proton gradient, the driving force of ATP synthesis (Nicholls and Ferguson, 1992). DCCD blocks specifically the ATPase by covalent binding, without disrupting the membrane potential (Sebald *et al*., 1980). Impeded ATP synthesis hampers, besides numerous biosynthetic reactions, adenylylation (activation) of the electron acceptor, sulfate. First, growth inhibition was tested with different concentrations of FCCP (20, 50 and 200 μM) and with DCCD (1 mM) in NaphS cultures with naphthalene (here supplied via carrier phase) and sulfate. Concentrations of 20 μM FCCP and 1 mM DCCD were sufficient to inhibit growth (Fig. S3) and were thus applied to harvested cells incubated with directly added naphthalene and sulfate. Indeed, those receiving FCCP or DCCD released 2-Npht directly upon addition of the inhibitor (Fig. 2). If FCCP was added to cells with naphthalene in the absence of sulfate, the released 2-Npht concentrations were even somewhat higher than those due to sulfate depletion alone. Again, 2-Npht release depended on the presence of naphthalene.

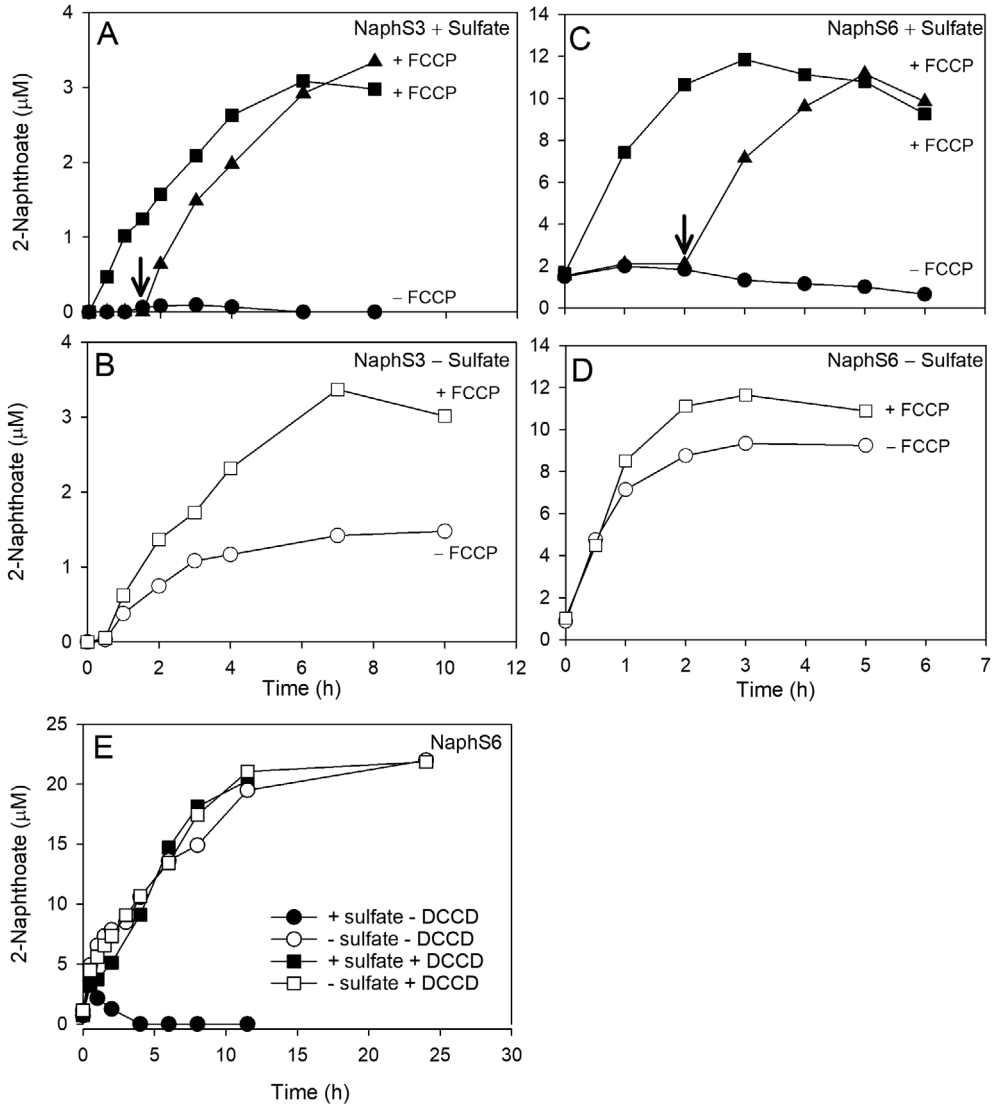

Fig 2. Influence of sulfate depletion and FCCP and DCCD addition on the formation of 2-naphthoate from naphthalene.
A,C. Addition of FCCP to concentrated cell suspensions of strain NaphS3 ($OD_{600} \approx 2.7$) or NaphS6 ($OD_{600} \approx 6.4$) in the presence of sulfate.
B,D. Addition of FCCP to NaphS3 ($OD_{600} \approx 2.7$) and NaphS6 ($OD_{600} \approx 6.4$) cell suspensions without sulfate.
E. Treatment of NaphS6 cell suspensions ($OD_{600} \approx 6.4$) with DCCD had similar effects. Arrows (A, C) indicate FCCP addition during the incubation. Naphthalene was the starting substrate in all assays.

Discussion

The present investigation sheds light on the external conditions and the energetic state of cells that may cause a release of metabolites during the anaerobic degradation of naphthalene. The excretion of metabolites is explained by an imbalance between production and subsequent consumption. Such disturbance may result in particular from the impeded channelling of the naphthalene-derived reducing equivalents into the anaerobic respiratory chain, due to the lack of sulfate. Excretion of H_4-2-Npht and acetate may reflect the tailback of reducing equivalents and acetyl-CoA (the substrate of the oxidative Woold–Ljungdahl pathway) respectively. Overall, the observed processing of naphthalene represents a dismutation to more reduced (H_4-2-Npht) and more oxidized (acetate) products. However, since the measured amounts of H_4-2-Npht and acetate did not account for the naphthalene consumed (in terms of carbon and reducing equivalents), determination of the dismutation balance would require an extended analysis of organic metabolites and also of CO_2.

From an energetic point of view, the processing of naphthalene to the metabolites detected in solution in the absence of sulfate or with the addition of FCCP or DCCD is more puzzling. The initial metabolic reactions of naphthalene, viz. conversion to 2-Npht, thioesterification to

2-naphthoyl-CoA and reduction of the aromatic rings (Eberlein *et al*., 2013b; Estelmann *et al*., 2015) depend on energy. Also, formulated dismutation reactions (Supporting Information Equations S6 and S7) of naphthalene leading to carboxylates are endergonic under standard conditions, unlike established fermentations. Energy from the reduction of residual sulfate is unlikely. The calculated concentration of sulfate remaining after washing and resuspension in sulfate-free medium is only 0.2–0.3 mM. It would have been consumed by the concentrated cell suspension within less than 1 h, whereas naphthalene and 2-Npht utilization continued over several days (details in Supporting Information). Among the driving forces allowing the metabolism of naphthalene without sulfate one may envisage 'energy-rich' co-reactants, either as residual pools still present from the preceding growth phase, or formed by reactions during the observed dismutation.

A hypothetical residual co-reactant could be an 'energy-rich' carboxyl donor (such as a carboxybiotin group) that was not affected by the energy-depriving treatments of the cells. Like the introduction of a carboxylate group from CO_2 at any non-activated organic compound (Glueck *et al*., 2010; Bar-Even *et al*., 2012), also direct carboxylation of naphthalene according to

$$C_{10}H_8\,(c) + CO_2\,(g) \rightarrow C_{10}H_7 - COO^-\,(aq) + H^+ \qquad (1)$$

is energetically not feasible under standard biochemical conditions at pH = 7; the free energy change (calculated from ΔG_f° data; see Supporting Information) is $\Delta G^{\circ\prime}$= +26.8 kJ mol^{-1}. According to the equilibrium constant (formally calculated from standard ΔG° = +66.6 kJ mol^{-1}),

$$K_{eq} = \left(\frac{\{C_{10}H_7COO^-\}\{H^+\}}{\{C_{10}H_8\}\{CO_2\}}\right)_{eq} = 2.13 \times 10^{-12} \qquad (2)$$

the maximum concentration of 2-Npht estimated from the activities (see Supplementary Information) that could be formed spontaneously under the given conditions (naphthalene at saturating concentration, which is energetically equivalent with crystalline naphthalene; pH = 7; CO_2 partial pressure of 10 kPa, corresponding to ca. 10% by volume in head space gas) is only about 2 μM. However, concentrations around 20 μM were observed (Figs 1A and 2E), indicating that catalysis of 2-Npht formation must have been driven by a net input of energy. Moreover, the concentrations of 2-Npht formed in whole-cell assays were rather depended on the amount of cells than on the added naphthalene concentrations, further supporting the assumption of a coupling energy-rich carboxyl donor (Fig. 3).

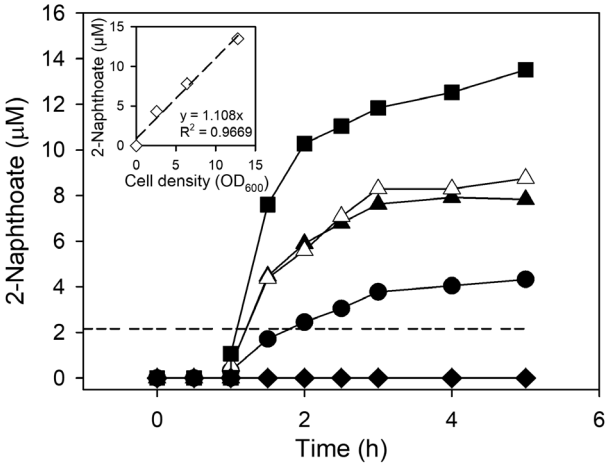

Fig 3. Influence of cell density and initial naphthalene concentration on the formation of 2-naphthoate.
Assays with the same concentration of naphthalene (100 μM) were supplied with 100 (■), 50 (▲) and 20 μl (●) of a strain NaphS6 cell suspension (theoretical $OD_{600} \approx 128$).
For comparison, assays with 50 μl cell suspension were provided with 200 μM naphthalene (△). In all assays, naphthalene was injected after 1 h of pre-incubation. 2-Npht was not detected in sterile controls (◆).
The concentration of formed 2-Npht showed a linear correlation with the cell density (inset). Dashed line indicates the predicted equilibrium concentration of 2-Npht (about 2 μM).

When 2-Npht was added at concentrations higher than observed for the excreted compound (ca. 1 vs. 0.02 mM), the 'disturbed' pool concentration led to a noticeable net reaction back to free naphthalene. The reversal of the carboxylation reaction per se is necessarily exergonic by the same amount of free energy that is needed to drive naphthalene carboxylation, with $\Delta G^{\circ\prime}$ = –26.8 kJ mol^{-1}. According to the equilibrium constant, an uncoupled reaction would leave a residual concentration of approx. 2 μM 2-Npht. Hence, the bulk would be converted to free naphthalene, accumulating fivefold higher than saturation. However, the proposed coupling mechanism that drives naphthalene carboxylation obviously counteracted naphthalene formation, and became dominant when the decreasing 2-Npht concentration no longer favoured the back reaction.

As energy-rich co-reactants formed during dismutation, one may envisage CoA-thioesters of H_4-2-Npht and acetate. Analysis of the genome of strain NaphS2 (DiDonato *et al*., 2010) predicts at least 13 CoA-transferases some of which may allow 2-Npht activation (Table S2) while leading to the free acid anions that are excreted.

Even though present explanations of the observed processing of naphthalene despite the experimentally hampered energy metabolism are still insufficient, the results obtained with the marine NaphS strains may add two kinds of perspectives to biogeochemical research of oil and coal reservoirs. First, formation of hydrocarbon-derived metabolites such as 2-Npht should not only be

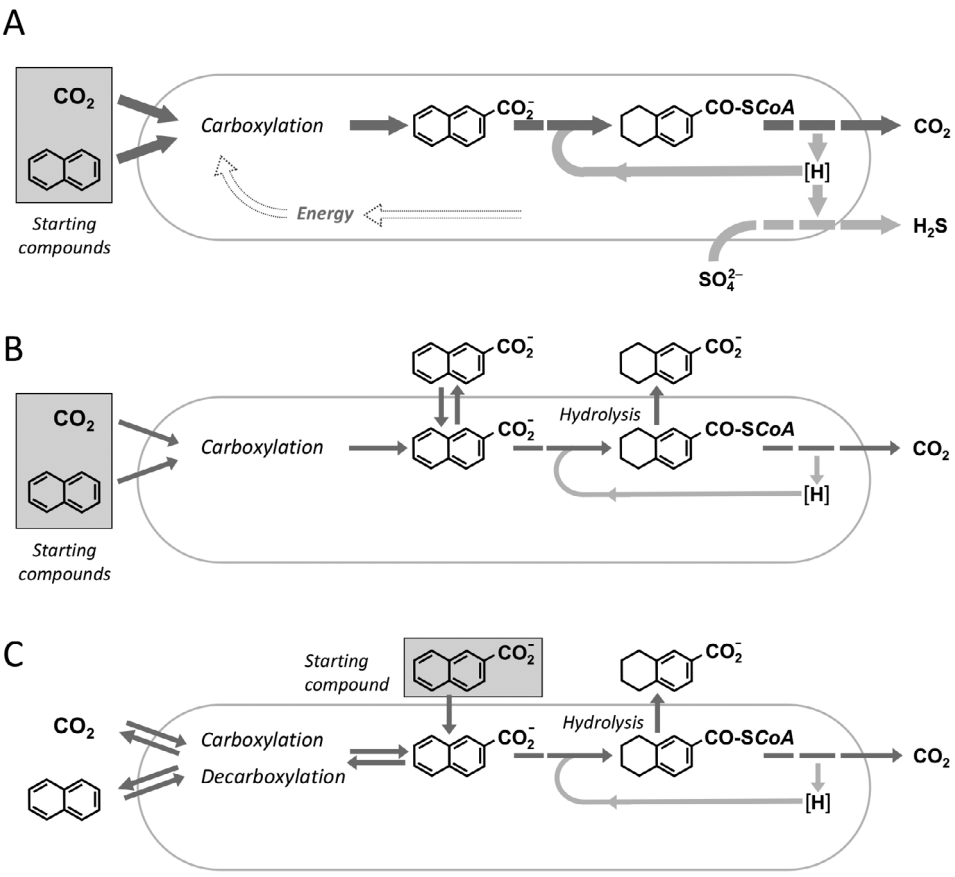

Fig 4. Proposed basic flow of carbon and reducing equivalents during growth with limiting and non-limiting sulfate.
Release of metabolites, not detectable in the culture medium during regular growth with naphthalene and non-limiting sulfate (A), was observed only under strong sulfate limitation or depletion (B).
Release of intermediates and even the net formation of the hydrocarbon naphthalene were observed with 2-Npht as the initial substrate, in the absence of sulfate (C). Resembling *in situ* conditions, our findings offer an alternative explanation for the occurrence of naphthalene-derived metabolites in petroleum reservoirs.

taken as an indicator of microbial activity, but also of stress and suboptimal conditions such as electron acceptor limitation. Absence of metabolites does not necessarily indicate the absence of hydrocarbon-degrading microorganisms; there may be periods of sulfate availability (for example introduced by injection of formation water), so that metabolites are not excreted or excreted metabolites are again taken up (Fig. 4). Still, there might be other factors that trigger metabolite release in situ, in particular the solvent-like, membrane-disturbing effects of oil hydrocarbons belonging to the volatile fraction (Sikkema *et al*., 1995). For more definite prediction and interpretation of metabolite release, additional, refined experiments would be needed, for instance growth with continuous supply of strongly limiting sulfate instead of the sulfate-limited batch incubations. Second, formation by the NaphS strains of naphthalene from 2-Npht shows that microbial net conversion of a monocarboxylic acid to an aromatic hydrocarbon is in principle possible (Fig. 4). Strictly anaerobic hydrocarbon-utilizing microorganisms thrive with relatively low amounts of free energy (i.e. with small differences in chemical potentials of reactants vs. products; Widdel and Musat, 2010). Their catabolism can only function if reactions are without much energy dissipation, that is, not far from the equilibrium. Such reactions necessarily include noticeable reverse reactions (back fluxes; Holler *et al*., 2011; Mouttaki *et al*., 2012; Hahn *et al*., 2020). Back fluxes may turn into net reversal if thermodynamic settings are in favour of such a process. In the NaphS strains, the net reversal (decarboxylation) was transient, forced by the unusual incubation conditions, and not associated with observable growth. However, fermentative microorganisms are known that conserve energy for growth by coupling decarboxylation of aliphatic dicarboxylates with Na^+ translocation across cell membranes, or with secondary transport systems (Dimroth and Hilpert, 1984; Poolman and Konings, 1993; Dimroth and Schink, 1998). The long-term process of in situ hydrocarbon formation represents mostly a loss of carboxyl groups from components in kerogen (Tissot and Welte, 1984; Seewald, 2003). One may thus speculate that some of these reactions are not abiotic, as commonly assumed, but catalysed by yet unknown genuine decarboxylating anaerobic microorganisms.

Experimental procedures

Chemicals

Naphthalene (purity 99%), 2-naphthoic acid (98%), [5,6,7,8]-tetrahydro-2-naphthoic acid (97%), 2,2,4,4,6,8,8-heptamethylnonane (HMN, 98%), FCCP (≥ 98%), and

DCCD (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Radiolabeled [1,4,5,8-^{14}C]-naphthalene and ^{14}C-sodium bicarbonate ($NaH^{14}CO_3$) were purchased from Hartmann Analytic (Braunschweig, Germany).

Microorganisms and cultivation techniques

The naphthalene-degrading sulfate-reducing bacterium strain NaphS2 was isolated from anoxic sediments of a North Sea harbour near Wilhelmshaven, Germany (Galushko *et al*., 1999). Strains NaphS3 and NaphS6 were isolated from marine sediments of a Mediterranean lagoon (Musat *et al*., 2009). Since isolation, all strains were maintained in the laboratory as active cultures by repeated transfers in fresh culture medium with naphthalene as a substrate. The artificial seawater (ASW) medium, and the anaerobic cultivation techniques used were as described elsewhere (Widdel and Bak, 1992; Widdel, 2010). Routine cultivation was performed in 100-ml flat bottles, containing 50 ml of $NaHCO_3/CO_2$-buffered ASW medium and inoculated with 10 ml of a grown culture. The bottles were sealed with butyl-rubber stoppers under a headspace of N_2:CO_2 (9:1 vol/vol), and provided with 2.5 ml HMN as an inert carrier phase containing 20 mg ml^{-1} naphthalene. For preparation of cell suspensions cultures were routinely prepared in 2 l bottles with a side arm (Glasgeraetebau Ochs, Bovenden/Lenglern, Germany) containing 1.6 l ASW medium, and 200 ml inoculum from a fully grown culture; the bottles were provided with 100 ml of anoxic HMN containing naphthalene (20 mg ml^{-1}). The development of the cultures was routinely monitored by photometric measurements (λ = 480 nm) of sulfide concentrations via the formation of colloidal CuS, according to the reaction $Cu^{2+} + H_2S \rightarrow CuS + 2\ H^+$, as previously described (Cord-Ruwisch, 1985).

Incubation of cell suspensions without sulfate

Cultures were harvested under anoxic conditions when sulfide concentrations had reached 10–15 mM. In order to avoid contact with oxygen, all manipulations were done anoxically under a continuous stream of N_2 gas, or inside an anoxic tent with an atmosphere of N_2:CO_2 (9:1, vol/vol). Volumes of 1.6 l of grown cultures were transferred to separatory funnels, separated from the overlying HMN carrier phase, and collected in 400 ml centrifuge beakers. Cells were collected by centrifugation for 25 min at 16,000*g* (Beckman Coulter Avanti J-26XP), 10°C.

After harvesting, cell pellets were washed once with 300 ml sulfate-free bicarbonate-buffered (30 mM) artificial seawater medium, and finally suspended in 20 ml of the same medium. Cell suspensions were divided into two 10-ml aliquots, one supplied with naphthalene, while the second one was serving as a substrate-free control. With 2-naphthoate as a starting substrate the incubations were conducted with 5 ml cell suspensions, in duplicate. Reactions were started by addition of the substrate (naphthalene or 2-naphthoate). Naphthalene was added at working concentrations of 200 μM from stock solutions in methanol (200 mM) using N_2-flushed syringes; during the slow addition the transient formation of a whitish cloud (naphthalene microcrystals) was observed. A similar volume of pure methanol was added to substrate-free controls to exclude potential artefacts generated by methanol.

At defined time points, volumes of 300 μl were taken with N_2-flushed syringes, from which aliquots of 50 μl were mixed with 50 μl of 70% acetonitrile in water and acidified by adding 1 μl of H_2SO_4 (1 M); the mixtures were then centrifuged and filtered through 0.2 μm nylon filters (WICOM, Germany) for subsequent Ultra Performance Liquid Chromatography (UPLC) analysis. The rest of the sample (ca. 250 μl) was also acidified with concentrated HCl, centrifuged to remove cells, filtered through 0.45 μm nylon filters, and used for organic acid analysis.

Whole-cell assays, application of protonophore FCCP and ATPase inhibitors DCCD

For whole-cell assays, cultures (volume = 1.6 l) of strains NaphS2, NaphS3 or NaphS6 were harvested, cell pellets were washed as described above, and were finally suspended in 5 ml sulfate-free medium. The assays were performed in an anoxic tent, in 3-ml serum vials closed with Teflon-lined septa fixed by aluminium crimps. A defined volume (50 μl, unless otherwise indicated) of the cell suspension was diluted with ASW medium with or without sulfate to a final volume of 1 ml. The cell density in whole-cell assays was estimated as OD_{600} units considering the OD_{600} values of the cultures before harvesting and the concentration of the cells following centrifugations. Reactions were started by addition of naphthalene (ca. 200 μM working concentration) or 2-naphthoate (ca. 1 mM working concentration). Naphthalene was added from a 200 mM stock solution in methanol, and 2-naphthoate from 100 mM stock solution in water. FCCP was injected from a 1 mM stock solution in methanol to a final concentration of 20 μM (unless otherwise indicated). In control assays without the addition of FCCP, equal volumes of methanol were added to exclude possible artefacts caused by methanol. Whole-cell assays with the application of DCCD were conducted in a similar manner. DCCD was added to concentrations of 50 μM or 1 mM.

For sampling, 50 μl aliquots were withdrawn at defined time points with a microliter syringe (Hamilton, Bonaduz, Switzerland), mixed with 50 μl of a 70% (vol/vol) acetonitrile solution in water, and acidified by adding 1 μl H_2SO_4 (1 M). The samples were centrifuged at 16,000*g* for 5 min (Eppendorf Centrifuge 5415 R, Eppendorf, Hamburg, Germany) and filtered through 0.2 μm nylon filters (WICOM, Heppenheim, Germany) in order to remove the cells. The concentrations of naphthalene, 2-Npht, and H_4-2-Npht were determined on an UPLC system.

To investigate the effects of FCCP and DCCD on the growth of strain NaphS3, experiments were carried out in 20 ml cultivation tubes containing 15 ml ASW medium, 3 ml of a fully grown culture as an inoculation, and 0.6 ml HMN containing naphthalene (20 mg ml^{-1}). FCCP (at the concentrations of 20, 50 and 200 μM) or DCCD (at the concentration of 1 mM) was injected into the cultures at the start or during the incubation period (i.e., 25 days). To exclude the influence of methanol, equal volumes of pure methanol were also injected in control incubations without FCCP and DCCD. Sulfate reduction was monitored by measuring the formed sulfide.

Chemical analyses

Naphthalene, 2-naphthoate, and [5,6,7,8]-tetrahydro-2-naphthoate were analysed on an UPLC system (Acquity H-Class, Waters) equipped with an Acquity UPLC BEH shield reverse phase C_{18} column (1.7 μm Ethylene Bridged Hybrid [BEH] particle; 2.1 mm inner diameter × 50 mm length) and a photo diode array (PDA) detector. The oven temperature was maintained at 30°C and a gradient of 10% to 70% (vol/vol) acetonitrile containing 1 mM H_3PO_4 was delivered at a flow rate of 0.6 ml min^{-1} for 5 min. Naphthalene was detected at 220 nm, while 2-naphthoate and tetrahydro-2-naphthoate were detected at 235 nm. Identification was done based on co-elution with authentic standards, and quantification was based on external calibration curves acquired under the same conditions.

Organic acids in samples were analysed on a high-performance liquid chromatography (HPLC, Sykam, Germany) system equipped with an anion exchange column (Aminex HPX-87H; 300 × 7.8 mm, Bio-Rad) and a UV–VIS detector (Sapphire). The column temperature was set at 60°C, and an eluent of 5 mM H_2SO_4 was delivered isocratically at a rate of 0.6 ml min^{-1}. All standard fatty acids, including succinate, lactate, formate, acetate, propionate and butyrate, were well separated and detected at 210 nm. The concentrations were calculated based on standard calibration curves acquired under the same conditions.

Radiolabeled metabolites (i.e. ^{14}C-2-Npht) formed from incubations with ^{14}C-naphthalene or unlabeled naphthalene with ^{14}C-sodium bicarbonate were identified by co-elution with authentic standards in a HPLC system (Sykam, Germany) equipped with an UV–VIS detector (Linear 206 PHD, USA) and a consecutively connected online radioflow detector (Berthold LB509, Germany).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Naphthalene was rapidly degraded (within about 12 h of incubation) in 80-fold concentrated cell suspensions of strain NaphS in the presence of sulfate.

Fig. S2. Formation of ^{14}C-labelled 2-naphthoate in cell suspensions of strain NaphS incubated with unlabeled 2-naphthoate and $H^{14}CO_3^-$.

Fig. S3. Additions of FCCP or DCCD effectively inhibited sulfate reduction to sulfide in incubations of strain NaphS with naphthalene as a growth substrate.

Appendix S1. Thermodynamic calculations, including thermodynamic equilibrium constant and energetics of hypothetical naphthalene dismutation reactions.

Table S1. Free energies of formation used, and calculation of free energies of formation based on the group contribution approach.

Table S2. Coenzyme A transferases in the genome of strain NaphS2.