AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

BIODEGRADATION

The 5,6,7,8-Tetrahydro-2-Naphthoyl-Coenzyme A Reductase Reaction in the Anaerobic Degradation of Naphthalene and Identification of Downstream Metabolites

Philip Weyrauch,[a,b] Isabelle Heker,[a] Andrey V. Zaytsev,[c]* Christian A. von Hagen,[a] Meike E. Arnold,[a] Bernard T. Golding,[c] Rainer U. Meckenstock[a]

[a]Biofilm Centre, University of Duisburg-Essen, Essen, Germany
[b]Institute of Groundwater Ecology, Helmholtz Zentrum München, Neuherberg, Germany
[c]School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom

ABSTRACT Anaerobic degradation of polycyclic aromatic hydrocarbons has been investigated mostly with naphthalene as a model compound. Naphthalene degradation by sulfate-reducing bacteria proceeds via carboxylation to 2-naphthoic acid, formation of a coenzyme A thioester, and subsequent reduction to 5,6,7,8-tetrahydro-2-naphthoyl-coenzyme A (THNCoA), which is further reduced to hexahydro-2-naphthoyl-CoA (HHNCoA) by tetrahydronaphthoyl-CoA reductase (THNCoA reductase), an enzyme similar to class I benzoyl-CoA reductases. When analyzing THNCoA reductase assays with crude cell extracts and NADH as electron donor via liquid chromatography-mass spectrometry (LC-MS), scanning for putative metabolites, we found that small amounts of the product of an HHNCoA hydratase were formed in the assays, but the downstream conversion by an NAD^+-dependent β-hydroxyacyl-CoA dehydrogenase was prevented by the excess of NADH in those assays. Experiments with alternative electron donors indicated that 2-oxoglutarate can serve as an indirect electron donor for the THNCoA-reducing system via a 2-oxoglutarate:ferredoxin oxidoreductase. With 2-oxoglutarate as electron donor, THNCoA was completely converted and further metabolites resulting from subsequent β-oxidation-like reactions and hydrolytic ring cleavage were detected. These metabolites indicate a downstream pathway with water addition to HHNCoA and ring fission via a hydrolase acting on a β'-hydroxy-β-oxo-decahydro-2-naphthoyl-CoA intermediate. Formation of the downstream intermediate *cis*-2-carboxycyclohexylacetyl-CoA, which is the substrate for the previously described lower degradation pathway leading to the central metabolism, completes the anaerobic degradation pathway of naphthalene.

IMPORTANCE Anaerobic degradation of polycyclic aromatic hydrocarbons is poorly investigated despite its significance in anoxic sediments. Using alternative electron donors for the 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase reaction, we observed intermediary metabolites of anaerobic naphthalene degradation via *in vitro* enzyme assays with cell extracts of anaerobic naphthalene degraders. The identified metabolites provide evidence that ring reduction terminates at the stage of hexahydro-2-naphthoyl-CoA and a sequence of β-oxidation-like degradation reactions starts with a hydratase acting on this intermediate. The final product of this reaction sequence was identified as *cis*-2-carboxycyclohexylacetyl-CoA, a compound for which a further downstream degradation pathway has recently been published (P. Weyrauch, A. V. Zaytsev, S. Stephan, L. Kocks, et al., Environ Microbiol 19:2819–2830, 2017, https://doi.org/10.1111/1462-2920.13806). Our study reveals the first ring-cleaving reaction in the anaerobic naphthalene degradation pathway. It closes the gap between the reduction of the first ring of 2-naphthoyl-CoA by 2-napthoyl-CoA reductase and the lower degradation pathway starting from *cis*-2-carboxycyclohexylacetyl-CoA, where the second ring cleavage takes place.

Citation Weyrauch P, Heker I, Zaytsev AV, von Hagen CA, Arnold ME, Golding BT, Meckenstock RU. 2020. The 5,6,7,8-tetrahydro-2-naphthoyl-coenzyme A reductase reaction in the anaerobic degradation of naphthalene and identification of downstream metabolites. Appl Environ Microbiol 86:e00996-20. https://doi.org/10.1128/AEM.00996-20.

Editor Rebecca E. Parales, University of California, Davis

Address correspondence to Rainer U. Meckenstock, rainer.meckenstock@uni-due.de.

* Present address: Andrey V. Zaytsev, Health and Life Sciences, Northumbria University, Newcastle upon Tyne, UK.

Received 30 April 2020
Accepted 15 May 2020
Accepted manuscript posted online 22 May 2020
Published 20 July 2020

KEYWORDS THNCoA reductase, anaerobic catabolic pathways, naphthalene, polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) can derive from mineral oil products or incomplete combustion processes and are omnipresent in nature (2). Aerobic degradation of PAHs involving ring-opening dioxygenases is well described (3, 4), whereas the extremely slow, anaerobic degradation of PAHs is less well understood, and even the degradation pathway of the simplest PAH, naphthalene, is still not fully elucidated (5, 6). Anaerobic bacteria cannot rely on oxygen (O_2) as reactive cosubstrate and, after the initial activation reaction, channel aromatic substrates to central intermediates such as benzoyl-CoA (7) for monocyclic aromatic compounds, such as toluene, and 2-naphthoyl-CoA for naphthalene and 2-methylnaphthalene (6, 8). In contrast to aerobic pathways, the resonance energy of the aromatic ring system is overcome by reduction via dearomatizing aryl-CoA reductases (9–11). Three different strategies for aromatic ring reduction are known to date.

(i) Class I benzoyl-CoA reductases consist of four different subunits and their catalytic activities are ATP-dependent and oxygen sensitive (12). They are usually found in facultative anaerobic bacteria like *Thauera aromatica* (formerly known as *Pseudomonas* strain K172) (12–14), *Azoarcus* strain CIB (15), or *Rhodopseudomonas palustris* (16). All class I benzoyl-CoA reductases hydrolyze one ATP to ADP + P_i per electron transferred and use ferredoxin as the natural electron donor (17). The reduced ferredoxins delivering the required low-potential electrons are generated by electron-donor: ferredoxin oxidoreductases and genes coding for an oxidoreductase and a ferredoxin are typically found within the gene clusters containing the benzoyl-CoA reductase genes (15, 16, 18).

(ii) Class II benzoyl-CoA reductases are also sensitive toward oxygen and, in contrast to the class I reductases, are ATP-independent W-enzymes (19). The vast majority of strict anaerobes that can utilize aromatic compounds employ a class II reductase (20). These enzymes were, for example, studied in the sulfate reducer *Desulfococcus multivorans* (21), the iron reducer *Geobacter metallireducens* (22, 23), and the fermenting bacterium *Syntrophus aciditrophicus* (24). Class II reductases are huge enzyme complexes (ca. 1 MDa) that are proposed to have a modular composition of eight different subunits (19). ATP-independent reduction of the aromatic system is most likely achieved via an electron bifurcation mechanism (25) in which one low-potential electron from ferredoxin is transferred to the substrate and a second is transferred from ferredoxin to the high-potential electron acceptor $NAD(P)^+$, making the overall process exergonic (19, 26).

(iii) A third type of aryl-CoA reductase was recently discovered in sulfate-reducing naphthalene degraders and is only known for anaerobic PAH degradation (27). These class III reductases belong to the "old yellow enzyme" (OYE) family and are ATP-independent and insensitive toward oxygen. In the initial steps of the anaerobic degradation pathway of naphthalene (Fig. 1), two distinct OYE-like enzymes catalyze the reduction of 2-naphthoyl-CoA to 5,6-dihydro-2-naphthoyl-CoA and the subsequent reduction of the latter to 5,6,7,8-tetrahydro-2-naphthoyl-CoA (28).

The subsequent reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) in this pathway is probably catalyzed by enzymes similar to the class I benzoyl-CoA reductases of the *Azoarcus*-type that were identified in the anaerobic naphthalene-degrading bacteria deltaproteobacterium strain NaphS2 and *Desulfobacterium* strain N47 (29, 30). In cell extracts of enrichment culture N47, THNCoA was reduced to a hexahydro-2-naphthoyl-CoA with an as-yet-unknown positioning of the diene moiety (31). This reaction was dependent on ATP and an electron donor, preferably NADH. However, a putative oxidoreductase as well as a ferredoxin are encoded directly downstream of the genes coding for the proposed THNCoA reductase subunits in the genome of strains NaphS2 and N47 (29, 30). These enzymes might be involved in NAD(P)H-dependent

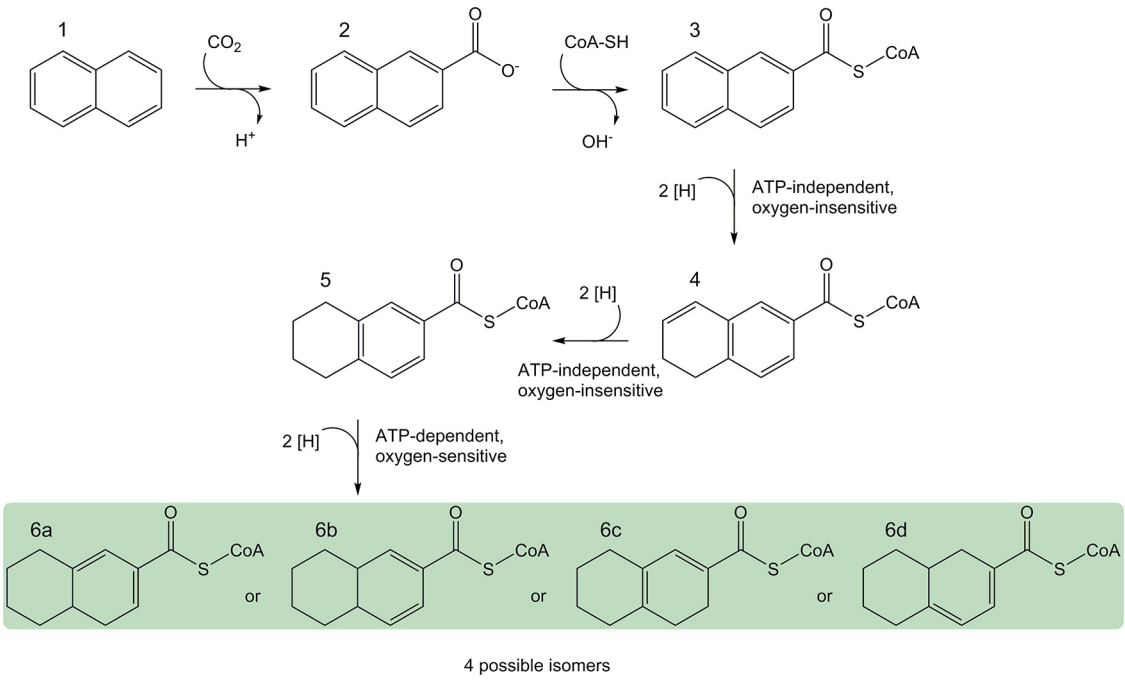

FIG 1 First steps of the anaerobic naphthalene degradation pathway. Naphthalene (1) is initially carboxylated to 2-naphthoate (2) followed by the formation of 2-naphthoyl-CoA (3). The latter is stepwise reduced to 5,6-dihydro-2-naphthoyl-CoA (4) and 5,6,7,8-tetrahydro-2-naphthoyl-CoA (5) via ATP-independent and oxygen-insensitive class III aryl-CoA reductases. The subsequent reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA via an ATP-dependent and oxygen-sensitive class II aryl-CoA reductase yields a hexahydro-2-naphthoyl-CoA (6) with the diene moiety being one of the possibilities shown.

electron transfer to THNCoA via ferredoxin in analogy to the electron-generating systems of benzoyl-CoA reduction described above.

It remains an open question whether the hexahydro-2-naphthoyl-CoA (HHNCoA) observed in THNCoA reductase assays (31) is the final product of the reductase reaction, or whether the reduction proceeds to octahydro-2-naphthoyl-CoA (OHNCoA). The latter would be analogous to cyclohex-1-ene-1-carboxyl-CoA, which is known as intermediate of the benzoyl-CoA pathway in *R. palustris* (16). The similarity of the identified THNCoA reductase to benzoyl-CoA reductases of the *Azoarcus*-type might be an indication for HHNCoA as final product of the reductase reaction, because known reductases of this type most likely transfer two electrons to their substrate (15, 32). However, the number of electrons transferred by the reductase cannot be unequivocally deduced from sequence homologies alone because the two electrons transferring benzoyl-CoA reductase from *T. aromatica* (33) and the four electrons transferring reductase from *R. palustris* (7) are very similar at the protein sequence level (32).

Given the uncertainties described above, the objective of the present work was to identify the final product of THNCoA reduction in anaerobic naphthalene catabolism. Furthermore, we set out to identify subsequent metabolic intermediates of the pathway in order to elucidate the underlying enzyme reactions. Unlike the aerobic degradation pathways, metabolic pathways of anaerobic degradation of mono- and polycyclic aromatic compounds do not converge at the same central intermediate. The downstream degradation pathway of naphthalene, which can serve as an exemplar for polycyclic aromatic hydrocarbons, does not proceed via benzoyl-CoA, but via metabolites with a cyclohexane skeleton (8). Our research aimed to elucidate the pathway further and to close the gap between the THNCoA reductase and the recently described downstream pathway starting from 2-carboxycyclohexylacetyl-CoA (1).

(This research was conducted by Philip Weyrauch in partial fulfillment of the requirements for a doctoral degree from the University of Duisburg-Essen [34].)

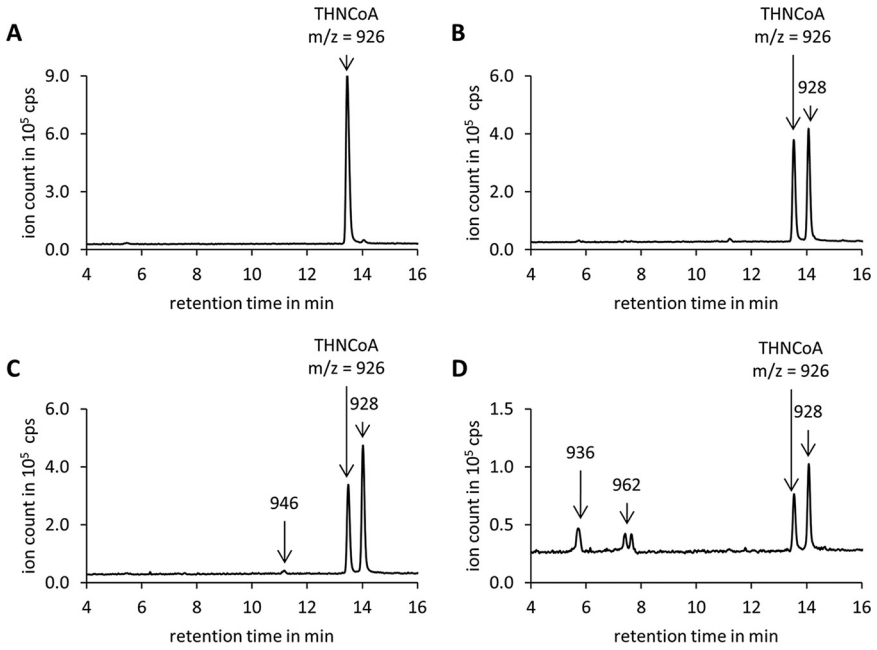

FIG 2 LC-MS chromatograms of reductase assays with cell extracts of culture N47 and 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) as the substrate. Standard assays contained 50 μM THNCoA, 5 mM ATP, and 5 mM NADH. (A) 0 min incubation time. (B) 90 min incubation time. In a second experiment, 5 mM NAD^+ was added to the standard assay after a preincubation time of 30 min. (C) Assay directly after addition of NAD^+ (30 min). (D) 60 min after addition of NAD^+. Note different *y*-axis scales in panels C and D. LC-MS chromatograms obtained in single-ion mode show accumulated ion counts of expected metabolites.

RESULTS

THNCoA reductase reaction in cell extract of culture N47. In order to further study the THNCoA reductase, we measured the reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) to hexahydro-2-naphthoyl-CoA (HHNCoA) with NADH as electron donor as reported previously (31) (Fig. 2A and B).

When NAD^+ was added to the assay mixture after a preincubation time of 30 min, further conversion of the previously formed HHNCoA could be detected (Fig. 2C and D). Already after 30 min, more than 50% of the initially added THNCoA ($m/z = 926$, retention time 13.6 min) was converted to HHNCoA ($m/z = 928$, peak at retention time 14.1 min) (Fig. 2C). The HHNCoA/THNCoA ratio slightly increased during further incubation and a new peak with $m/z = 946$ (retention time 11.3 min) appeared (Fig. 2B). The mass of $m/z = 946$ indicates that this metabolite could either represent the product of a hexahydro-2-naphthoyl-CoA hydratase, β-hydroxyoctahydro-2-naphthoyl-CoA (compound 7, see Fig. 7), or a metabolite resulting from water addition to octahydro-2-naphthoyl-CoA and a subsequent β-hydroxyacyl-CoA dehydrogenase reaction, namely, β-oxodecahydro-2-naphthoyl-CoA. After further incubation with NAD^+, the metabolite with $m/z = 946$ was completely converted and two new peaks arose ($m/z = 962$, Fig. 2D). NAD^+ therefore seems to be an essential cosubstrate for an enzyme, most likely a β-hydroxyacyl-CoA dehydrogenase, involved in the conversion steps downstream of the THNCoA reductase. Since the metabolite with $m/z = 946$ only accumulated in the absence of NAD^+ (Fig. 2B), it is presumably the substrate for a β-hydroxyacyl-CoA dehydrogenase rather than the product of this enzyme reaction, which points to β-hydroxyoctahydro-2-naphthoyl-CoA (compound 7, see Fig. 7). This also indicates that the β-oxidation-like downstream pathway starts with a hydratase reaction on hexahydro-2-naphthoyl-CoA (HHNCoA pathway).

Among the downstream metabolites formed after addition of NAD^+ were two compounds each with $m/z = 962$ (the pair of peaks in the LC-MS chromatogram, Fig. 2D). This mass-to-charge ratio corresponds to the substrate of a ring-opening hydrolase in an HHNCoA pathway, i.e., β'-hydroxy-β-oxodecahydro-2-naphthoyl-

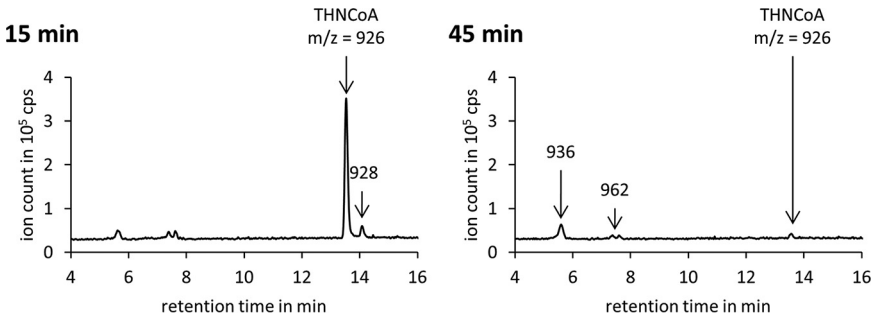

FIG 3 LC-MS chromatograms of a 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) reductase assay with cell extract of culture N47. The assay contained 50 μM THNCoA, 5 mM ATP, and 5 mM 2-oxoglutarate as electron donor. Samples were analyzed via LC-MS in single-ion mode scanning for expected metabolites. Chromatograms show accumulated ion counts for those metabolites.

CoA (compound 9, see Fig. 7), present as two diastereoisomers (in this case and for other chiral compounds, the configuration at a stereogenic center is not defined). Alternatively, this product pair could be a CoA thioester of 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid or 3-(2-[carboxymethyl]cyclohexyl)acrylic acid (compounds A1 and A2, see Fig. 8) derived by the action of a hydrolase in a β-oxidation-like downstream pathway starting from octahydro-2-naphthoyl-CoA (OHNCoA pathway). The other peak (retention time 5.8 min, $m/z = 936$) arising from NAD^+ action was identified as a CoA thioester of *cis*-2-(carboxymethyl)cyclohexanecarboxylic acid (compound 12, see Fig. 7) by comparison with a chemically synthesized reference. This compound was previously identified as an intermediate in anaerobic naphthalene degradation and a downstream degradation pathway starting from this metabolite was recently described (1, 8).

In reductase assays with culture N47 cell extracts that contained only ATP and NADH as cosubstrates, the reduction of THNCoA to HHNCoA typically stopped at a conversion of ca. 60%, and the metabolite with $m/z = 946$ was the only downstream metabolite that could be detected. Subsequent experiments with an increased concentration of THNCoA indicated that the reduction of the latter was not limited to a certain percentage of conversion but to a fixed amount of THNCoA that could be reduced under the given assay conditions, so most likely a cosubstrate necessary for the reduction was depleted. However, when 2-oxoglutarate was added as electron donor instead of NADH, a complete conversion of THNCoA within 45 min was observed (Fig. 3).

HHNCoA was only transiently detected and was obviously further converted to products giving rise to peaks with $m/z = 962$ and $m/z = 936$, which were also observed when NAD^+ was added to reductase assays with NADH as electron donor after a preincubation time of 30 min (see above). These observations indicate that 2-oxoglutarate can indirectly serve as electron donor for the THNCoA reductase, most likely because reduced ferredoxin can be regenerated via a 2-oxoglutarate:ferredoxin oxidoreductase comparable to the ferredoxin-reducing system reported previously for *T. aromatica* (35). Surprisingly, the reductase reaction with 2-oxoglutarate as electron donor did not require the addition of CoA-SH, as would be expected for such a system (a 2-oxoglutarate:ferredoxin oxidoreductase forms succinyl-CoA and CO_2 from 2-oxoglutarate and CoA-SH), and supplementation with free CoA-SH did not affect the product pattern observed in those assays. It appears that culture N47 cell extract either contains high levels of free CoA-SH or can regenerate CoA-SH from acetyl-CoA, succinyl-CoA, or via nonspecific thioesterases hydrolyzing acyl-CoA compounds in the assay mixture. This is in accordance with results for 2-naphthoate:CoA ligase assays conducted in our group, where up to 100 μM 2-naphthoyl-CoA was produced from 2-naphthoate in culture N47 cell extract supplemented with ATP, but without external addition of CoA-SH (unpublished results). Only when the THNCoA reductase assays

TABLE 1 Oxidoreductase rates observed in cell extracts of culture N47 for different combinations of electron donors and acceptors[a]

Electron donor	Electron acceptor	Oxidoreductase rate in μM min^{-1}
NADH	Methyl viologen	ND
2-oxoglutarate	NAD^+	ND
2-oxoglutarate	Methyl viologen	0.22
2-oxoglutarate and methyl-viologen as mediator	NAD^+	1.85

[a]ND, not detected.

were performed with cell extracts of strain NaphS2 or with culture N47 extracts that were harvested during a later growth phase did the THNCoA reductase reaction with 2-oxoglutarate as electron donor indeed require coenzyme A as an additional cosubstrate, confirming the proposed oxidoreductase reaction.

Oxidoreductase assays with different electron donors and acceptors. As demonstrated above, different compounds can serve as electron donor for the THNCoA reductase reaction in cell extracts. Assuming that the reductase itself should be specific for one kind of electron donor, the observed versatility might be a result of oxidoreductase activities in the extracts which can mediate electron transfer between different combinations of electron donors and acceptors. Therefore, spectrophotometric oxidoreductase assays were performed in order to elucidate electron transfer capabilities of the extracts (Table 1).

No electron transfer could be observed from NADH to methyl viologen, which served as a proxy for ferredoxin, or from 2-oxoglutarate to NAD^+. In contrast, a 2-oxoglutarate-dependent reduction of methyl viologen occurred at a rate of 0.22 μM min^{-1}, proving the activity of a 2-oxoglutarate:ferredoxin oxidoreductase. An even higher oxidoreductase rate of 1.85 μM min^{-1} was observed when 2-oxoglutarate, methyl viologen, and NAD^+ were combined so methyl viologen would serve as a mediator for the indirect electron transfer from 2-oxoglutarate to NAD^+ via 2-oxoglutarate:ferredoxin and a presumed ferredoxin: NAD^+ oxidoreductase, resulting in the production of NADH.

THNCoA reductase assays with various NADH and NAD^+ concentrations. The results from THNCoA reductase assays described above indicated that levels of NADH and NAD^+ need to be balanced to allow for both the NADH-dependent THNCoA reductase reaction and a NAD^+-dependent β-hydroxyacyl-CoA dehydrogenase reaction to take place. We therefore conducted further THNCoA reductase assays supplemented with different ratios of NADH and NAD^+ (Fig. 4).

In assays with 5 mM concentrations of both NADH and NAD^+ (Fig. 4A), some downstream metabolites were produced, but conversion of THNCoA was incomplete and HHNCoA accumulated. Less THNCoA remained unconverted and more HHNCoA accumulated in assays with 5 mM NADH and 0.2 mM NAD^+ (Fig. 4B). Furthermore, no downstream metabolites were detected under those conditions, indicating that this NAD^+/NADH ratio did not allow for any downstream reaction to occur and, as a consequence, accumulation of HHNCoA inhibited further conversion of THNCoA. In contrast, an almost complete conversion of 50 μM THNCoA and production of the downstream metabolites could be achieved with 0.2 mM NADH and 5 mM NAD^+ (Fig. 4C). The lower NADH concentration was apparently sufficient for THNCoA reduction and the elevated NAD^+/NADH ratio enabled further conversion of HHNCoA.

THNCoA reductase rates with different electron donors. After observing that compounds other than NADH can also be used as electron donors for THNCoA reduction in cell extracts of anaerobic naphthalene degraders, the conversion rates of THNCoA in standard THNCoA reductase assays were determined as a function of the electron donor (Fig. 5).

Within the first 30 min of the reaction, about 40 μM THNCoA was converted for the three electron donors tested: NADH, 2-oxoglutarate, and sodium citrate. However, with

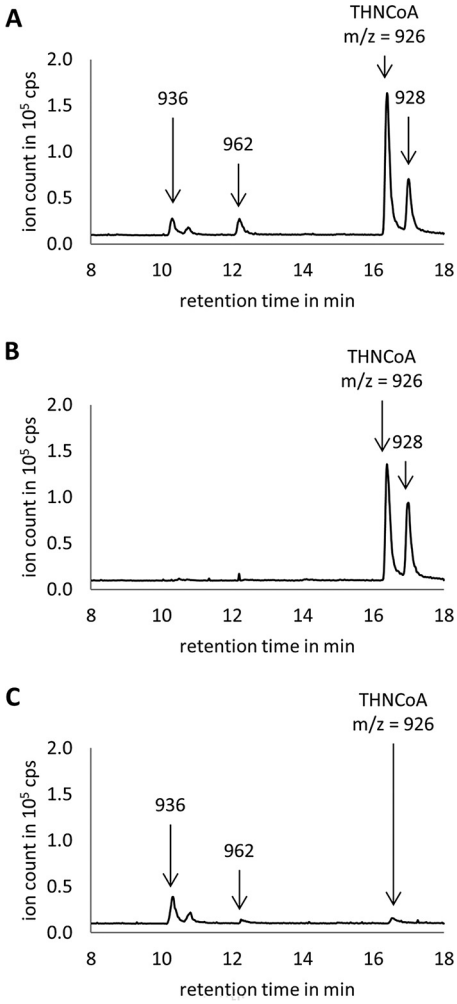

FIG 4 LC-MS chromatograms (accumulated ion counts of single-ion mode scans) of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) reductase assays in cell extract of culture N47. The assays contained 50 μM THNCoA, 5 mM ATP, and different ratios of NADH and NAD^+ as follows: 5 mM NADH and 5 mM NAD^+ (A); 5 mM NADH and 0.2 mM NAD^+ (B); and 0.2 mM NADH and 5 mM NAD^+ (C). Samples were taken after 90 min and analyzed via LC-MS in single-ion mode scanning for expected metabolites.

NADH as electron donor, the consumption of THNCoA was accompanied by an accumulation of HHNCoA, whereas in assays with 2-oxoglutarate or citrate no accumulation of HHNCoA was observed. The HHNCoA concentration in assays with NADH reached a maximum of ca. 25 μM after 45 to 60 min and remained almost constant during further incubation. Coinciding with the accumulation of HHNCoA, the conversion rate of THNCoA decreased after 45 min of incubation and less than 60 μM THNCoA was consumed after 120 min. In contrast, with 2-oxoglutarate or citrate as electron donor, no decrease of the THNCoA conversion rate after 45 min was observed and a total of 80 μM THNCoA could be converted during the 120 min incubation time without any accumulation of HHNCoA.

THNCoA reductase reaction in cell extract of strain NaphS2. In contrast to cell extract of culture N47, which showed a clear preference for NADH over NADPH as electron donor for the THNCoA reducing system, strain NaphS2 extract produced almost the same THNCoA conversion rates with either of the two cosubstrates. The maximum conversion rate was, however, only ca. 30%, which is significantly lower than the rate obtained with culture N47 extract. Again, a metabolite with $m/z = 946$ appeared at a retention time of 11.3 min in the LC-MS chromatograms, which had already

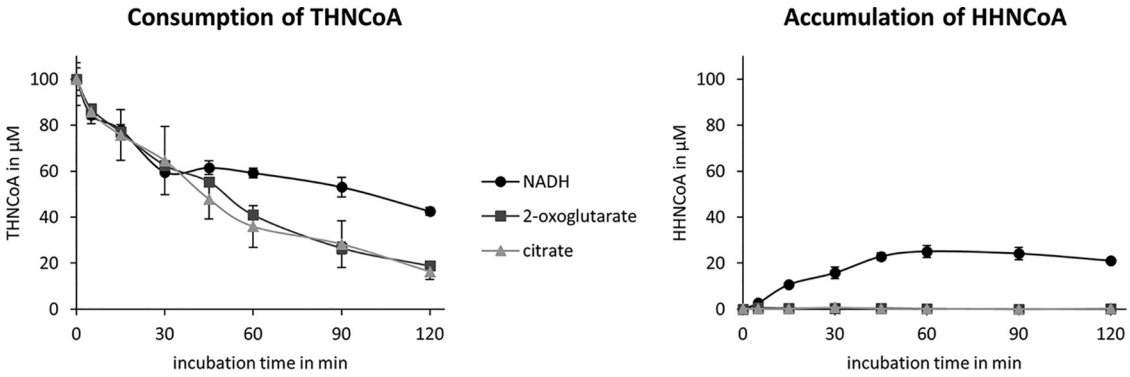

FIG 5 Rates of THNCoA consumption or HHNCoA accumulation in THNCoA reductase assays with cell extracts of culture N47 using NADH, 2-oxoglutarate, or citrate as electron donor (5 mM each). Error bars indicate standard deviations of triplicate incubations for each electron donor.

been detected in THNCoA reductase assays with culture N47 extract. Since the reductase reaction with NADPH was not inhibited by the presence of NAD^+, the two cosubstrates could be added simultaneously to the assay mixture at a concentration of 5 mM each. This led to a further conversion of the HHNCoA formed during THNCoA reduction and the production of metabolites with $m/z = 962$ and $m/z = 936$, which had the same retention times as the respective compounds formed in assays with culture N47 extract. Hence, the downstream pathways in strains N47 and NaphS2 most likely proceed via the same intermediates.

THNCoA reductase assays with other electron donors in strain NaphS2 cell extract gave similar results to the ones with culture N47 extract (data not shown). The best THNCoA conversion with strain NaphS2 extract was obtained when 2-oxoglutarate was added as electron donor, matching the results obtained with culture N47 extract, albeit with the difference that the 2-oxoglutarate-dependent reduction in strain NaphS2 cell extract indeed required CoA-SH as an additional cofactor, as expected for a 2-oxoglutarate:ferredoxin oxidoreductase. Without addition of CoA-SH, no significant conversion of THNCoA took place. Only if both 2-oxoglutarate (5 mM) and coenzyme A (1 mM) were added to the standard assay mixture was THNCoA converted to yield HHNCoA, as well as the metabolites with $m/z = 962$ and $m/z = 936$. A similar pattern was observed if the assays were additionally dosed with NAD^+. In this case no HHNCoA, but only residual THNCoA and the downstream metabolites, were detectable after 90 min of incubation. In contrast, addition of NADH to assays containing 2-oxoglutarate and CoA-SH caused an accumulation of HHNCoA and seemed to prevent further conversion of this intermediate. The two latter observations indicate that the further conversion of THNCoA depends on the oxidation of an intermediate by a β-hydroxyacyl-CoA dehydrogenase which requires NAD^+. In the presence of excess of NADH, the equilibrium of this reaction shifts toward the reduced substrate, which prevents further conversion and leads to the accumulation of upstream metabolites.

Identification of the downstream metabolites. In a previous study, a β-oxidation-like downstream pathway was proposed starting from octahydro-2-naphthoyl-CoA with a CoA thioester of 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid (compound A1) or 3-(2-[carboxymethyl]cyclohexyl)acrylic acid (compound A2) as intermediate (8). The expected mass-to-charge ratio of these coenzyme A thioesters is 962, which is identical to the pair of downstream metabolites that occurred in THNCoA reductase assays (see above). LC-MS chromatograms of samples from these assays were compared under two different pH conditions, with CoA thioesters of chemically synthesized reference compounds A1 and A2 (Fig. 6).

At pH 7.0, the unknown metabolite appeared as two close peaks with retention times of 7.4 and 7.7 min. CoA thioesters of compound A1 eluted significantly different, showing two peaks with retention times of 7.6 and 7.9 min, whereas the retention times

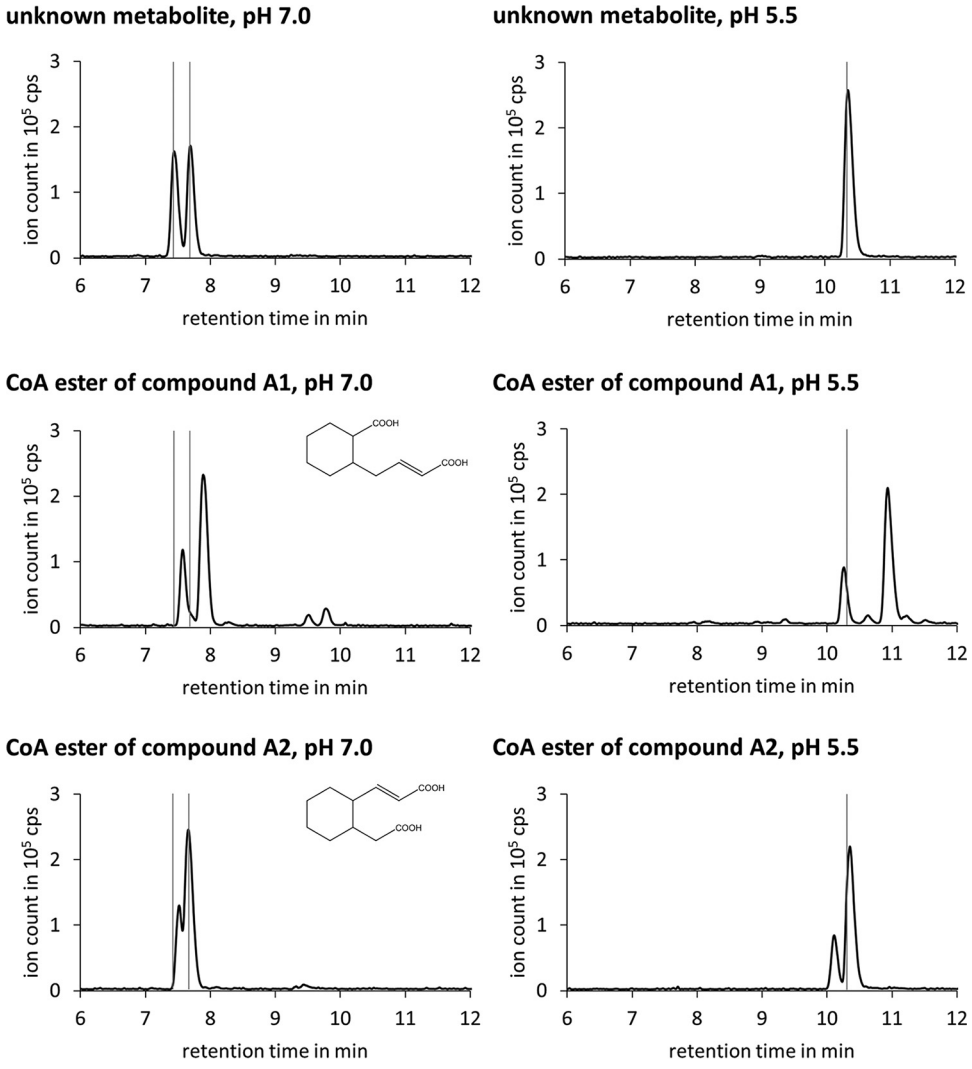

FIG 6 Comparison of the LC-MS chromatograms of the downstream metabolite with $m/z = 962$ observed in THNCoA reductase assays with 2-oxoglutarate (5 mM) as electron donor and chemically synthesized reference compounds (A1 and A2, both obtained as a mixture of *cis*-isomers with the CoA thioester formed on either carboxyl group). LC-MS analyses were conducted at pH 5.5 and pH 7.0 for each compound. Gray lines indicate the retention times of peaks of the unknown metabolite. The compounds with $m/z = 962$ were detected via the MS unit in single-ion mode.

of the two peaks of CoA thioesters of compound A2 (7.5 min and 7.7 min) were identical in one case and similar in the other to those observed for the unknown metabolite. In the case of the reference compounds, the two peaks represent isomers differing in the position of the CoA thioester on the dicarboxylic acid. At pH 5.5, the distance between the two isomer peaks of the reference compounds was increased (10.3 and 11.0 min for compound A1 and 10.1 and 10.4 min for compound A2). In contrast, the unknown metabolite appeared as a single peak at pH 5.5 (retention time 10.4 min). The elution patterns of the unknown metabolite and the reference compounds were confirmed by analyses of several independent samples and by spiking of biological samples with reference standards. Thus, we can tentatively conclude that neither compound A1 nor compound A2 CoA thioesters represent the naturally occurring metabolite with $m/z = 962$.

The CoA thioesters of the two reference compounds were also tested for conversion in cell extracts of N47 or NaphS2 cultures grown with naphthalene or 2-naphthoate, respectively. The naturally occurring metabolite should be converted by an enoyl-CoA hydratase, which is assumed not to require any cosubstrate but water. LC-MS analysis

of the assay mixtures revealed no conversion of either of the two compounds during incubation in cell extracts (data not shown), which indicates that the metabolite with $m/z = 962$ has a different structure from the two reference compounds, despite the similarities in elution patterns described above. Hence, it is unlikely that either octahydro-2-naphthoyl-CoA or the CoA thioesters of 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid or 3-(2-[carboxymethyl]cyclohexyl)acrylic acid are intermediates of the naphthalene degradation pathway. Although we propose (see Discussion section) that the unknown metabolite is a β-keto CoA thioester, the uncertainty in the precise identity of the metabolite pair giving rise to $m/z = 962$ requires further investigation. The observed pH-dependent chromatographic behavior of metabolite and reference compounds could be explained by the different ionization states of the CoA moiety, as well as, in the case of the unknown metabolite, of a pH-dependent shift in the equilibrium between the keto-enol pair of tautomers.

DISCUSSION

HHNCoA is the final product of the THNCoA reductase. The reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) to a hexahydro-2-naphthoyl-CoA (HHNCoA) was previously demonstrated with cell extracts of the N47 enrichment culture. The reaction was catalyzed by an enzyme similar to the class I benzoyl-CoA reductases and NADH was described as the best working electron donor (31). However, a complete conversion of THNCoA could not be achieved in these assays and no further metabolites downstream of HHNCoA were detected. THNCoA conversion stopped at approximately 50% conversion in conjunction with an accumulation of HHNCoA. The high excess of NADH in those assays presumably shifted the equilibrium of an NAD^+-dependent β-hydroxyacyl-CoA dehydrogenase involved in the downstream pathway backward, leading to an accumulation of the upstream metabolites. A better conversion of THNCoA was achieved by the further addition of NAD^+, the supposed dehydrogenase cosubstrate. We observed a novel downstream metabolite with $m/z = 946$, presumably β-hydroxyoctahydro-2-naphthoyl-CoA (compound 7, Fig. 7). Thus, we propose a downstream pathway involving a hydratase reaction on HHNCoA and a subsequent β-hydroxyacyl-CoA dehydrogenase, which acts analogously to cyclohexa-1,5-diene-1-carboxyl-CoA hydratase (36) and 6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase (37) from the benzoyl-CoA pathway in *T. aromatica*.

Despite the fact that downstream reactions were inhibited in THNCoA reductase assays with high concentrations of NADH, whereas complete conversion of THNCoA and production of downstream metabolites could be achieved in assays with 2-oxoglutarate or citrate, our data still suggest that NADH is the natural electron donor for THNCoA reductase. This is based on the following two observations. First, THNCoA reductase assays with reduced NADH levels and a high NAD^+/NADH ratio allowed for both THNCoA reduction as well as downstream reactions to take place (Fig. 4). Second, indirect electron transfer from 2-oxoglutarate to NAD^+ took place in oxidoreductase assays, but in turn NADH did not serve as electron donor for the reduction of methyl viologen, which was used as a proxy for ferredoxin (Table 1). Therefore, the observed NADH-dependent reduction of THNCoA should only be possible if NADH rather than ferredoxin was the natural electron donor for the reductase. Citrate or 2-oxoglutarate-dependent THNCoA reduction was most likely an artifact of the assay conditions, where 2-oxoglutarate served as indirect electron donor via oxidoreductase-mediated electron transfer, yielding NADH. *Desulfobacterium* strain N47 operates a modified citric acid cycle in which the ordinary 2-oxoglutarate dehydrogenase (38, 39) is replaced by a 2-oxoglutarate:ferredoxin oxidoreductase (29). A similar oxidoreductase was also identified in strain NaphS2 (30). Such enzymes can generate reduced ferredoxin upon conversion of 2-oxoglutarate and CoA-SH to succinyl-CoA and CO_2 (35). Another oxidoreductase would then use the reduced ferredoxin as electron donor for the reduction of NAD^+ to NADH (35). As a result, NADH can be continuously regenerated from the pool present in cell extracts without requirement for external addition of NADH. Notably, the observed rate of 1.85 μM would be sufficient to supply the NADH

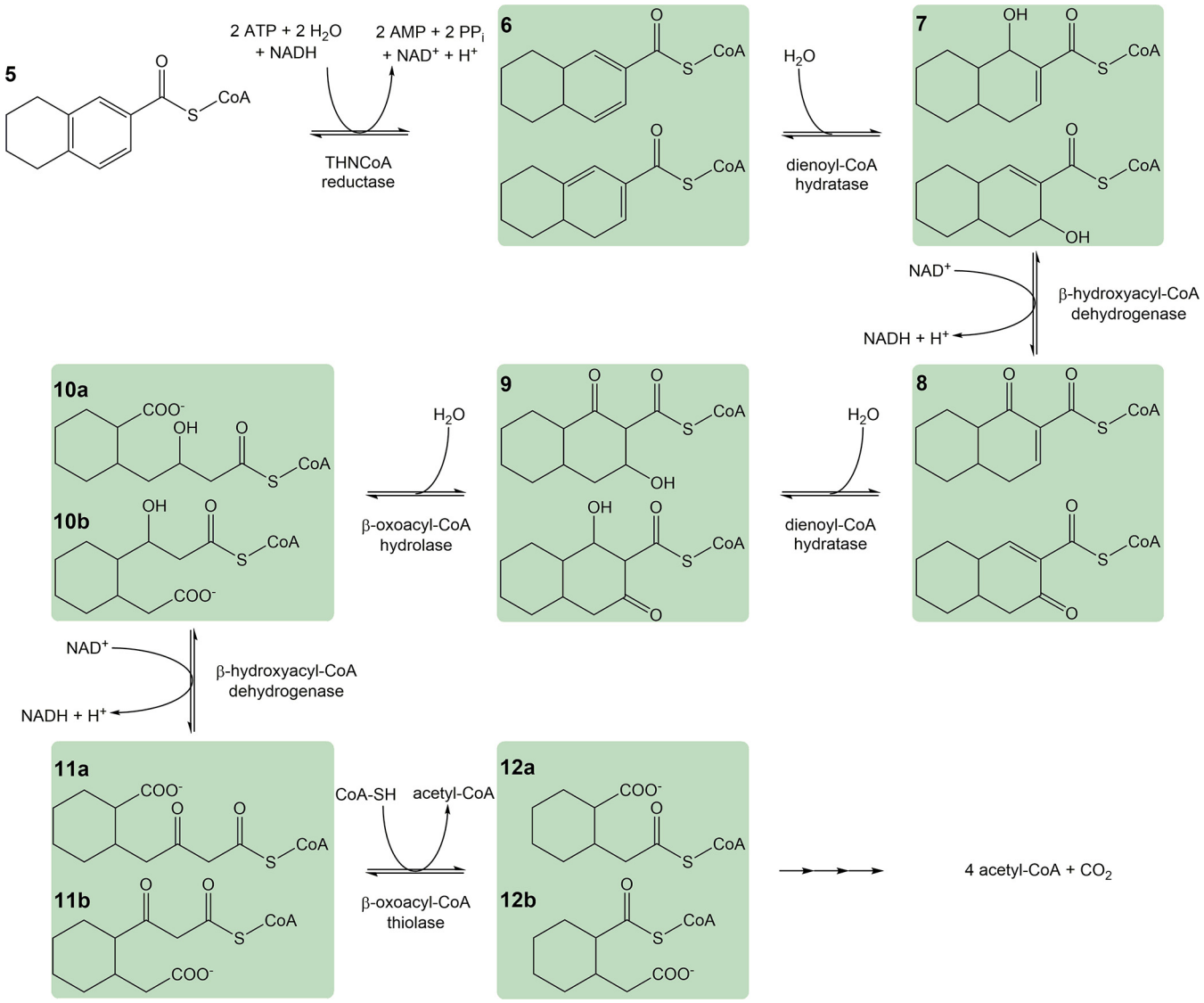

FIG 7 Proposed degradation pathway of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA, compound 5, $m/z = 926$) assuming the product of the THNCoA reductase to be a hexahydro-2-naphthoyl-CoA (HHNCoA, $m/z = 928$) with double bonds in α,β- or β',γ'-position (7). The subsequent β-oxidation-like pathway proceeds via β-hydroxyoctahydro-2-naphthoyl-CoA (8) ($m/z = 946$), β-oxooctahydro-2-naphthoyl-CoA (9) ($m/z = 944$), and β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA (10) ($m/z = 962$). Hydrolytic opening of ring I gives 4-(2-carboxycyclohexyl)-3-hydroxybutyryl-CoA (10a) or 3-(2-[carboxymethyl]cyclohexyl)-3-hydroxypropionyl-CoA (10b) (both $m/z = 980$). Another β-oxidation-like sequence then continues via 4-(2-carboxycyclohexyl)-3-oxobutyryl-CoA (11a) or 3-(2-[carboxymethyl]cyclohexyl)-3-oxopropionyl-CoA (11b) (both $m/z = 978$), and 2-(2-carboxycyclohexyl)acetyl-CoA (12a) or 2-(carboxymethyl)cyclohexane-1-carboxyl-CoA (12b) (both $m/z = 936$), from where a previously described downstream pathway starts.

required for the reduction of 100 μM THNCoA within 90 min. This continuous regeneration of NADH presumably resembles intracellular conditions much better than external addition of excess NADH, and therefore does not interfere with downstream reactions.

Earlier studies with the THNCoA reductase could not detect any metabolites downstream of HHNCoA, which was surprising because the comparable reactions of the benzoyl-CoA reductase and the subsequent cyclohexa-1,5-diene-1-carboxyl-CoA hydratase had previously been measured in one assay (33). In fact, a peak presumably representing the HHNCoA hydratase product was detected in our assays, albeit in very low concentrations, even with NADH as electron donor. Due to its very low concentration, this peak could only be detected via liquid chromatography-mass spectrometry (LC-MS) in single-ion mode and was overlooked in previous studies that used HPLC for analyzing the *in vitro* assays (31). Our results indicate that if the equilibrium of the

subsequent β-hydroxyacyl-CoA dehydrogenase is shifted due to high NADH concentrations, mainly HHNCoA rather than its hydratase product accumulated as upstream metabolite. Hence, the equilibrium of the hydratase reactions seems to be far on the side of the educt HHNCoA. Since the cosubstrate of a hydratase—water—is present in vast excess in aqueous assays, the equilibrium of such a reaction is usually expected to be in favor of the product. For the cyclohexa-1,5-diene-1-carboxyl-CoA hydratase, equilibrium concentrations of substrate and product were, however, reported to be almost equal (36), which makes the very low concentration of HHNCoA hydratase product observed in our assays therefore appear realistic. Assuming that water addition occurs in the β,γ′-position (i.e., 1,4- or 3,8a-) by analogy with cyclohexa-1,5-diene-1-carboxyl-CoA hydratases, the HHNCoA hydratase could be handicapped sterically by the already-reduced ring, depending on the nature of HHNCoA.

Identification of downstream metabolites. Further novel metabolites identified in our assays ($m/z = 962$ and $m/z = 936$) presumably represented the CoA thioesters of compounds that were identified earlier in culture extracts of the N47 enrichment culture (8). While $m/z = 936$ matches the mass of 2-(carboxymethyl) cyclohexane-1-carboxylic acid CoA thioester (compound 12 in Fig. 7), the metabolite with $m/z = 962$ is most likely not a CoA thioester of the earlier postulated 3-(2-[carboxymethyl]cyclohexyl)acrylic acid or 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid but rather a β′-hydroxy-β-oxodecahydro-2-naphthoyl-CoA (compound 9). This was deduced from the chromatographic behavior (Fig. 6) of this metabolite, which indicated a structure that (i) is different from the previously postulated structures and (ii) can undergo keto-enol tautomerism. In the benzoyl-CoA pathway, the analogous β′-hydroxy-β-oxo-intermediate is normally not detected as a free metabolite since the Oah enzyme, which acts on 6-oxocyclohex-1-ene-1-carboxyl-CoA in this pathway, has both enoyl-CoA hydratase and ring-hydrolyzing activity (37, 40). As described before, no Oah-like enzyme is encoded by the *thn*-operon that codes for enzymes of the downstream pathway of anaerobic naphthalene degradation (5). We assume that β-oxooctahydro-2-naphthoyl-CoA hydratase and β′-hydroxy-β-oxodecahydro-2-naphthoyl-CoA hydrolase are two separate enzymes in this pathway (Fig. 7), which is in accordance with the detection of β′-hydroxy-β-oxodecahydro-2-naphthoyl-CoA as a free metabolite.

In this pathway, up to five acetyl-CoA are formed via β-oxidation-like reactions and can be fed into a modified citric acid cycle or the Wood-Ljungdahl pathway, which are both expressed during growth with naphthalene (29).

Implications for the downstream pathway. Previous studies identified an intermediate of the β-oxidation-like downstream pathway in cell extracts that was interpreted as 3-(2-[carboxymethyl]cyclohexyl)acrylic acid or 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid according to its m/z in gas chromatography-mass spectrometry (GC-MS) analyses (8). Since the further downstream metabolite 2-(carboxy-methyl) cyclohexane-1-carboxylic acid was found to occur only as the *cis*-isomer (8) and β-oxidation-like reactions usually proceed via *E*-unsaturated CoA thioester intermediates (41), we tested the chemically synthesized CoA thioesters of the *cis*-(*E*)-isomers of the two compounds for conversion in cell extracts of culture N47 and strain NaphS2. However, none of the tested CoA thioesters was metabolized. Although it cannot be completely excluded that the pathway proceeds via the *trans*-isomers or that the double bond occurs in *Z*- rather than in *E*-configuration, the observed persistence of *cis*-(*E*)-3-(2-[carboxymethyl]cyclohexyl)acrylyl-CoA and *cis*-4-(2-carboxycyclohexyl)-crotonyl-CoA (i.e., CoA thioesters of 3-[2-[carboxymethyl]cyclohexyl]acrylic acid and 2-[3-carboxyallyl]cyclohexane-1-carboxylic acid) in cell extracts of sulfate-reducing naphthalene degraders casts doubt on the previous interpretation of the identified mass.

In the work referred to, metabolites were extracted as free acids (hydrolysis of CoA thioesters via NaOH treatment) from N47 cultures and the acids were derivatized with trimethylsulfonium hydroxide, which methylates carboxyl groups (42). High-resolution

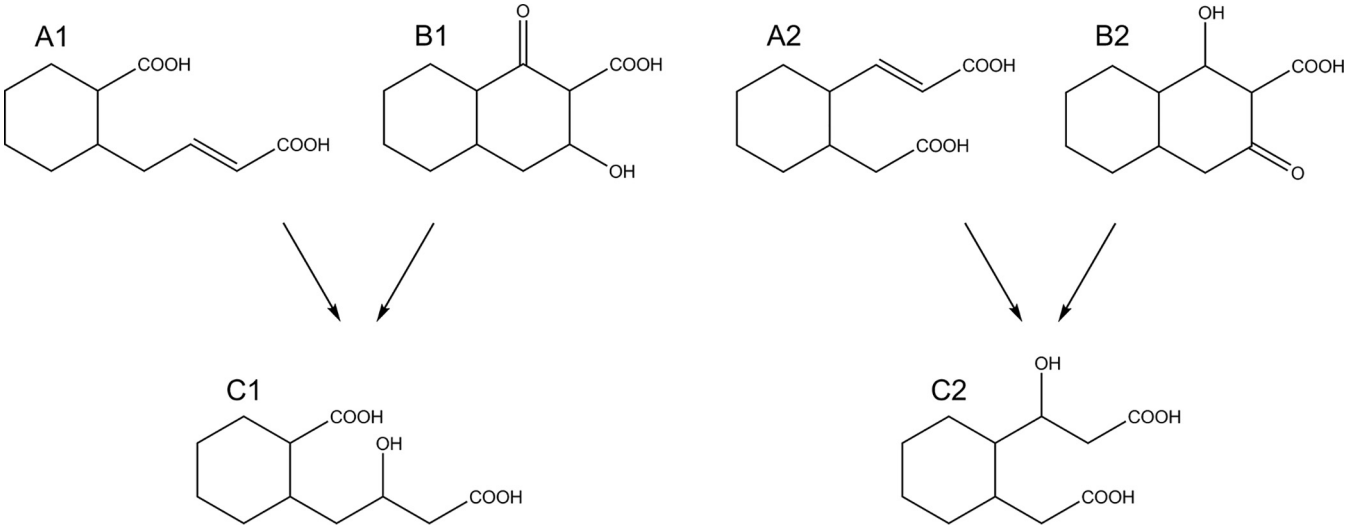

FIG 8 Potential intermediates (as free acids) of anaerobic naphthalene degradation with $m/z = 962$ (as CoA thioester) correlating with the previously observed elemental composition $C_{11}H_{16}O_4$ (A and B) and their downstream metabolites (C). A1: 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid; A2: 3-(2-[carboxymethyl]cyclohexyl)acrylic acid; B1: 3-hydroxy-1-oxodecahydro-2-naphthoic acid; B2: 1-hydroxy-3-oxodecahydro-2-naphthoic acid; C1: 4-(2-carboxycyclohexyl)-3-hydroxybutyric acid; C2: 3-(2-[carboxymethyl]cyclohexyl)-3-hydroxypropionic acid.

GC-MS could confirm the elemental composition of the respective metabolite as $C_{11}H_{16}O_4$ (nonmethylated form) but could not prove the presence of two methylated carboxyl groups. Thus, the observed metabolite could also represent β′-hydroxy-β-oxodecahydro-2-naphthoic acid, which exhibits the same elemental composition (Fig. 8).

Assuming hexahydro-2-naphthoyl-CoA as the final product of reductive dearomatization (31), β′-hydroxy-β-oxodecahydro-2-naphthoyl-CoA could result from a second water addition to the remaining double bond in β-oxooctahydro-2-naphthoyl-CoA prior to first ring cleavage (Fig. 7). This would represent a reaction scheme similar to the 6-oxocyclohex-1-enecarboxyl-CoA hydratase/hydrolase from anaerobic benzoate-degrading bacteria (40). A hydrolytic ring cleavage at β′-hydroxy-β-oxodecahydro-2-naphthoyl-CoA would result in the same downstream metabolite as a hydratase reaction at 3-(2-[carboxymethyl]cyclohexyl)-acrylyl-CoA or 4-(2-carboxycyclohexyl) crotonyl-CoA, respectively (Fig. 8). Regarding the apparent absence of an enoyl-CoA hydratase activity toward cyclohexane derivatives with unsaturated C_3- or C_4-side chains, we postulate that the previously proposed CoA thioester of 3-(2-[carboxymethyl]cyclohexyl)acrylic acid or 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid is not an intermediate of anaerobic naphthalene degradation. In fact, our data suggest that β′-hydroxy-β-oxodecahydro-2-naphthoyl-CoA (compound 9 in Fig. 7) is the true intermediate.

This is in perfect agreement with our analysis of downstream metabolites in THNCoA reductase assays (see above). The postulated pathway via HHNCoA and β′-hydroxy-β-oxodecahydro-2-naphthoyl-CoA (Fig. 7) is therefore supported by the results of two independent experimental approaches (metabolite analysis and negative conversion tests with previously postulated metabolites), indicating a β-oxidation-like downstream pathway starting from HHNCoA rather than from OHNCoA as proposed previously.

MATERIALS AND METHODS

Growth of bacterial cells. The enrichment culture N47 and deltaproteobacterium strain NaphS2 (DSM 14454) were grown anaerobically in artificial freshwater (N47) or seawater (NaphS2) medium with naphthalene (N47) or 2-naphthoate (NaphS2) as the sole source of carbon and electrons, as described previously (1). A freshly harvested 6-week-old (N47) or 4-week-old (NaphS2) 1.6 liter culture was used for each set of assays.

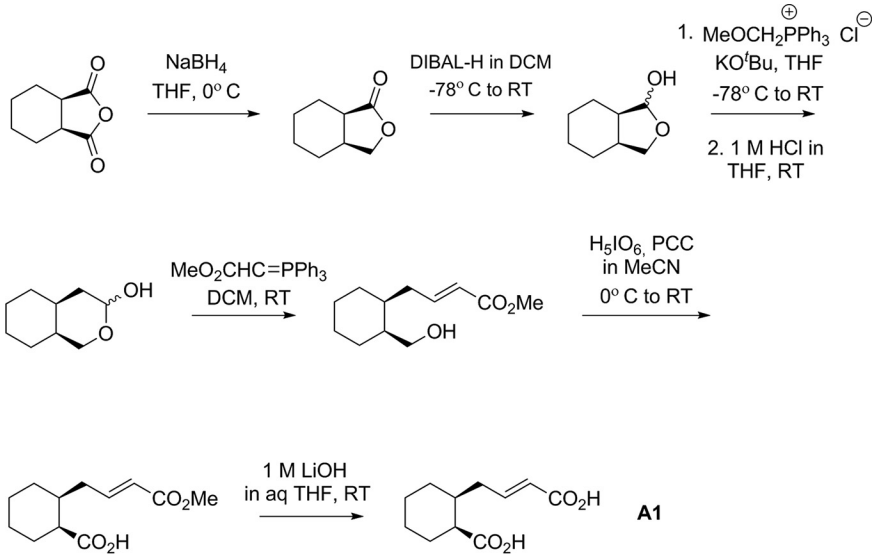

FIG 9 Synthesis of putative metabolite A1 with only one enantiomer shown. DIBAL-H, di-isobutylaluminum hydride; DCM, dichloromethane; THF, tetrahydrofuran; PCC, pyridinium chlorochromate.

Anaerobic preparation of cell extracts. Cultures were transferred into air-tight centrifuge beakers (4 × 400 ml) in an anaerobic LABstar Glove Box Workstation (M. Braun, Garching, Germany) with an N_2 atmosphere and cells were harvested by centrifugation for 30 min at 17,700 × *g* and 4°C. The supernatant was discarded, the cell pellets were washed once with enzyme test buffer (see below), and then suspended in the same buffer (1 ml buffer per 1 liter initial culture). The cells were opened by using a French press (Thermo Electron, Waltham, MA, USA) operated at 6.9 MPa. To obtain the cell extract, cell debris was afterwards segregated by centrifugation for 15 min at 20,800 × *g* and 4°C. Protein concentrations in cell extract were typically 5 to 10 mg/ml. To keep the extract anoxic, the following steps were conducted inside a glove box: resuspension and washing of cell pellets, loading of French press mini cell, and transfer of crude extract into air-tight screw cap microcentrifuge tubes after French press passage.

Synthesis of potential downstream metabolites. (i) Racemic (1*S*,2*S*/1*R*,2*R*)-2-([*E*]-3-carboxyallyl) cyclohexane-1-carboxylic acid (A1). Two consecutive reductions (43) of commercially available 1,2-*cis*-cyclohexanedicarboxylic anhydride with $NaBH_4$ followed by di-isobutylaluminum hydride yielded an intermediate hemiacetal (see Fig. 9). Wittig reaction of the hemiacetal with (methoxymethyl)triphenylphosphonium chloride (44) and subsequent acidic hydrolysis of an intermediate methyl enol ether led to the homologous hemiacetal, which was subjected to a Wittig reaction with methyl triphenylphosphoranylideneacetate. The resulting hydroxy-ester was oxidized to a carboxylic acid, which was saponified to give the desired dicarboxylic acid (A1) after acidification (Fig. 9).

(ii) Racemic (*E*)-3-([1*R*,2*R*/1*S*,2*S*]-2-[carboxymethyl]cyclohexyl)acrylic acid (A2). The hemiacetal (Fig. 10) was reacted with methyl triphenylphosphoranylideneacetate to give a hydroxy-ester, which was converted into the corresponding triflate for subsequent S_N2 reaction with cyanide to afford an intermediate nitrile. Acidic methanolysis of the nitrile followed by basic hydrolysis (1 M LiOH in THF/water) of the methyl ester group afforded the desired dicarboxylic acid (A2) (Fig. 10) after acidification.

Synthesis and purification of CoA thioesters. Compound 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) and the CoA thioesters of 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid and 3-(2-[carboxymethyl]cyclohexyl)acrylic acid were synthesized from the free acid via their succinimidyl esters and purified as described earlier (1). A Strata phenyl SPE column (Phenomenex, Aschaffenburg, Germany)

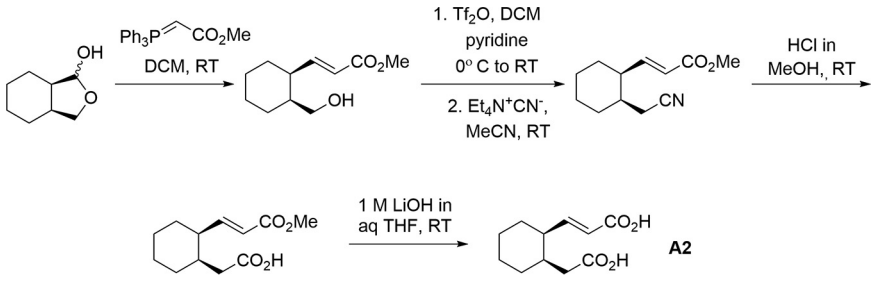

FIG 10 Synthesis of putative metabolite A2 with only one enantiomer shown. Tf, CF_3SO_2.

TABLE 2 Mass-to-charge ratios of metabolites expected in 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase assays[a]

m/z	Metabolite of putative HHNCoA pathway	Metabolite of putative OHNCoA pathway
926	5,6,7,8-tetrahydro-2-naphthoyl-CoA	5,6,7,8-tetrahydro-2-naphthoyl-CoA
928	Hexahydro-2-naphthoyl-CoA	Hexahydro-2-naphthoyl-CoA
930	NA	Octahydro-2-naphthoyl-CoA
946	β-hydroxyoctahydro-2-naphthoyl-CoA	β-oxodecahydro-2-naphthoyl-CoA
944	β-oxooctahydro-2-naphthoyl-CoA	NA
948	NA	β-hydroxydecahydro-2-naphthoyl-CoA
962	β′-hydroxy-β-oxodecahydro-2-naphthoyl-CoA	3-(2-(carboxymethyl)cyclohexyl)acrylyl-CoA or 4-(2-carboxycyclohexyl)crotonyl-CoA
980	3-(2-[carboxymethyl]cyclohexyl)-3-hydroxypropionyl-CoA or 4-(2-carboxycyclohexyl)-3-hydroxybutyryl-CoA	3-(2-[carboxymethyl]cyclohexyl)-3-hydroxypropionyl-CoA or 4-(2-carboxycyclohexyl)-3-hydroxybutyryl-CoA
978	3-(2-[carboxymethyl]cyclohexyl)-3-oxopropionyl-CoA or 4-(2-carboxycyclohexyl)-3-oxobutyryl-CoA	3-(2-[carboxymethyl]cyclohexyl)-3-oxopropionyl-CoA or 4-(2-carboxycyclohexyl)-3-oxobutyryl-CoA
936	2-(carboxymethyl)cyclohexanecarboxyl-CoA 2-(2-carboxycyclohexyl)-acetyl-CoA	2-(carboxymethyl)cyclohexanecarboxyl-CoA 2-(2-carboxycyclohexyl)-acetyl-CoA

[a]Depending on the final product of the reductase reaction, i.e., hexahydro-2-naphthoyl-CoA (HHNCoA) or octahydro-2-naphthoyl-CoA (OHNCoA), and on the position of the first ring-opening, i.e., between C-1 and C-2 or between C-2 and C-3, different downstream metabolites should emerge. NA, not applicable.

was used for purification of THNCoA and a HyperSep C_{18} SPE column (Thermo Fisher Scientific, Ulm, Germany) was used for the purification of the CoA thioesters of 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid and 3-(2-[carboxymethyl]cyclohexyl)acrylic acid. Identity of the synthesized CoA thioester compounds was verified by LC-MS analysis.

Discontinuous reductase assays. THNCoA reductase activity was measured under strictly anoxic conditions in 100 mM MOPS (morpholinepropanesulfonic acid)/KOH buffer, pH 7.3 with 15 mM $MgCl_2$ as described previously (31). The 200 μl reaction mixture typically contained 60 to 100 μl of anaerobic cell extract of enrichment culture N47 or deltaproteobacterium strain NaphS2, 5 mM ATP, 5 mM electron donor (NADH, NADPH, sodium citrate, or 2-oxoglutarate) and 50 μM THNCoA. Assays with 2-oxoglutarate as electron donor were conducted with and without the addition of 1 mM CoA-SH. Some assays additionally contained 0.2 to 5 mM electron acceptors like NAD^+ or $NADP^+$, or a combination of two electron donors (see the Results section for a detailed composition of the respective assays). The reactions were started by the addition of THNCoA and samples for HPLC analysis (see below) were taken at different time points as described elsewhere (1).

Spectrophotometric oxidoreductase assays. Cell extract-mediated electron transfer between different donors and acceptors was tested at 30°C under anoxic conditions in air-tight glass cuvettes using a Specord 200 photometer (Analytik Jena, Jena, Germany) according to published procedures (35, 45). The 1 ml reaction mixture in 100 mM MOPS/KOH buffer, pH 7.3 with 15 mM $MgCl_2$ contained 250 μl of anaerobic cell extract of enrichment culture N47, 0.5 mM CoA-SH, and 0.025 mM sodium dithionite. NADH (1 mM) or 2-oxoglutarate (2.5 mM) was used as the electron donor and methyl viologen (1 mM) or NAD^+ (1 mM) as the electron acceptor. The assays were monitored at 600 nm (methyl viologen reduction) or 365 nm (NAD^+ reduction) and reaction rates were calculated using the following molar absorption coefficients (ε): NADH, $\varepsilon_{365} = 3.4\ mM^{-1}\ cm^{-1}$; methyl viologen, $\varepsilon_{600} = 13\ mM^{-1}\ cm^{-1}$. Control assays were performed without the respective electron donor.

Assays with potential downstream metabolites. The CoA thioesters of the chemically synthesized compounds *cis*-2-([*E*]-3-carboxy-allyl)cyclohexane-1-carboxylic acid and *cis*-(*E*)-3-(2-[carboxymethyl]cyclohexyl)acrylic acid, which had earlier been proposed as possible intermediates of the anaerobic naphthalene degradation pathway in culture N47 (8), were tested for conversion by enoyl-CoA hydratases in cell extracts of culture N47 and strain NaphS2 as described previously (1). Due to the two carboxyl groups present in these compounds, a mixture of two isomers differing in the attachment sites of the CoA thioester was obtained upon synthesis of the respective CoA thioesters. The complete mixture was used as the substrate for the hydratase tests. However, only the isomers with a double bond next to the carboxyl-CoA residue, namely, *cis*-(*E*)-3-(2-[carboxymethyl]-cyclohexyl)acrylyl-CoA and *cis*-4-(2-carboxycyclohexyl)crotonyl-CoA, were expected to be converted in a β-oxidation-like manner.

LC-MS analysis. LC-MS analyses (liquid chromatography coupled to mass spectrometry) were performed with an LC-2040C system coupled to a LCMS-2020 single quadrupole mass spectrometer (Shimadzu Deutschland, Duisburg, Germany). Samples were separated via a Nucleodur C_{18} Gravity-SB column, 100 × 3 mm, 5 μm particle size or a Nucleodur C_{18} Pyramid column, 100 × 3 mm, 5 μm particle size (both Macherey-Nagel, Dueren, Germany). The column oven was set to 35°C. Eluent A was water with 0.1% (wt/vol) ammonium acetate, eluent B was acetonitrile. Eluent B increased from 5% up to 35% over 15 min at a flow rate of 0.7 ml/min. Mass spectrometric analysis was carried out with an ESI system in positive mode. The voltage of the ESI system was set to 4.5 kV and the temperature to 350°C. Nebulizing gas flow was 1.5 liters/min, drying gas flow 12 liters/min. Heat block temperature was 200°C and the desolvation line was operated at 0 V and 250°C.

Mass-to-charge ratios (*m/z*) of expected metabolites (Table 2) were detected in single-ion mode with ionization conditions as optimized for 5,6,7,8-tetrahydro-2-naphthoyl-CoA. Settings for the Qarray ion guide were as follows: DC voltage 0 V, RF voltage 105 to 120 V (compound specific).

ACKNOWLEDGMENTS

We thank the anonymous reviewers for their helpful suggestions that have led to a substantial improvement of our manuscript.

REFERENCES

1. Weyrauch P, Zaytsev AV, Stephan S, Kocks L, Schmitz OJ, Golding BT, Meckenstock RU. 2017. Conversion of *cis*-2-carboxycyclohexylacetyl-CoA in the downstream pathway of anaerobic naphthalene degradation. Environ Microbiol 19:2819–2830. https://doi.org/10.1111/1462-2920.13806.
2. Wilkes H, Schwarzbauer J. 2010. Hydrocarbons: an introduction to structure, physico-chemical properties and natural occurrence, p 1–48. *In* Timmis KN (ed), Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, Germany.
3. Gibson DT, Parales RE. 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr Opin Biotechnol 11:236–243. https://doi.org/10.1016/s0958-1669(00)00090-2.
4. Habe H, Omori T. 2003. Genetics of polycyclic aromatic hydrocarbon metabolism in diverse aerobic bacteria. Biosci Biotechnol Biochem 67:225–243. https://doi.org/10.1271/bbb.67.225.
5. Meckenstock RU, Boll M, Mouttaki H, Koelschbach JS, Cunha Tarouco P, Weyrauch P, Dong X, Himmelberg AM. 2016. Anaerobic degradation of benzene and polycyclic aromatic hydrocarbons. J Mol Microbiol Biotechnol 26:92–118. https://doi.org/10.1159/000441358.
6. Meckenstock RU, Mouttaki H. 2011. Anaerobic degradation of non-substituted aromatic hydrocarbons. Curr Opin Biotechnol 22:406–414. https://doi.org/10.1016/j.copbio.2011.02.009.
7. Harwood CS, Burchhardt G, Herrmann H, Fuchs G. 1998. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. FEMS Microbiol Rev 22:439–458. https://doi.org/10.1111/j.1574-6976.1998.tb00380.x.
8. Annweiler E, Michaelis W, Meckenstock RU. 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. https://doi.org/10.1128/aem.68.2.852-858.2002.
9. Boll M, Fuchs G, Heider J. 2002. Anaerobic oxidation of aromatic compounds and hydrocarbons. Curr Opin Chem Biol 6:604–611. https://doi.org/10.1016/s1367-5931(02)00375-7.
10. Gibson J, Harwood CS. 2002. Metabolic diversity in aromatic compound utilization by anaerobic microbes. Annu Rev Microbiol 56:345–369. https://doi.org/10.1146/annurev.micro.56.012302.160749.
11. Buckel W, Kung JW, Boll M. 2014. The benzoyl-coenzyme A reductase and 2-hydroxyacyl-coenzyme A dehydratase radical enzyme family. Chembiochem 15:2188–2194. https://doi.org/10.1002/cbic.201402270.
12. Boll M, Fuchs G. 1995. Benzoyl-coenzyme A reductase (dearomatizing), a key enzyme of anaerobic aromatic metabolism. ATP dependence of the reaction, purification and some properties of the enzyme from *Thauera aromatica* strain K172. Eur J Biochem 234:921–933. https://doi.org/10.1111/j.1432-1033.1995.921_a.x.
13. Koch J, Eisenreich W, Bacher A, Fuchs G. 1993. Products of enzymatic reduction of benzoyl-CoA, a key reaction in anaerobic aromatic metabolism. Eur J Biochem 211:649–661. https://doi.org/10.1111/j.1432-1033.1993.tb17593.x.
14. Koch J, Fuchs G. 1992. Enzymatic reduction of benzoyl-CoA to alicyclic compounds, a key reaction in anaerobic aromatic metabolism. Eur J Biochem 205:195–202. https://doi.org/10.1111/j.1432-1033.1992.tb16768.x.
15. López Barragán MJ, Carmona M, Zamarro MT, Thiele B, Boll M, Fuchs G, García JL, Díaz E. 2004. The *bzd* gene cluster, coding for anaerobic benzoate catabolism, in *Azoarcus* sp. strain CIB. J Bacteriol 186:5762–5774. https://doi.org/10.1128/JB.186.17.5762-5774.2004.
16. Egland PG, Pelletier DA, Dispensa M, Gibson J, Harwood CS. 1997. A cluster of bacterial genes for anaerobic benzene ring biodegradation. Proc Natl Acad Sci U S A 94:6484–6489. https://doi.org/10.1073/pnas.94.12.6484.
17. Boll M, Fuchs G. 1998. Identification and characterization of the natural electron donor ferredoxin and of FAD as a possible prosthetic group of benzoyl-CoA reductase (dearomatizing), a key enzyme of anaerobic metabolism. Eur J Biochem 251:946–954. https://doi.org/10.1046/j.1432-1327.1998.2510946.x.
18. Breese K, Boll M, Alt-Mörbe J, Schägger H, Fuchs G. 1998. Genes coding for the benzoyl-CoA pathway of anaerobic aromatic metabolism in the bacterium *Thauera aromatica*. Eur J Biochem 256:148–154. https://doi.org/10.1046/j.1432-1327.1998.2560148.x.
19. Boll M, Löffler C, Morris BEL, Kung JW. 2014. Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme A esters: organisms, strategies and key enzymes. Environ Microbiol 16:612–627. https://doi.org/10.1111/1462-2920.12328.
20. Löffler C, Kuntze K, Vazquez JR, Rugor A, Kung JW, Böttcher A, Boll M. 2011. Occurrence, genes and expression of the W/Se-containing class II benzoyl-coenzyme A reductases in anaerobic bacteria. Environ Microbiol 13:696–709. https://doi.org/10.1111/j.1462-2920.2010.02374.x.
21. Peters F, Rother M, Boll M. 2004. Selenocysteine-containing proteins in anaerobic benzoate metabolism of *Desulfococcus multivorans*. J Bacteriol 186:2156–2163. https://doi.org/10.1128/jb.186.7.2156-2163.2004.
22. Wischgoll S, Heintz D, Peters F, Erxleben A, Sarnighausen E, Reski R, Van Dorsselaer A, Boll M. 2005. Gene clusters involved in anaerobic benzoate degradation of *Geobacter metallireducens*. Mol Microbiol 58:1238–1252. https://doi.org/10.1111/j.1365-2958.2005.04909.x.
23. Kung JW, Loffler C, Dorner K, Heintz D, Gallien S, Van Dorsselaer A, Friedrich T, Boll M. 2009. Identification and characterization of the tungsten-containing class of benzoyl-coenzyme A reductases. Proc Natl Acad Sci U S A 106:17687–17692. https://doi.org/10.1073/pnas.0905073106.
24. McInerney MJ, Rohlin L, Mouttaki H, Kim U, Krupp RS, Rios-Hernandez L, Sieber J, Struchtemeyer CG, Bhattacharyya A, Campbell JW, Gunsalus RP. 2007. The genome of *Syntrophus aciditrophicus*: life at the thermodynamic limit of microbial growth. Proc Natl Acad Sci U S A 104:7600–7605. https://doi.org/10.1073/pnas.0610456104.
25. Buckel W, Thauer RK. 2013. Energy conservation via electron bifurcating ferredoxin reduction and proton/Na^+ translocating ferredoxin oxidation. Biochim Biophys Acta 1827:94–113. https://doi.org/10.1016/j.bbabio.2012.07.002.
26. Boll M, Einsle O, Ermler U, Kroneck PMH, Ullmann GM. 2016. Structure and function of the unusual tungsten enzymes acetylene hydratase and class II benzoyl-coenzyme A reductase. J Mol Microbiol Biotechnol 26:119–137. https://doi.org/10.1159/000440805.
27. Eberlein C, Estelmann S, Seifert J, von Bergen M, Muller M, Meckenstock RU, Boll M. 2013. Identification and characterization of 2-naphthoyl-coenzyme A reductase, the prototype of a novel class of dearomatizing reductases. Mol Microbiol 88:1032–1039. https://doi.org/10.1111/mmi.12238.
28. Estelmann S, Blank I, Feldmann A, Boll M. 2015. Two distinct old yellow enzymes are involved in naphthyl ring reduction during anaerobic naphthalene degradation. Mol Microbiol 95:162–172. https://doi.org/10.1111/mmi.12875.
29. Bergmann F, Selesi D, Weinmaier T, Tischler P, Rattei T, Meckenstock RU. 2011. Genomic insights into the metabolic potential of the polycyclic aromatic hydrocarbon degrading sulfate-reducing *Deltaproteobacterium* N47. Environ Microbiol 13:1125–1137. https://doi.org/10.1111/j.1462-2920.2010.02391.x.
30. DiDonato RJ, Young ND, Butler JE, Chin KJ, Hixson KK, Mouser P, Lipton MS, DeBoy R, Methe BA. 2010. Genome sequence of the deltaproteobacterial strain NaphS2 and analysis of differential gene expression during anaerobic growth on naphthalene. PLoS One 5:e14072. https://doi.org/10.1371/journal.pone.0014072.
31. Eberlein C, Johannes J, Mouttaki H, Sadeghi M, Golding BT, Boll M, Meckenstock RU. 2013. ATP-dependent/-independent enzymatic ring reductions involved in the anaerobic catabolism of naphthalene. Environ Microbiol 15:1832–1841. https://doi.org/10.1111/1462-2920.12076.
32. Song B, Ward BB. 2005. Genetic diversity of benzoyl coenzyme A reductase genes detected in denitrifying isolates and estuarine sediment communities. Appl Environ Microbiol 71:2036–2045. https://doi.org/10.1128/AEM.71.4.2036-2045.2005.
33. Boll M, Laempe D, Eisenreich W, Bacher A, Mittelberger T, Heinze J, Fuchs G. 2000. Nonaromatic products from anoxic conversion of benzoyl-CoA with benzoyl-CoA reductase and cyclohexa-1,5-diene-1-carbonyl-CoA

hydratase. J Biol Chem 275:21889–21895. https://doi.org/10.1074/jbc.M001833200.
34. Weyrauch P. 2016. Mikrobieller Abbau von Naphthalin unter anaeroben Bedingungen. Doctoral dissertation. University of Duisburg-Essen, Duisburg, Germany.
35. Dörner E, Boll M. 2002. Properties of 2-oxoglutarate:ferredoxin oxidoreductase from *Thauera aromatica* and its role in enzymatic reduction of the aromatic ring. J Bacteriol 184:3975–3983. https://doi.org/10.1128/jb.184.14.3975-3983.2002.
36. Laempe D, Eisenreich W, Bacher A, Fuchs G. 1998. Cyclohexa-1,5-diene-1-carboxyl-CoA hydratase, an enzyme involved in anaerobic metabolism of benzoyl-CoA in the denitrifying bacterium *Thauera aromatica*. Eur J Biochem 255:618–627. https://doi.org/10.1046/j.1432-1327.1998.2550618.x.
37. Laempe D, Jahn M, Fuchs G. 1999. 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase and 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase, enzymes of the benzoyl-CoA pathway of anaerobic aromatic metabolism in the denitrifying bacterium *Thauera aromatica*. Eur J Biochem 263:420–429. https://doi.org/10.1046/j.1432-1327.1999.00504.x.
38. Ochoa S. 2006. Enzymic mechanisms in the citric acid cycle, p 183–270. *In* Toone EJ (ed). Advances in enzymology and related areas of molecular biology. John Wiley & Sons, Inc.
39. Sanadi DR, Littlefield JW, Bock RM. 1952. Studies on α-ketoglutaric oxidase. II. Purification and properties. J Biol Chem 197:851–862.
40. Kuntze K, Shinoda Y, Moutakki H, McInerney MJ, Vogt C, Richnow HH, Boll M. 2008. 6-Oxocyclohex-1-ene-1-carbonyl-coenzyme A hydrolases from obligately anaerobic bacteria: characterization and identification of its gene as a functional marker for aromatic compounds degrading anaerobes. Environ Microbiol 10:1547–1556. https://doi.org/10.1111/j.1462-2920.2008.01570.x.
41. Schulz H. 1991. Beta oxidation of fatty acids. Biochim Biophys Acta 1081:109–120. https://doi.org/10.1016/0005-2760(91)90015-a.
42. Yamauchi K, Tanabe T, Kinoshita M. 1979. Trimethylsulfonium hydroxide: a new methylating agent. J Org Chem 44:638–639. https://doi.org/10.1021/jo01318a037.
43. Cran JW, Krafft ME, Seibert KA, Haxell TFN, Wright JA, Hirosawa C, Abboud KA. 2011. The intramolecular Morita–Baylis–Hillman-type alkylation reaction. Tetrahedron 67:9922–9943. https://doi.org/10.1016/j.tet.2011.09.061.
44. Miyafuji A, Ito K, Katsuki T. 2000. Application of oxidative desymmetrization of meso-tetrahydrofurans: syntheses of functionalized chiral building blocks and of (-)-alloyohimbane. Heterocycles 52:261–272. https://doi.org/10.3987/COM-99-S20.
45. Ebenau-Jehle C, Boll M, Fuchs G. 2003. 2-oxoglutarate:NADP$^+$ oxidoreductase in *Azoarcus evansii*: properties and function in electron transfer reactions in aromatic ring reduction. J Bacteriol 185:6119–6129. https://doi.org/10.1128/jb.185.20.6119-6129.2003.