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Offen im Denken

Mikrobieller Abbau von Naphthalin unter anaeroben Bedingungen

Dissertation

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“I think perhaps the most important problem is that we are trying to understand the fundamental workings of the universe via a language devised for telling one another when the best fruit is.”

Sir Terry Pratchett

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1. General introduction

1.01. Environmental occurrence and toxicity of naphthalene

Naphthalene is a polycyclic aromatic hydrocarbon (PAH) with the sum formula C_8H_{10} and consisting of two fused aromatic rings with ten delocalised π-electrons. Like other PAHs, naphthalene is formed during geochemical combustion of biomass and is contained in petroleum and in petroleum-derived products (Tissot and Welte, 1978; Neff, 2002). There is also a direct biological production of naphthalene for instance by endophytic fungi (Daisy *et al.*, 2002; Ezra *et al.*, 2004), *Magnolia* flowers (Azuma *et al.*, 1996) or by termites (Chen *et al.*, 1998a, b). However, its incidence in the environment typically originates from anthropogenic sources such as pollutions from manufactured gas plant sites (Harkins *et al.*, 1988; Luthy *et al.*, 1994), from oil spills and also from engine exhausts (Clark *et al.*, 1982). Additionally, PAHs are contained in coal tar, from which especially naphthalene, the smallest PAH, partitions to considerable amounts into water (Lee *et al.*, 1992). Human exposure to naphthalene is mainly attributed to inhalation of vehicles' exhaust and of cigarette smoke (Schmeltz *et al.*, 1976; Griego *et al.*, 2008; Jia and Batterman, 2010).

Naphthalene is hazardous both to natural habitats and to human health (Preuss *et al.*, 2003). As shown in toxicity assays with *Vibrio fisheri*, it has higher toxic effects towards bacteria than other PAHs due to its comparably high bioavailability (Loibner *et al.*, 2004). For humans and mammals, naphthalene is known to cause anaemia which is due to products formed by its metabolic oxidation, naphthols and, to a lesser extent, naphthoquinones (Zuelzer and Apt, 1949; Mackell *et al.*, 1951). Furthermore, naphthalene most likely has a carcinogenic effect on humans and animals (National Toxicology Program, 1992; Bogen *et al.*, 2008; North *et al.*, 2008). Again this effect is caused by metabolic transformation products of naphthalene, namely naphthoquinones, which can disturb cellular and inter-cellular signalling processes by chemically modifying regulatory proteins (Brusick *et al.*, 2008; Kumagai *et al.*, 2012; Klotz *et al.*, 2014). In contrast, mutagenic activity was not observed for naphthalene or its transformation products (Flowers, 2004) although naphthoquinones and enzyme activated naphthols were shown to form adducts with purine nucleotides in vitro (Saeed *et al.*, 2007). However,

larger PAHs are known to be mutagenic due to their ability to form DNA adducts (Chu and Chen, 1985; Pufulete *et al.*, 2004).

Regarding the described environmental and health related risks of naphthalene, its microbial degradation as potential tool of bioremediation is of great interest. Naphthalene, being the smallest polycyclic aromatic hydrocarbon, may furthermore serve as a model compound for studying degradation strategies for PAHs in general.

1.02. Biodegradation at contaminated sites

PAHs are regularly found in low concentrations in water systems and soils where they tend to accumulate due to their low water-solubility and high hydrophobicity (Wilkes and Schwarzbauer, 2010). Their natural attenuation is mainly achieved by microbes which are capable of PAH degradation under various environmental conditions (Lu *et al.*, 2011a). Anaerobic microbial degradation is of special environmental relevance since PAHs, like the majority of environmental pollutants, typically end up in anoxic zones due to the rapid oxygen depletion within a plume (Christensen *et al.*, 1994; Christensen *et al.*, 2001). Biological attenuation of pollutants therefore mainly occurs at the plume fringes where they can be metabolised by microbes that use alternative terminal electron acceptors like nitrate, sulphate, iron(III), manganese(IV) or have an methanogenic lifestyle (Jobelius *et al.*, 2011; Meckenstock *et al.*, 2015).

For the estimation of the self-cleaning potentials of contaminated sites, monitoring of biodegradation of specific pollutants is required. This is typically achieved via a combination of the following three approaches:

Specific metabolites that exclusively occur during biological degradation of compounds and are not produced by anaerobic processes can serve as **key metabolites** indicating biodegradation (Callaghan, 2013). For example, benzylsuccinate derivatives were identified as specific metabolites of the anaerobic microbial catabolism of toluene, ethylbenzene or xylenes, which is initiated by fumarate-addition on a methyl group of the substrate (Beller, 2000; Elshahed *et al.*, 2001a). Furthermore, the anaerobic degradation or co-metabolisation of naphthalene and other PAHs produces carboxylated

metabolites which are indicative for this pathway (Safinowski *et al.*, 2006) and also 5,6,7,8-tetrahydro-2-naphthoic acid formed during anaerobic naphthalene degradation could be detected at PAH contaminated sites (Griebler *et al.*, 2004).

Alternatively, genes coding for key enzymes of known degradation pathway can serve as **marker genes** which make it possible to analyse if distinct pollutants are metabolised at a given site. The *bssA* genes, coding for the enzyme benzylsuccinate synthase, which catalyses the aforementioned formation of benzylsuccinate derivatives from alkylbenzenes, are widely used for detection of alkylbenzene degraders at contaminated sites (Winderl *et al.*, 2007; Callaghan *et al.*, 2010; Staats *et al.*, 2011). Analogously, the related *nmsA* genes, which code for the naphthyl-2-methysuccinate synthase, serve as gene markers for bacteria with the ability to degrade the bicyclic compound 2-methylnaphthalene (von Netzer *et al.*, 2013; von Netzer *et al.*, 2016). Degraders of naphthalene, 2-methylnaphthalene and 2-naphthoate can also be detected via their 2-naphthoyl-CoA reductase genes since these enzymes are completely different from aryl-CoA reductases involved in other pathways (Morris *et al.*, 2014).

Biodegradation can also be monitored via **stable isotope probing** (Elsner *et al.*, 2005; Vogt *et al.*, 2016). For this technique the isotope ratios of a distinct pollutant in anaerobic samples are analysed and compared to reference data. If the rate limiting step of a degradation pathway involves breaking of a covalent bond within the target molecule, molecules with a heavier isotope at this specific position react more slowly than those exhibiting a lighter isotope. Molecules with heavier isotopes are therefore slightly enriched during the majority of biological attenuation processes. Stable isotope probing was also proven to be a suitable tool for the monitoring of biodegradation of aromatic compounds (Lollar *et al.*, 1999; Meckenstock *et al.*, 1999; Bergmann *et al.*, 2011c).

Since all these monitoring techniques depend on a detailed knowledge about the degrading organisms and the respective metabolic pathways, basic research on these topics is essential for the detection of biodegradation of a given contaminant and for the development of remediation strategies for contaminated sites.

1.03. Anaerobic naphthalene degrading cultures

While the degradation of naphthalene under aerobic conditions, that usually occurs via dioxygenases (Gibson and Parales, 2000; Karlsson *et al.*, 2003), is nowadays well understood (Cerniglia, 1992; Habe and Omori, 2003), the research on the anaerobic degradation of naphthalene is still ongoing (Meckenstock and Mouttaki, 2011; Meckenstock *et al.*, 2016). Anaerobic microbial degradation of naphthalene has first been reported under nitrate reducing conditions (Mihelcic and Luthy, 1988; McNally *et al.*, 1998a; Rockne and Strand, 1998) and later on also under sulphate (Coates *et al.*, 1996a; Bedessem *et al.*, 1997; Zhang and Young, 1997; Rockne and Strand, 1998), ferric iron (Coates *et al.*, 1996b) and manganese reducing (Langenhoff *et al.*, 1996) as well as under methanogenic conditions (Christensen *et al.*, 2004; Chang *et al.*, 2005; Chang *et al.*, 2006). Currently there are two sulphate reducing cultures with the ability to grow anaerobically on naphthalene or 2-methylnaphthalene being investigated, namely the strain NaphS2 belonging to the *Deltaproteobacteria* (Galushko *et al.*, 1999) and the freshwater enrichment culture N47 (Meckenstock *et al.*, 2000), which is dominated by a *Desulfobacterium* accompanied by a member of the *Spirochaetes* (Selesi *et al.*, 2010). Strains related to N47 seem to be widespread and were recently found in various groundwater environments (Kuemmel *et al.*, 2015). Furthermore, two other naphthalene-degrading sulphate-reducing strains named NaphS3 and NaphS6, that are closely related to NaphS2, have been isolated from a Mediterranean lagoon (Musat *et al.*, 2009), but nothing is published about further investigations on these strains. In analogy to N47, also NaphS2-like strains seem to occur recently in contaminated environments since bacteria related to NaphS2 were found in several marine sediments with a long history of pollution by PAHs (Hayes and Lovley, 2002).

For anaerobic bacteria utilising naphthalene, best energy yield is expected with nitrate as terminal electron acceptor (Meckenstock *et al.*, 2016). Therefore, degradation of naphthalene or PAHs in general under nitrate-reducing conditions might be an important process in the environment and the corresponding microorganisms are of great scientific interest. Pioneering studies on the degradation of monocyclic aromatic compounds were also conducted with a denitrifying bacterium, namely *Thauera aromatica* (Boll and Fuchs, 1995; Biegert *et al.*, 1996).

The first reports on the bio-degradation of naphthalene and other PAHs under nitrate-reducing conditions came from experiments with artificial soil-water systems (Mihelcic and Luthy, 1988; Langenhoff *et al.*, 1996). Additionally, enrichment cultures with the ability to grow on naphthalene under nitrate-reduction were obtained from marine sediments (Rockne and Strand, 1998, 2001; Lu *et al.*, 2011b), contaminated Artic soils (Eriksson *et al.*, 2003) and coal-tar contaminated sediment from a former coal gasification plant (Ramsay *et al.*, 2003). There were also some pure denitrifying strains isolated that could use naphthalene as sole source of carbon and energy (McNally *et al.*, 1998a; Rockne *et al.*, 2000; Mittal and Rockne, 2008). McNally *et al.* isolated three strains that were phylogenetically similar to *Pseudomonas* spp. from freshwater sediments and creosote contaminated soils. The isolates published by Rockne *et al.* originated from marine sediments (Rockne and Strand, 1998) and showed phylogenetical similarity to *Pseudomonas* spp. and *Vibrio* spp., respectively. Unfortunately all these pure cultures got lost so nowadays there is no known isolate of a denitrifying naphthalene degrader. Nevertheless, various publications showed that naphthalene degradation under nitrate-reducing conditions should be possible.

Recently it has been shown that bacteria can use dioxygen which is intracellulary produced from nitrous oxide to oxidise methane (Ettwig *et al.*, 2010; Wu *et al.*, 2011) or hexadecane (Zedelius *et al.*, 2011), respectively. Regarding the aforementioned difficulties to activate PAHs for biodegradation in absence of the highly reactive co-substrate dioxygen, this strategy could also be used for the degradation of naphthalene under nitrate-reducing conditions which would imply degradation pathways completely different from the ones used by the sulphate-reducing naphthalene degraders. This assumption is further supported by recently published studies that identified genes coding for a naphthalene dioxygenase within denitrifying enrichment cultures anaerobically grown on naphthalene and other PAHs (Lu *et al.*, 2011b; Lu *et al.*, 2012). However, to date there is no direct experimental evidence that naphthalene degradation under denitrifying conditions is initiated by dioxygenases.

1.04. Enrichment culture N47

The anaerobic naphthalene-degrading culture N47 has been enriched from soil material of a contaminated aquifer near Stuttgart, Germany, under sulphate-reducing conditions (Meckenstock *et al.*, 2000). It can grow on naphthalene, 2-methylnaphthalene, 1-naphthoate and 2-naphthoate as sole source of carbon and energy with sulphate as terminal electron acceptor, but it can neither metabolise 1-methylnaphthalene nor 2-(naphthalene-1-yl)acetate, 2-(naphthalene-2-yl)acetate and hydroxylated compounds like 1-naphthol, 2-naphthol, 1-hydroxy-2-naphthoate, 3-hydroxy-2-naphthoate and 2-hydroxy-1-naphthoate (Meckenstock *et al.*, 2000). Furthermore, N47 can utilise the monocyclic aromatic compounds benzoate, phenylacetate (Meckenstock *et al.*, 2000), phenol, 4-methylphenol, benzaldehyde and 3-hydroxybenzaldehyde (Selesi *et al.*, 2010), which is surprising since apparently the N47 genome does not contain any genes for the anaerobic benzoyl-CoA pathway (Bergmann *et al.*, 2011a).

Growing N47 on phenol lead to a shift of the bacterial community within this culture and the accompanying *Spirochaetes* became dominant (Bergmann *et al.*, 2011b). This could indicate that monocyclic compounds are actually degraded by other members of the enrichment culture, illustrating the uncertainties of statements concerning the substrate utilisation of highly enriched cultures. Since most of the previous substrate tests for the naphthalene degrader N47 were not verified by a community structure analysis, these results need to be regarded with caution. Other monocyclic aromatic hydrocarbons like phthalate (benzene-1,2-dicarboxylate), homophthalate (2-carboxyphenylacetate), salicylate (2-hydroxybenzoate), benzene (Meckenstock *et al.*, 2000) or toluene (Annweiler *et al.*, 2000) do not serve as growth substrates for N47. More reduced substrates like cyclohexanecarboxylate, cyclohex-1-ene-1-carboxylate (Meckenstock *et al.*, 2000) and 1,2,3,4-tetrahydronaphthalene (Annweiler *et al.*, 2002) can serve as growth substrate, whereas this is not the case for cyclohexane, 1,2,3,4-tetrahydro-2-naphthoate, *trans*-cyclohexane-1,2-dicarboxylate, cyclohexylacetate and decahydro-2-naphthoate (Meckenstock *et al.*, 2000).

Besides sulphate, N47 can also use elemental sulphur as terminal electron acceptor (Selesi *et al.*, 2010), which is a rarely spread feature within sulphate-reducing bacteria (Rabus *et al.*, 2006). Genes for the reduction of nitrate to ammonium were also found

within the genome of the naphthalene-degrading *Desulfobacterium* dominating the enrichment culture N47, but nevertheless the culture seems not to be able to grow with nitrate as sole terminal electron acceptor (Bergmann *et al.*, 2011a).

1.05. *Deltaproteobacterium* strain NaphS2

The strain NaphS2 has been isolated from an anoxic, black sediment from a small North Sea harbour (Horumersiel) near Wilhelmshaven, Germany, that had been incubated with naphthalene as sole carbon source under sulphate-reducing conditions (Galushko *et al.*, 1999). Besides naphthalene, NaphS2 was able to utilise 2-naphthoate, benzoate, acetate and pyruvate as carbon source linked to sulphate reduction (Galushko *et al.*, 1999), but it was not able to grow on other aromatic compounds like 1-naphthoate, 1-naphthol, 2-naphthol or benzene (Galushko *et al.*, 1999). Furthermore, NaphS2 was also able to grow on 2-methylnaphthalene, but not on 1-methylnaphthalene (Musat *et al.*, 2009).

1.06. Metabolites in anaerobic naphthalene degrading cultures

Since the aforementioned sulphate-reducing strains N47 and NaphS2 are the only stable pure or highly enriched cultures capable of anaerobic growth on naphthalene that are known so far, all detailed metabolomic investigation on the anaerobic naphthalene degradation pathway were based on either of these two cultures. Metabolite analysis was typically conducted via gas-chromatography coupled to mass-spectrometry (GC-MS) in previous studies (Meckenstock *et al.*, 2000; Annweiler *et al.*, 2002). This technique requires an alkaline lysis of cells contained in the sample, followed by a titration of the sample to pH 2. Organic metabolites are then extracted from the aqueous phase with a water-immiscible solvent, for example hexane or ethyl acetate.

According to recent knowledge about the anaerobic naphthalene degradation, most metabolites emerging in the pathway contain a carboxyl group which forms a thioester with coenzyme A (Meckenstock *et al.*, 2016). Following the uptake of naphthalene or 2-naphthoate into the cell, the corresponding CoA-ester, 2-naphthoyl-CoA is formed, presumably by a 2-naphthoate:CoA ligase (Figure 1).

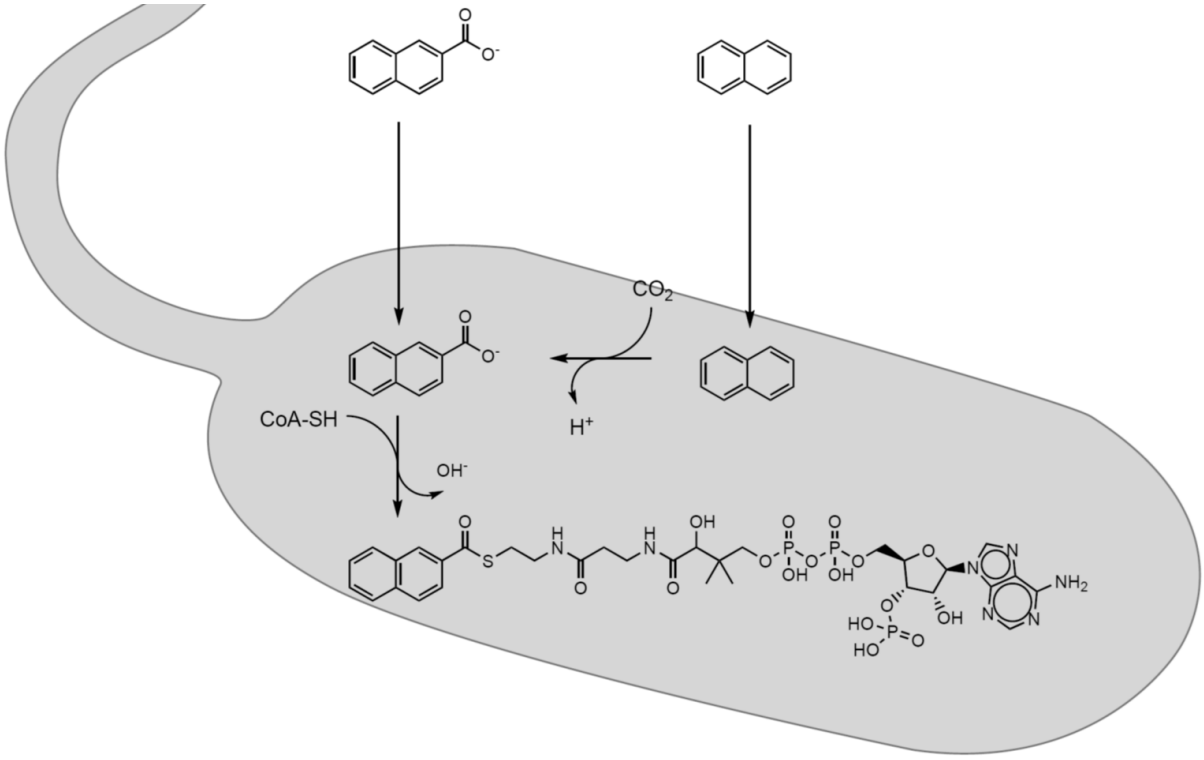


Figure 1 Uptake and initial activation of naphthalene or 2-naphthoate, respectively, by sulphate-reducing naphthalene degraders. Naphthalene is activated inside the cell by a naphthalene carboxylase to 2-naphthoate. The latter is converted to 2-naphthoyl-CoA, presumably by a 2-naphthoate:CoA ligase. All further metabolites are expected to occur in the form of coenzyme A thioesters.

All subsequent metabolites are expected to occur intracellular since the bulky carboxyl-CoA residue prevents diffusion through the cytoplasmic membrane (Figure 1). Therefore, alkaline cell lysis releasing intracellular metabolites is a prerequisite for the analysis of the whole metabolom. However, CoA-esters get hydrolysed during the aforementioned sample treatment, so metabolites are typically detected in the form of free acids and the information about attachment sites of the CoA-thioesters gets lost. For this reason, all metabolites referred to in the following paragraph will be regarded as free acids reflecting the direct results of GC-MS analyses.

A metabolite regularly detected in several anaerobic naphthalene- or 2-methyl-naphthalene-degrading cultures was 2-naphthoic acid (Zhang and Young, 1997; Sullivan

et al., 2001; Galushko *et al.*, 2003; Kleemann and Meckenstock, 2011). As proven by recent studies, the conversion of naphthalene to 2-naphthoic acid is realised via carboxylation (Figure 1, details will be given in section 1.07). Furthermore, 2-naphthoic acid has been proven to be the central metabolite of N47 cultures grown on naphthalene or 2-methylnaphthalene (Annweiler *et al.*, 2000; Meckenstock *et al.*, 2000). It is therefore assumed that 2-naphthoic acid or 2-naphthoyl-CoA, respectively, is the central intermediate of the anaerobic degradation of the two-ringed PAHs naphthalene and 2-methylnaphthalene like benzoyl-CoA is the central intermediate of anaerobic degradation pathways of monoaromatic compounds (Heider and Fuchs, 1997).

Derived from 2-methylnaphthalene, the metabolites 2-(naphthalene-2-ylmethyl)succinic acid and 2-(naphthalene-2-ylmethylidene)succinic acid could be detected, which indicates a conversion by fumarate addition and a subsequent dehydrogenase reaction (Annweiler *et al.*, 2000). The latter was assumed to undergo β-oxidation-like reactions leading to the central intermediate 2-naphthoic acid (Figure 2).

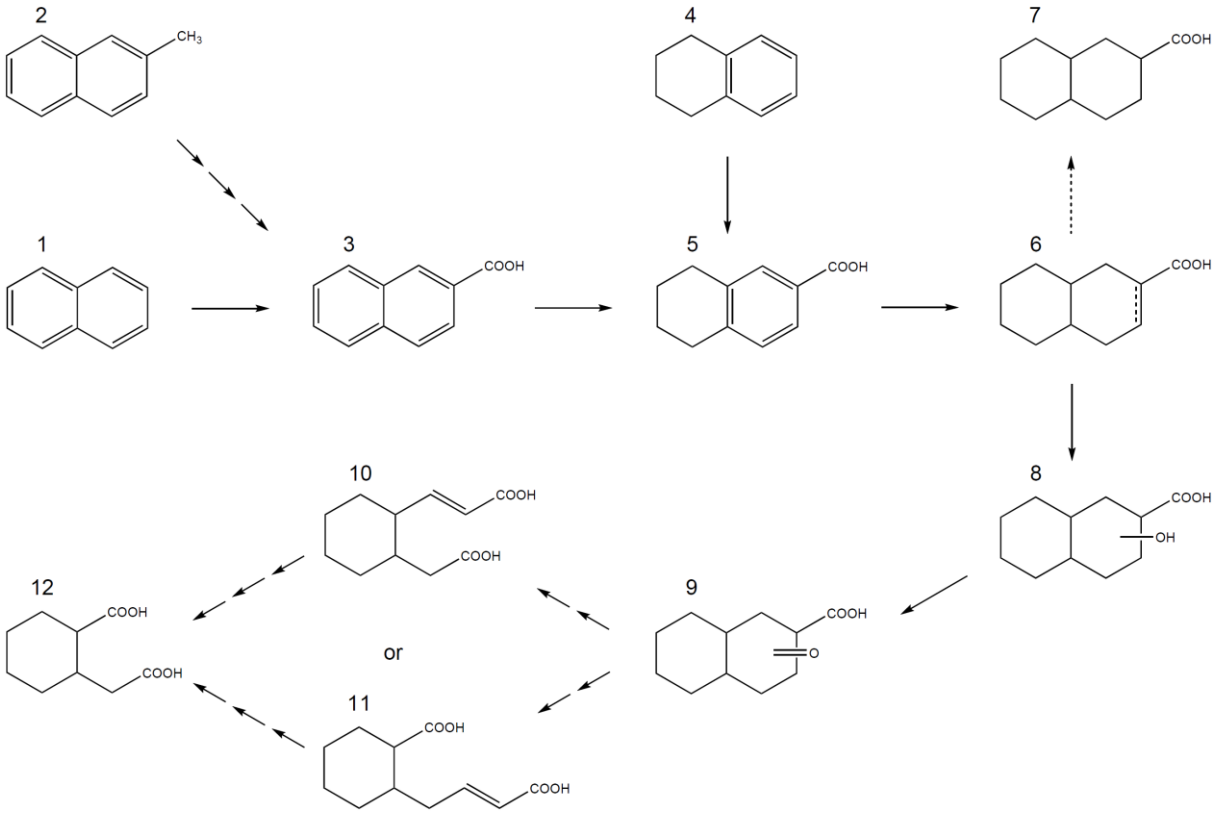


Figure 2 Metabolic pathway implied by metabolites that have so far been identified for the anaerobic degradation of naphthalene, 2-methylnaphthalene or tetralin (1,2,3,4-tetrahydronaphthalene) by the sulphate-reducing enrichment culture N47. (1): naphthalene. (2): 2-methylnaphthalene. (3): 2-naphthoic acid. (4): tetralin; (5): 5,6,7,8-tetrahydro-2-naphthoic acid. (6): decahydro-2-naphthoic acid. (7) octahydro-2-naphthoic acid. (8): postulated β-hydroxydecahydro-2-naphthoic acid. (9): postulated β-oxo-octahydro-2-naphthoic acid. (10): 3-(2-(carboxymethyl)cyclohexyl)acrylic acid. (11): 2-(3-carboxyallyl)cyclohexane-1-carboxylic acid. (12): 2-(carboxymethyl)cyclohexane-1-carboxylic acid.

The next metabolite detected downstream of 2-naphthoic acid was 5,6,7,8-tetrahydro-2-naphthoic acid, indicating reductase reactions at ring II (Annweiler *et al.*, 2000; Meckenstock *et al.*, 2000; Annweiler *et al.*, 2002). 5,6,7,8-tetrahydro-2-naphthoic acid as well as decahydro-2-naphthoic acid were also detected in a sulphate-reducing mixed culture growing on naphthalene (Zhang *et al.*, 2000). Tetralin (1,2,3,4-tetrahydronaphthalene), a reduced derivative of naphthalene, can enter the anaerobic degradation pathway of naphthalene at the stage of tetrahydro-2-naphthoic acid (Annweiler *et al.*, 2002). When the enrichment culture N47 was grown on, this substrate was carboxylated

only to 5,6,7,8-tetrahydro-2-naphthoic (Figure 2) acid but not to 1,2,3,4-tetrahydro-2-naphthoic acid (Annweiler *et al.*, 2002).

The pathway seems to proceed via further reductions at ring I yielded, since octahydro-2-naphthoic acid and decahydro-2-naphthoic acid were observed as subsequent metabolites (Annweiler *et al.*, 2000; Meckenstock *et al.*, 2000; Annweiler *et al.*, 2002). The latter was assumed to be a dead-end product rather than a metabolite of the anaerobic naphthalene degradation pathway. It was postulated that octahydro-2-naphthoic acid is further metabolised via β-hydroxydecahydro-2-naphthoic acid and β-oxodecahydro-2-naphthoic acid (Figure 2) and finally cleavage of ring I by a β-oxo-acyl-CoA hydrolase occurs next to the carboxyl group. This implies that the further degradation pathway goes via monocyclic compounds with a cyclohexane backbone. Indeed, two cyclohexane-derivatives were detected in N47 cultures (Annweiler *et al.*, 2002). According to its molecular composition, one of these metabolites could either be 2-(3-carboxy-allyl)cyclohexane-1-carboxylic acid or 3-(2-(carboxymethyl)cyclohexyl)-acrylic acid (compounds 10 and 11 in Figure 2). The first isomer would result from a cleavage of ring I in between C2 and C3 (compound 10), the latter from ring-cleavage between C1 and C2 (compound 11). The other detected metabolite was identified as 2-(carboxymethyl)cyclohexane-1-carboxylic acid (compound 12, termed 2-carboxy-cyclohexylacetic acid in the referred study) and it could even be demonstrated that this metabolite occurred only in *cis*- [i.e. (1*R*,2*R*)- or (1*S*,2*S*)-] conformation (Annweiler *et al.*, 2002). To date it is still unknown in which position the first ring cleavage occurs and how *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid is further degraded.

Another metabolomic study (Safinowski and Meckenstock, 2006) led to the transient assumption that methylation to 2-methylnaphthalene rather than carboxylation is the initial reaction in anaerobic naphthalene degradation. This was based on the observations that (i) N47 cultures transferred from naphthalene to 2-methylnaphthalene could use this substrate without a recognisable lag-phase while they showed a lag-phase of around 100 days when transferred from 2-methylnaphthalene to naphthalene and (ii) metabolites and enzyme activities specific for 2-methylnaphthalene degradation where also found during growth on naphthalene.

However, experiments with labelled substrates that were conducted with *Deltaproteobacterium* NaphS2 yielded contradictory results (Musat *et al.*, 2009). Cultures grown on naphthalene and 2-methylnaphthalene both showed the common intermediate 2-naphthoic acid and when deuterated naphthalene and unlabelled 2-methylnaphthalene were co-metabolically converted by NaphS2, it was shown that the major proportion of 2-naphthoic acid derived from naphthalene while almost all 2-(naphthalene-2-ylmethyl)succinic acid derived from 2-methylnaphthalene (Musat *et al.*, 2009). Therefore, these experimental data strongly indicated that naphthalene is directly carboxylated to form 2-naphthoic acid, while 2-methylnaphthalene is degraded similar to the anaerobic toluene degradation pathway mentioned above. This was further confirmed by the finding that proteins connected to the catabolic pathway of 2-methylnaphthalene were only present in cultures grown with 2-methylnaphthalene and not in naphthalene-grown cultures (Musat *et al.*, 2009; DiDonato *et al.*, 2010).

1.07. Comparison to the anaerobic benzoyl-CoA pathway

The metabolites identified in sulphate-reducing naphthalene-degrading cultures (see chapter 1.06) show similarities to the ones occurring in the benzoyl-CoA-pathway. This pathway is the common catabolic strategy for the anaerobic degradation of monoaromatic compounds (Heider and Fuchs, 1997; Harwood *et al.*, 1999; Boll *et al.*, 2002). After activation of benzoate to a CoA-thioester, the subsequent steps of the upper benzoyl-CoA degradation pathway are slightly different in the two model organisms *T. aromatica* and *Rhodopseudomonas palustris*, but both pathways converge to 3-hydroxypimeloyl-CoA as shown in Figure 3.

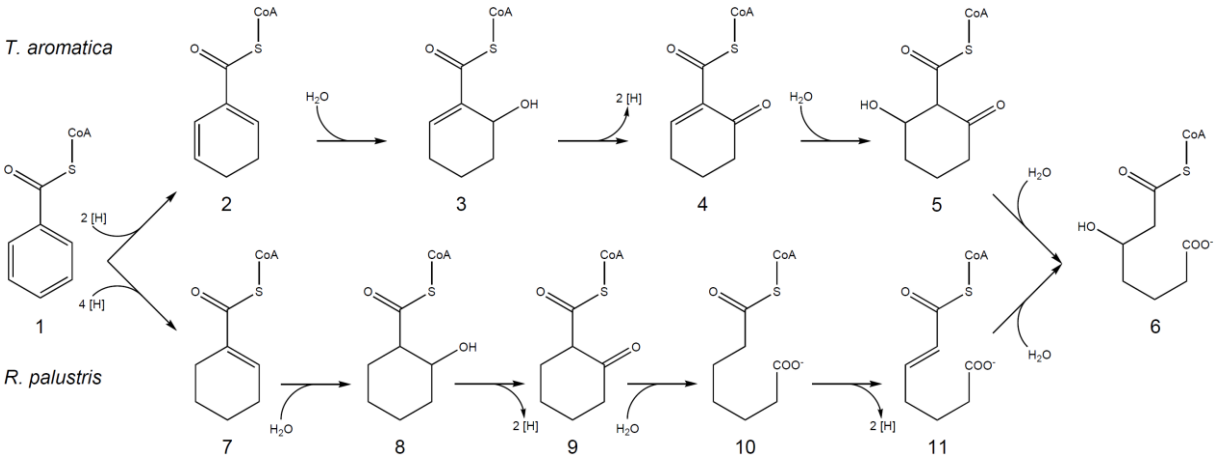


Figure 3 Benzoyl-CoA degradation pathways in *Thauera aromatica* (top) and *Rhodopseudomonas palustris* (bottom). (1): benzoyl-CoA. (2): cyclohexa-1,5-diene-1-carboxyl-CoA. (3): 6-hydroxycyclohex-1-ene-1-carboxyl-CoA. (4): 6-oxocyclohex-1-ene-1-carboxyl-CoA. (5): 2-hydroxy-6-oxocyclohexane-1-carboxyl-CoA. (6): 3-hydroxypimeloyl-CoA. (7): cyclohex-1-ene-1-carboxyl-CoA. (8): 2-hydroxycyclohexane-1-carboxyl-CoA. (9): 2-oxocyclohexane-1-carboxyl-CoA. (10): pimeloyl-CoA. (11): 2,3-didehydropimeloyl-CoA.

In *T. aromatica*, an oxygen-sensitive, ATP-dependant two-electron reduction (class I aryl-CoA reductase) of benzoyl-CoA yields cyclohexa-1,5-diene-1-carboxyl-CoA, which is transformed by subsequent reactions containing hydratisation, oxidation and hydrolytic cleavage to 3-hydroxypimeloyl-CoA via formation of 6-oxocyclohex-1-ene-1-carboxyl-CoA and 2-hydroxy-6-oxocyclohex-1-ene-1-carboxyl-CoA (Boll and Fuchs, 1995; Breese *et al.*, 1998; Laempe *et al.*, 1998; Laempe *et al.*, 1999). The mineralisation of benzoyl-CoA by *R. palustris* goes through the formation of 3-hydroxypimeloyl-CoA as well, but since in this case the initial reaction is an ATP-dependant four-electron reduction step leading to cyclohex-1-ene-1-carboxyl-CoA, the further intermediates formed are 6-oxocyclohexane-1-carboxyl-CoA and pimeloyl-CoA (Dutton and Evans, 1969; Perrotta and Harwood, 1994; Laempe *et al.*, 1998; Pelletier and Harwood, 1998).

In contrast to the nitrate-reducing *T. aromatica* and the phototrophic *R. palustris*, strict anaerobes like the sulphate reducer *Desulfococcus multivorans* (Peters *et al.*, 2004), the iron reducer *Geobacter metallireducens* (Wischgoll *et al.*, 2005) or the fermenting benzoate degrader *Syntrophus aciditrophicus* (Peters *et al.*, 2007) use another type of benzoyl-CoA reductases (class II) that do not require ATP (Peters *et al.*, 2004; Kung *et*

al., 2009; Loeffler *et al.*, 2011). It is assumed (Kung *et al.*, 2010; Loeffler *et al.*, 2011; Boll *et al.*, 2016), that these organisms drive the endergonic electron-transfer to benzoyl-CoA by a so-called "bifurcation mechanism", which means that the endergonic electron transfer is driven by coupling it to a second exergonic transfer (Herrmann *et al.*, 2008; Li *et al.*, 2008; Buckel and Thauer, 2013). This assumption is further supported by the finding that dearomatisation of benzoyl-CoA is fully reversible in *G. metallireducens* (Kung *et al.*, 2010) as well as in *S. aciditrophicus* (Mouttaki *et al.*, 2007). Regarding the low energy yields of sulphate-reducing naphthalene degraders (Meckenstock and Mouttaki, 2011), it should be expected that they use a similar ATP-saving process for the dearomatising reduction of 2-naphthoyl-CoA. According to recent results, this seems indeed to be the case for the 2-naphthoyl-CoA reductase but, surprisingly, not for the subsequent 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase (see chapter 1.08).

1.08. Enzyme reactions in cell free extracts

The aforementioned metabolic steps converting 2-methylnaphthalene to 2-naphthoyl-CoA via fumarate addition and subsequent β-oxidation-like reactions were the first enzyme reactions to be measured with crude cell extracts of sulphate-reducing PAH degraders (Annweiler *et al.*, 2000; Safinowski and Meckenstock, 2004). This conversion occurs through a reaction sequence similar to the one used in the anaerobic degradation pathway of toluene (Biegert *et al.*, 1996; Kube *et al.*, 2004), where toluene is activated by the addition of fumarate using a radical mechanism catalysed by benzylsuccinate synthase (Leuthner *et al.*, 1998; Heider *et al.*, 2016). During 2-methyl-naphthalene degradation, fumarate addition yields 2-(naphthalene-2-ylmethyl)-succinate, which is then activated to 2-(naphthalene-2-ylmethyl)succinyl-CoA by a CoA-transferase. Consecutive β-oxidation-like steps yield 2-naphthoyl-CoA and succinyl-CoA (Figure 4).

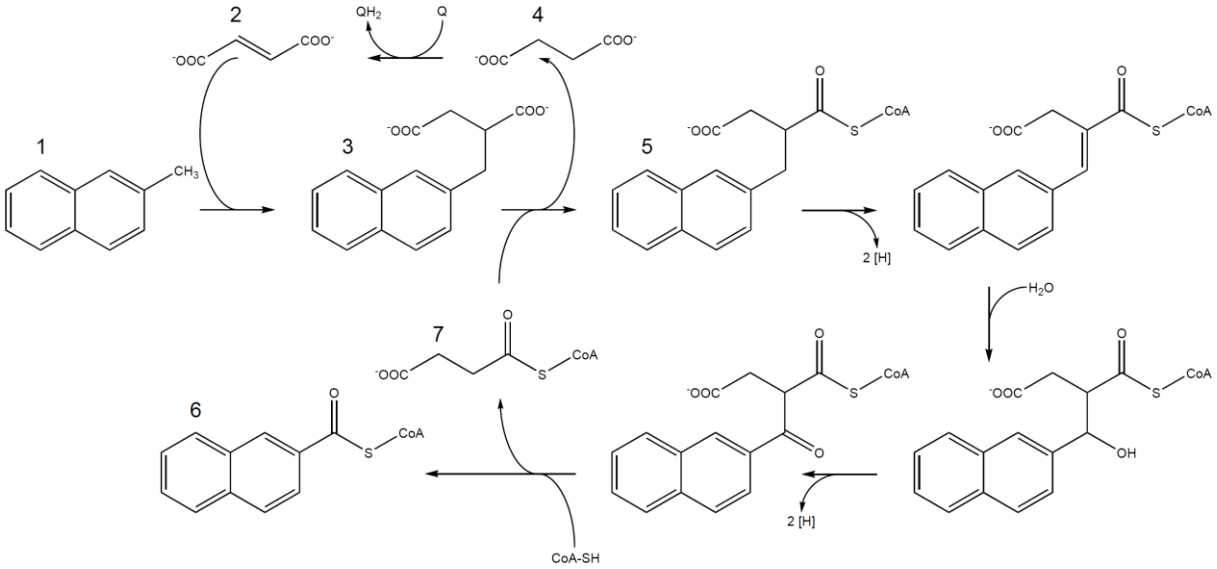


Figure 4 Catabolic pathway from 2-methylnaphthalene to 2-naphthoyl-CoA as described for the sulphate-reducing enrichment culture N47. (1): 2-methylnaphthalene. (2): fumarate. (3): 2-(naphthalene-2-yl-methyl)succinate. (4): succinate. (5): 2-(naphthalene-2-ylmethyl)succinyl-CoA. (6): 2-naphthoyl-CoA. (7): succinyl-CoA. (Q): quinone.

While 2-naphthoyl-CoA gets subsequently reduced through aryl-CoA reductases (see below), succinyl-CoA is recycled to fumarate via two additional steps: First, a succinyl-CoA:2-(naphthalene-2-ylmethyl)succinate CoA-transferase uses the CoA group of succinyl-CoA for the activation of 2-(naphthalene-2-ylmethyl)succinate, forming 2-(naphthalene-2-ylmethyl)succinyl-CoA and succinate. The latter is then oxidised to fumarate by a quinone-dependant dehydrogenase and fumarate can be used for another reaction cycle (Figure 4). The enzymes involved in this pathway are very similar to the ones responsible for anaerobic toluene degradation to benzoyl-CoA (Selesi *et al.*, 2010). Further details will be given in the subsequent section "Proteomic and transcriptomic approaches" (1.09).

The debate whether initial activation of naphthalene occurs via carboxylation or via methylation was ended by a study proving direct carboxylation of naphthalene to 2-naphthoate in crude cell extracts of N47 (Mouttaki *et al.*, 2012). Furthermore, a gene cluster probably coding for subunits of a naphthalene-carboxylase have been identified within the N47 genome (Bergmann *et al.*, 2011b). Therefore, earlier findings of metabo-

lites of 2-methylnaphthalene degradation when N47 was grown with naphthalene only could be explained by the possible reversibility of the 2-methylnaphthalene degradation pathway starting from 2-naphthoyl-CoA.

The naphthalene carboxylase reaction initially seemed to be independent of ATP (Mouttaki *et al.*, 2012), but recent assays with cell free extracts rather indicate an ATP-dependant reaction (J. Koelschbach, unpublished results). The reaction mechanism of the naphthalene carboxylase is still being investigated. As deduced from an analysis of the subunits forming the naphthalene carboxylase complex (Koelschbach, 2015), the mechanism might include cycloaddition to a prenylated flavin co-factor analogously to the recently discovered mechanism of ubiquinone decarboxylases (Payne *et al.*, 2015; White *et al.*, 2015).

Following the naphthalene carboxylase and a postulated 2-naphthoate:coenzyme A ligase, 2-naphthoyl-CoA is stepwise reduced to 5,6,7,8-tetrahydro-2-naphthoyl-CoA (Figure 5) which could be demonstrated in assays with cell free extracts of N47 and with heterologously produced enzymes (Eberlein *et al.*, 2013b; Eberlein *et al.*, 2013a; Estelmann *et al.*, 2015).

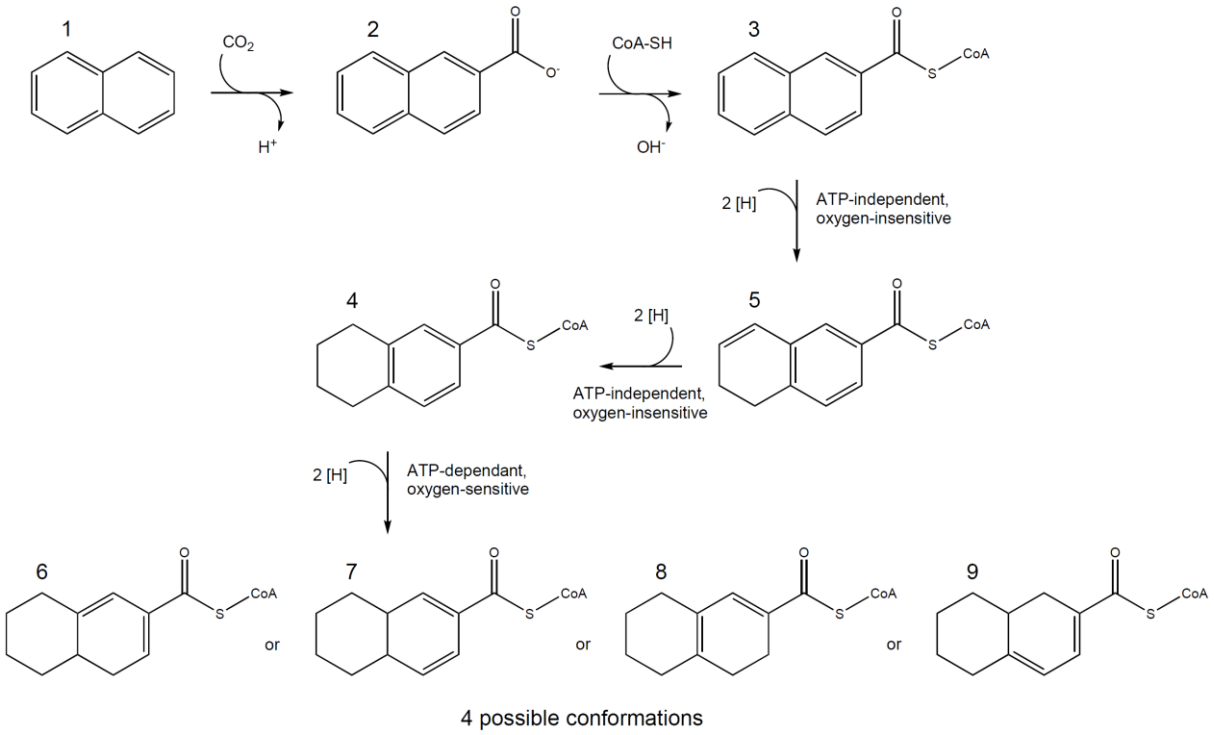


Figure 5 Initial steps of the anaerobic naphthalene degradation pathway. (1): naphthalene. (2): 2-naphthoate. (3): 2-naphthoyl-CoA. (4): 5,6,7,8-tetrahydro-2-naphthoyl-CoA. (5): 5,6-dihydro-2-naphthoyl-CoA. (6): 3,4a,5,6,7,8-hexahydro-2-naphthoyl-CoA. (7): 4a,5,6,7,8,8a-hexahydro-2-naphthoyl-CoA. (8): 3,4,5,6,7,8-hexahydro-2-naphthoyl-CoA. (9): 1,5,6,7,8,8a-hexahydro-2-naphthoyl-CoA.

The conversion of 2-napthoyl-CoA to 5,6,7,8-tetrahydro-2-naphthoyl-CoA was observed to be ATP-independent and insensitive towards oxygen and is catalysed by two enzymes belonging to the "old yellow enzyme" (OYE) family (Eberlein *et al.*, 2013b; Estelmann *et al.*, 2015). The first enzyme reduces 2-naphthoyl-CoA to 5,6-dihydro-2-naphthoyl-CoA (Figure 5), which is then further reduced to 5,6,7,8-tetrahydro-2-naphthoyl-CoA by the second OYE-like enzyme (Estelmann *et al.*, 2015). There seems to be a tight coupling of the two reductase reactions, so when both enzymes were present in the assay mixture only the final product 5,6,7,8-tetrahydro-2-naphthoyl-CoA could be observed but not the intermediate 5,6-dihydro-2-naphthoyl-CoA (Estelmann *et al.*, 2015).

In general, old yellow enzymes are oxidoreductases containing a flavin co-factor which typically use NAD(P)H as electron donor and catalyse a hydride transfer to their substrate (Williams and Bruce, 2002). It is therefore assumed that the reductions of

2-naphthoyl-CoA and 5,6-dihydro-2-naphthoyl-CoA, respectively, also proceed via hydride transfer (Estelmann *et al.*, 2015; Sawers, 2015). This is in contrast to the benzoyl-CoA reductases which are known to use a radical mechanism with sequential transfer of electrons and protons for the reduction of benzoyl-CoA (Buckel *et al.*, 2014). However, the significantly more positive redox potential for the reduction of the bicyclic aromatic system of 2-naphthoyl-CoA compared to the monocyclic system of benzoyl-CoA might allow for a reduction mechanism via hydride transfer in this case. To achieve these reductions without energy input by ATP, at least the first one presumably needs to be coupled to an exergonic electron transfer (Estelmann *et al.*, 2015; Sawers, 2015) in a so-called electron bifurcation scenario (Buckel and Thauer, 2013).

The subsequent two-electron reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA was found to proceed in an ATP-dependant and oxygen-sensitive manner and is therefore most likely catalysed by an enzyme similar to the class I benzoyl-CoA reductases (Eberlein *et al.*, 2013a). The product of this reaction was identified as one of the four fully conjugated isomers of hexahydro-2-naphthoyl-CoA (Figure 5) but the specific conformation could so far not be identified due to the very low amounts of product formed in these assays which did not allow for detailed analyses.

1.09. Proteomic and transcriptomic approaches

When extracts of NaphS2 cells grown either on naphthalene or 2-methylnaphthalene were analysed via denaturing gel electrophoresis, a 2-methylnaphthalene-specific protein band appeared (Musat *et al.*, 2009). Further analysis revealed that the corresponding protein is closely related to the aforementioned (1.02) benzylsuccinate synthases (BssA) from *T. aromatica* (Leuthner *et al.*, 1998) and it was therefore described as napthyl-2-methylsuccinate synthase NmsA. This was also confirmed by a subsequent genome analysis which additionally identified genes coding for two other proteins that form the NmsABC complex together with NmsA (DiDonato *et al.*, 2010).

A combination of proteomic, transcriptomic and confirmative real-time quantitative PCR analysis has been conducted for the comparison of NaphS2 cells grown on naphthalene,

benzoate and pyruvate (DiDonato *et al.*, 2010). Genes most likely coding for a 2-naphthoyl-CoA reductase and therefore named *ncrABCD* were up-regulated in naphthalene-grown cultures in comparison to cells grown on benzoate or pyruvate, respectively. The same held true for a gene cluster surrounding the *ncrABCD* genes that mainly consists of genes coding for β-oxidation-like proteins. Recent studies (see chapter 1.08) showed that NcrABCD is actually the 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase and that the reduction of 2-naphthoyl-CoA is achieved by completely different enzymes, the OYE-like class III (ATP-independent and insensitive towards oxygen) aryl-CoA reductases (Eberlein *et al.*, 2013b; Estelmann *et al.*, 2015). The latter enzymes are encoded by another cluster of genes (*NPH_5472 – NHP_5476*) that was up-regulated during growth on naphthalene vs. benzoate and naphthalene vs. pyruvate, respectively. It was assumed that 2-naphthoyl-CoA is stepwise reduced by the proteins encoded by the two reductase clusters and that further degradation steps are catalysed by enzymes encoded by downstream components of the cluster containing *ncrABCD* (DiDonato *et al.*, 2010). However, around 200 genes up-regulated during growth on naphthalene were detected via transcriptomic analyses, which makes it difficult to identify the ones directly involved in naphthalene degradation out of this data set. Proteomic data from this study partially support the involvement of some of the identified genes in naphthalene degradation, while other genes postulated to be involved in naphthalene degradation rather seem to be up-regulated during growth on benzoate (DiDonato et al., 2010, supporting material). Proteomic data comparing naphthalene- vs. pyruvate-grown cells have not been published.

For the enrichment culture N47, proteomic experiments were so far hampered by the fact that the naphthalene-degrading *Deltaproteobacteria* of this culture were not available as a pure culture, so when growing the culture on other substrates than naphthalene or 2-methylnaphthalene there is always the risk of a population-shift. Nevertheless, two proteomic approaches have been conducted with this culture: One was done with 2-methylnaphthalene-grown cells and the dominant proteins within the soluble fraction were sequenced and linked to genes of the N47 metagenome (Selesi *et al.*, 2010). This study identified genes similar to *nmsABC* from NaphS2 (Musat *et al.*, 2009) and genes for a potential activating protein NmsD. Besides, a gene cluster consist-

ing of eight genes (*bnsABCDEFGH*) was identified that was closely related to gene clusters of anaerobic toluene degradation to benzoyl-CoA via β-oxidation-like steps in *Aromatoleum aromaticum* strain EbN1 (Kube *et al.*, 2004), *Azoarcus* sp. strain T (Achong *et al.*, 2001) and *T. aromatica* strain K172 (Leuthner and Heider, 2000; Hermuth *et al.*, 2002). Genes coding for a putative 2-naphthoyl-CoA reductase (*ncrABCD*), that were most similar to the genes for the benzoyl-CoA reductase subunits of *Azoarcus* sp. strain CIB (Lopez Barragan *et al.*, 2004), were also identified within this study.

In another experiment, the soluble fraction of the proteome of N47 cultures grown on naphthalene was compared to the one of 2-methylnaphthalene-grown ones to identify genes involved in the initial activation of naphthalene (Bergmann *et al.*, 2011b). The most remarkable finding of this study was the identification of a gene coding for a putative subunit of a naphthalene-carboxylase, which was two-fold up-regulated in naphthalene grown cultures and showed highest similarity to a postulated anaerobic benzene carboxylase from an iron-reducing, benzene degrading culture (Abu Laban *et al.*, 2010) as well as to the α-subunit of phenylphosphate carboxylase of *A. aromaticum* strain EbN1 (Schuehle and Fuchs, 2004). A gene coding for another subunit of the putative naphthalene-carboxylase has been identified as well. The abundances of subunits of the naphthoyl-CoA reductase described earlier (Selesi *et al.*, 2010) and an enoyl-CoA hydratase (N47_E41500), potentially involved in subsequent hydrolytic ring cleavage, were significantly higher in naphthalene-grown cells than in cells grown on 2-methylnaphthalene. This study also gave a hint to some enzyme candidates possibly representing the 2-naphthoate-CoA-ligase, since they showed similarities to known CoA-ligases and were present upon growth on naphthalene but not upon growth on 2-methylnaphthalene.

1.10. Anaerobic degradation of larger PAHs

Some of the enrichment cultures capable of the anaerobic degradation of naphthalene could also degrade the unsubstituted, three-ringed PAH phenanthrene (Figure 6), so phenanthrene degraders were discovered under nitrate-reducing (Rockne and Strand,

1998, 2001; Eriksson *et al.*, 2003), sulphate-reducing (Coates *et al.*, 1996a; Zhang and Young, 1997) and methanogenic conditions (Chang *et al.*, 2005; Chang *et al.*, 2006).

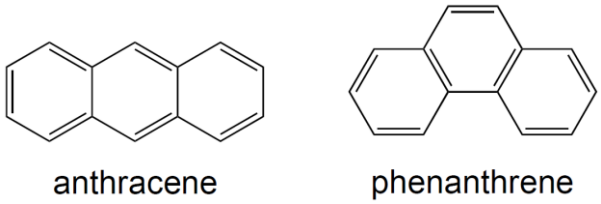


Figure 6 Molecular structures of the three-ringed PAHs anthracene (left) and phenanthrene (right).

In contrast, evidence for anaerobic degradation of anthracene, a regioisomer of phenanthrene (Figure 6), is rather scarce. There is only one culture published which can couple the mineralisation of anthracene to the reduction of nitrate, sulphate, and iron (Ramsay *et al.*, 2003) and another biphenyl-degrading culture was additionally able to grow on a mixture of anthracene and phenanthrene (Selesi and Meckenstock, 2009). Further indication of microbial degradation of anthracene was obtained from microcosms with aquifer sediments by molecular analysis of microbial communities (Wan *et al.*, 2012; Wang *et al.*, 2012) and stable isotope probing (Zhang *et al.*, 2012). The denitrifying naphthalene-degrading *Pseudomonas* strains published by McNally *et al.* were also able to utilise anthracene, phenanthrene and even larger PAHs like pyrene (McNally *et al.*, 1998b, 1999), but, as already mentioned, they unfortunately got lost (personal communication D. McNally).

Earlier studies with enrichment cultures capable of degrading a mixture of PAHs revealed that PAHs with more than three rings are not biodegradable under anaerobic conditions (Coates *et al.*, 1997; Sharak Genthner *et al.*, 1997), whereas other studies showed that PAHs with four or five rings like pyrene and benzo[a]pyrene can at least be co-metabolically degraded (Rothermich *et al.*, 2002; Maillacheruvu and Pathan, 2009). However, it seems like anaerobic microorganisms cannot use them as sole source of carbon and energy.

Interestingly, two independent enrichment cultures that could catabolise phenanthrene under sulphate reduction carboxylated this compound to the common intermediate phenanthrene-carboxylate whose carboxyl group was most likely situated at the C2 or C3 position (Zhang and Young, 1997; Davidova *et al.*, 2007). Thus it can be speculated that the anaerobic degradation pathways of larger PAHs resemble the anaerobic naphthalene-catabolism via 2-naphthoyl-CoA and are also initiated by carboxylation.

There might be further common strategies used for the anaerobic metabolisation of naphthalene as well as for the metabolisation of larger PAHs since the presence of additional rings requires adaptions of the well-known benzoyl-CoA pathway used for the degradation of monocyclic aromatic compounds. Studying the anaerobic degradation of naphthalene as model compound for PAHs in general might enable the identification of these specific strategies.

1.11. Aim of this work

The aim of this work was to gain further insights into the microbial degradation of naphthalene and to identify key enzymes and metabolites of this metabolic pathway. This knowledge is a prerequisite for the analysis of biological attenuation processes at contaminated sites. The work focussed on the following four topics:

- the activation of the known intermediate 2-naphthoate to 2-naphthoyl-CoA
- subsequent dearomatising ring reductions
- genes and enzymes involved in the downstream degradation pathway
- specific reactions required for the metabolisation of polycyclic compounds.

A special emphasis was laid on ring-opening reactions since these reactions are essential for channelling polycyclic compounds to intermediates of central metabolism which are all linear compounds.

2. Identification and characterisation of the 2-naphthoate:coenzyme A ligases of sulphate-reducing naphthalene degraders

Co-workers: Sandra Kolbeck, Andrey Zaytsev, Bernard Golding and Rainer Meckenstock

2.01. Abstract

The anaerobic degradation of naphthalene is known to proceed via coenzyme A thioesters. Candidate enzymes potentially catalysing the initial activation of the metabolite 2-naphthoate to 2-naphthoyl-CoA have been identified in the sulphate-reducing naphthalene degraders *Desulfobacterium* strain N47 and *Deltaproteobacterium* strain NaphS2. In this study, we could (i) measure 2-naphthoate:CoA ligase activity in cell free extracts of N47 and NaphS2 in aerobically conducted assays analysed by HPLC, (ii) characterise the affinity of the CoA-ligases towards substrate analogues and (iii) heterologously express the candidate gene from NaphS2 in *Escherichia coli* and proof the 2-naphthoate:CoA ligase activity of the gene-product. The activities in cell free extracts were dependant on the addition of ATP, which is in accordance with previous observations for the closely related benzoate:CoA ligases. A variety of substrate analogues were also activated to the respective CoA-thioester typically at a lower rate than the natural substrate. Like previously reported for benzoate:CoA ligases, activity towards fluorinated substrate analogues was similar to the activity towards the natural substrate whereas compounds with a hydroxyl group next to the carboxyl group could not be converted. N47 extract generally showed higher activities towards substituted or heteroaromatic bicyclic compounds than the NaphS2 extract, which in turn was more active towards compounds with a slightly altered carbon-backbone like 1-naphthoate or phenylacetate. The heterologously produced potential 2-naphthoate:CoA ligase from NaphS2 indeed conducted the respective reaction in an ATP- and Mg^{2+}-dependant manner. ADP could substitute for ATP which might provide an ATP-saving strategy. However, activity was only observed when

protein free extract of NaphS2 was added to the assay mixture. This indicates that 2-naphthoate:CoA ligase requires a so far unknown co-factor.

2.02. Introduction

2.02.01. Coenzyme A ligases involved in anaerobic degradation of aromatic compounds

Naphthalene represents the smallest member of the group of polycyclic aromatic hydrocarbons (PAHs) and is a pervasive environmental pollutant with potential carcinogenic effects (Preuss *et al.*, 2003). Its biodegradation at contaminated sites is usually limited by the availability of electron acceptors in the plume (Christensen *et al.*, 1994; Chapelle, 2000). According to recent studies, the plume-fringe is regarded as a hot-spot of biodegradation where organic contaminants are metabolised with the help of terminal electron acceptors like nitrate, sulphate, iron(III), manganese(IV) or under methanogenic conditions (Meckenstock *et al.*, 2015). Hence, the anaerobic microbial degradation of naphthalene is of high environmental importance. In sulphate-reducing bacteria like *Desulfobacterium* N47 or *Deltaproteobacterium* strain NaphS2, this process is initiated by carboxylation of naphthalene to 2-naphthoate (Mouttaki *et al.*, 2012; Cunha Tarouco *et al.*, 2013). Subsequently, the addition of a coenzyme A thioester via a 2-naphthoate:CoA ligase is expected, since the following ring-reductase reactions are known to occur on the level of 2-naphthoyl-CoA (Eberlein *et al.*, 2013b; Eberlein *et al.*, 2013a; Estelmann *et al.*, 2015). In analogy, the anaerobic degradation of monocyclic aromatic compounds like benzoate is also known to proceed via coenzyme A thioesters (Hutber and Ribbons, 1983).

Benzoate:CoA ligases are well characterised for example for the phototrophic bacterium *Rhodopseudomonas palustris* (Hutber and Ribbons, 1983; Geissler *et al.*, 1988) or the nitrate-reducer *Azoarcus evansii*, formerly known as *Pseudomonas* strain KB 740 (Altenschmidt *et al.*, 1991). For the latter organism, also a closely related enzyme for the specific activation of phenylacetate to phenylacetyl-CoA has been characterised (El-Said Mohamed and Fuchs, 1993). All these enzymes depend on energy delivered by the

hydrolysis of ATP to AMP and pyrophosphate (Geissler *et al.*, 1988; Altenschmidt *et al.*, 1991). Other nucleoside triphosphates or ADP could not be substituted for ATP (Auburger and Winter, 1992). Furthermore, these enzymes require the presence of the divalent cation Mg^{2+}. The latter can be replaced by Mn^{2+} (Geissler *et al.*, 1988; Altenschmidt *et al.*, 1991), whereas other divalent cations like Co^{2+}, Cu^{2+}, Fe^{2+}, Mo^{2+}, Ni^{2+} and Zn^{2+} had strong inhibitory effects (Altenschmidt *et al.*, 1991; Auburger and Winter, 1992; El-Said Mohamed and Fuchs, 1993). Another benzoate:CoA ligase from the denitrifier *Thauera aromatica* (Schuehle *et al.*, 2003), as well as the closely related phenylacetate:CoA ligases from *A. evansii* (El-Said Mohamed, 2000) and from *Pseudomonas putida* (Martínez-Blanco *et al.*, 1990), are known to be involved in aerobic and anaerobic pathways. Unlike the ring-reductases, these enzymes are therefore expected to be oxygen-insensitive.

The activity of a CoA:ligase facilitates substrate uptake into the cell since the conversion of the substrate allows to maintain a downhill substrate-concentration gradient between the cell cytoplasm and the cell surroundings (Harwood and Gibson, 1986). The formed CoA-thioester is trapped inside the cell since its bulky and polar structure prevents diffusion through the cytoplasmic membrane. The ring-reduction occurring during the anaerobic anabolism of aromatic compounds is also facilitated by the presence of a CoA-thioester due to its electron-drawing potential and its ability to stabilise radical anions produced as intermediates during the two one-electron transfer steps (Buckel and Keese, 1995), which greatly lowers the midpoint potential of the first electron transfer step and the overall process (Boll and Fuchs, 1995; Heider and Fuchs, 1997; Boll, 2005b). Furthermore, the CoA substituent is expected to support binding and correct positioning of the substrate (Boll and Fuchs, 1995).

2.02.02. Potential 2-naphthote:CoA ligases

In analogy to the aforementioned benzoate:CoA ligases, 2-naphthoate:CoA ligases are expected to couple the synthesis of 2-naphthoyl-CoA to the hydrolysis of ATP to AMP and pyrophosphate (Figure 7).

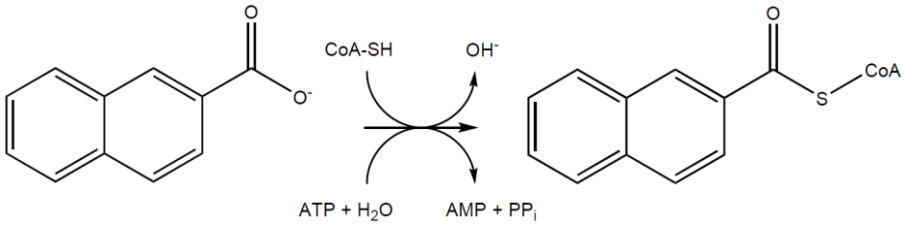


Figure 7 Proposed reaction scheme for the conversion of 2-naphthoate to 2-naphthoyl-CoA.

Three genes potentially coding for a 2-naphthoate:CoA ligase have been identified in the genome of the sulphate-reducing naphthalene degrader *Desulfobacterium* N47 (Bergmann *et al.*, 2011b). The respective gene products shared significant sequence similarities with the benzoate:CoA ligase from *R. palustris* and the genes were found to be expressed during growth on naphthalene. In *Deltaproteobacterium* strain NaphS2, another sulphate-reducing naphthalene degrader, the protein NPH_5477 has been identified as potential 2-naphthoate:CoA ligase because of its sequence similarity to the phenylacetate:CoA ligase from *Escherichia coli* (DiDonato *et al.*, 2010). This assumption is further supported by the fact that the NPH_5477 encoding gene clusters with genes coding for the recently identified 2-naphthoyl-CoA and 5,6-dihydro-2-naphthoyl-CoA reductases from the old yellow enzyme (OYE) family (DiDonato *et al.*, 2010; Eberlein *et al.*, 2013b; Estelmann *et al.*, 2015). A homologous protein, N47_I06840, is encoded by the N47 genome, but this gene is not part of a reductase encoding gene cluster.

The aim of the following work was to measure the 2-naphthoate:CoA ligase activity in cell free extracts of N47 and NaphS2 towards its natural substrate as well as towards substrate analogues and to heterologously produce the respective enzymes in *E. coli*.

2.03. Experimental procedures

2.03.01. Growth of bacterial cells

The sulphate-reducing enrichment culture N47 (Meckenstock *et al.*, 2000) was cultivated anaerobically in 200 mL serum bottles filled with 150 mL carbonate-buffered freshwater medium (Widdel *et al.*, 1983; Widdel and Bak, 1992). Sulphate (20 mM) was

added as terminal electron acceptor and 2% (v/v) of a 1.5% (w/v) naphthalene stock solution in 2,2,4,4,6,8,8-heptamethylnonane was added as carbon and electron source. The headspace contained N_2/CO_2 (80:20). The bottles were closed with butyl stoppers (Glasgeraetebau Ochs, Goettingen, Germany) and incubated at 30 °C in the dark after inoculation. Every four weeks, subcultures in fresh medium were inoculated with 15 mL of the previous culture.

Deltaproteobacterium strain NaphS2 (Galushko *et al.*, 1999) (DSM No. 14454) was obtained from the Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Cultures were grown anaerobically in butyl rubber-sealed 200 mL serum bottles containing 150 mL artificial sea water medium (Galushko *et al.*, 1999) with 21.1 mM sodium sulphate (3 g/L) as terminal electron acceptor and 2-naphthoate (2 mM) as carbon source.

For large volumes of the enrichment culture N47 or *Deltaproteobacterium* strain NaphS2, 2 L Schott bottles containing 1.6 L of the media described above were inoculated with 160 mL of a 4-weeks (NaphS2) or 5-weeks (N47) old pre-culture, sealed with butyl stoppers and incubated for 4 – 5 weeks (NaphS2) or 6 – 7 weeks (N47) before harvest. For large N47 cultures, paraffin oil was used as carrier phase for naphthalene instead of heptamethylnonane analogously to the description above.

2.03.02. Preparation of cell free extracts

Cells were harvested at ambient air 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 L initial culture). After washing, cell pellets could be stored for several months at -80 °C without significant loss of 2-naphthoyl-CoA ligase activity.

The cells were opened either by three freeze and thaw cycles or by using a French press (Thermo Electron, Waltham, USA) operated at 6.9 MPa (1000 psi) hydraulic pressure and a working pressure of 137 Mpa (20,000 psi) at the outlet of the miniature pressure

cell (Thermo Electron, Waltham, USA). To obtain the cell free extract, cell debris was afterwards segregated by centrifugation for 15 min at 20,800 × *g* and 4 °C.

2.03.03. Preparation of protein free extracts

For the preparation of protein free extracts, the proteins contained in cell free extracts (2.03.02) were precipitated by the addition of two volumes of methanol to one volume of cell free extract and subsequent centrifugation at 20,800 × *g* and 4 °C. The supernatant was diluted with the same volume of *Milli*-Q water, frozen at -80 °C and lyophilised. For the use as an additive in enzyme assays, the powder obtained after lyophilisation was dissolved in enzyme test buffer (see below).

2.03.04. Plasmid construction

Genomic DNA of the enrichment culture N47 and Deltaproteobacterium NaphS2 was isolated from mid-exponential cultures as described previously (Lueders *et al.*, 2004). Target genes were amplified from genomic DNA of N47 with the Phusion Hot Start II DNA Polymerase (Thermo Fisher Scientific, Ulm, Germany) following the manufacturer's protocol and using the primer pairs listed in Table 1.

Table 1 Primers used for the amplification of genes potentially coding for naphthoyl-CoA ligases

Gene name	Primer name	Primer sequence (5' → 3')	Restriction sites insert
N47_I06840	I06840-for	ATATACATGTGTCTATATACTCAGAAGGCC	PciI
	I06840-rev	TATACTCGAGTTTTCGTTTGCGGTGGTCAAAC	XhoI
NPH_5477	5477-for	ATATCCATGGGACAACTTTATAGTGATAAA-	NcoI
	5477-rev	ATATCTCGAGTTTCTTTTTTTCACGATGGTCAAATAC	XhoI

Primers were ordered from Eurofins Genomics, Ebersberg, Germany. The optimal annealing temperatures of each primer pair were determined by a temperature gradient PCR prior to the amplification reaction. The amplified genes were cloned into the pETM-

13 vector (European Molecular Biology Laboratory, Heidelberg, Germany) via the NcoI and XhoI restriction sites or via the PciI and XhoI sites, respectively (see Table 1). All restriction endonucleases used were FastDigest® enzymes from Thermo Fisher Scientific. The resulting plasmids enabled the heterologous expression of target proteins with a C-terminal His_6-tag under the control of the T7 promotor.

After ligation of the DNA fragments with T4 DNA Ligase (Thermo Fisher Scientific), *E. coli* strain DH5α was transformed with the resulting plasmids (Hanahan, 1985). Colony PCR with one of the primers used for gene amplification and one primer annealing either in the T7 promotor or the terminator region of the pETM-13 vector was performed in order to identify clones carrying the correct plasmid. The PCR reaction was performed with the DreamTaq Green DNA Polymerase (Thermo Fisher Scientific) according to the manufacturer's protocol (annealing at 64 °C) and analysed via agarose gel electrophoresis (Sambrook *et al.*, 1989). Single colonies of positive clones were grown in liquid LB medium (Bertani, 1951) with 30 µg/mL kanamycin sulphate at 30 °C and 200 rpm and plasmid DNA was isolated from these cultures using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). Plasmid identity was verified by sequencing (Eurofins Genomics, Ebersberg, Germany).

2.03.05. Heterologous production and purification of His_6-tagged proteins

The expression strains *E. coli* BL21(DE3) (Studier and Moffatt, 1986) and *E. coli* Rosetta™ 2(DE3) (Merck Millipore, Darmstadt, Germany) were transformed with the constructed plasmids (Hanahan, 1985). For gene expression, pre-cultures were grown in liquid LB medium (Bertani, 1951) with 30 µg/mL kanamycin sulphate and 34 µg/mL chloramphenicol (the latter only for *E. coli* Rosetta™ 2) at 37 °C and 200 rpm. Production cultures were inoculated with 0.1% (v/v) of a pre-culture with OD_{600nm} = 1 and grown in auto-induction medium ZYM-5052 (Studier, 2005) with 100 µg/mL kanamycin sulphate and, where required, 34 µg/mL chloramphenicol at 37 °C and 120 rpm (baffled flasks).When the main cultures reached an OD_{600nm} of 0.5, the temperature was lowered

to 20 °C and 10 mM (*S*)-cysteine were added to establish slightly reducing conditions during gene expression.

Cells were harvested by centrifugation for 15 min at 11,300 × *g* and 4 °C. The cell pellets were resuspended in 50 mM sodium phosphate buffer pH 8.0 containing 0.05% (v/v) 10 mM imidazole and opened by sonication on a UP50H homogeniser (Hielscher Ultrasonics GmbH, Teltow, Germany) for 2 x 10 min on ice. Instrument settings were cycle 0.6 and amplitude 60%. Cell debris was segregated by centrifugation for 15 min at 3,900 × *g* and 4 °C and the supernatant was loaded to a His GraviTrap™ TALON® column (GE Healthcare, Freiburg, Germany). Target enzymes were purified according to the manufacturer's protocol. All buffers used for the purification were based on 50 mM sodium phosphate buffer pH 8.0 with 0.05% (v/v) 2-mercaptoethanol. Washing buffers contained 20 or 50 mM imidazole, elution buffers 100 mM imidazole. Purity of the eluted enzymes was analysed via SDS-PAGE (Laemmli, 1970). For long term storage of purified enzymes, the buffer was changed to 50 mM TRIS/HCl pH 8.0 with 10% (v/v) glycerol and 2 mM dithiothreitol (DTT).

Prior to large scale production, a test production in small scale was performed. The subsequent test purification was conducted via His SpinTrap™ TALON® columns (GE Healthcare, Freiburg, Germany) according to the manufacturer's protocol and analysed via SDS-PAGE (Laemmli, 1970). The *E. coli* strain that gave better results in test production assays was used for large scale expression.

2.03.06. Synthesis of fluorinated analogues of 2-naphthoic acid

Three fluorinated analogues of 2-naphthoic acid, 1-, 3- and 4-fluoro-2-naphthoic acid, were synthesised by Dr. Andrey Zaytsev in the group of Prof. Dr. Bernard Golding, School of Chemistry, University of Newcastle, UK.

2.03.07. Discontinuous ligase assays

MOPS/KOH buffer (100 mM, pH 7.3) with 15 mM $MgCl_2$ was used as standard enzyme test buffer. The reaction mixture additionally contained 1 mM 2-naphthoate, 1 mM

CoA-SH, 5 mM ATP and 2 mM DTT and the reaction was started by the addition of 20% (v/v) cell free extract (2.03.02) or purified enzymes (2.03.05). Protein free extracts (2.03.03) were added to some assays with purified enzymes.

The assays were incubated at 30 °C and 900 rpm. Samples were taken at different time points and stopped by the addition of the double volume methanol containing 0.1% (v/v) formic acid. Samples were centrifuged for 15 min at 20,800 × *g* and 4 °C and the supernatant was subsequently analyses by HPLC (see below).

To test the substrate range of the 2-naphthoyl-CoA ligase, some assays contained chemical analogues of 2-naphthoate (1-naphthoate, 1-, 3-, 4- or 6-fluoro-2-naphthoate, 2-, 3 or 6-quinolinecarboxylate, 3-isoquinolinecarboxylate, 3- or 6-amino-2-naphthoate, 1-, 3- or 6-hydroxy-2-naphthoate, 1,2,3,4- or 5,6,7,8-tetrahydro-2-naphthoate, phenyl-acetate, benzoate, 3,4-dimethylbenzoate or cyclohexanecarboxylate) instead of the natural substrate. In further experiments ADP, CTP, GTP, ITP or UTP were added instead of ATP or Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+} or Zn^{2+} instead of Mg^{2+}.

2.03.08. HPLC analysis

HPLC analyses were carried out with a universal, modular Bischoff HPLC system (Bischoff Chromatography, Leonberg, Germany) using an Eclipse-XDB-C18 column, 150 × 4.6 mm, 5 µm particle size (Agilent Technologies, Waldbronn, Germany). Eluent A was 10 mM potassium phosphate buffer pH 6.0, eluent B acetonitrile. A gradient from 5 – 30% B was applied over 25 min at a flow rate of 1 mL/min. CoA-esters were detected via a diode array detector (DAD) at 260 nm.

2.04. Results

2.04.01. Ligase assays in cell free extracts

The ATP-dependant formation of 2-naphthoyl-CoA from 2-naphthoate and free coenzyme A could be observed in cell free extracts of aerobically harvested N47 and NaphS2 cultures via a discontinuous *in vitro* assay analysed via HPLC. As shown exemplarily for

the conversion in cell free extract of NaphS2 (NaphS2-cfe), a peak representing 2-naphthoate (retention time ca. 11 min) decreased during the incubation and a peak representing 2-naphthoyl-CoA (retention time ca. 14 min) increased simultaneously (Figure 8).

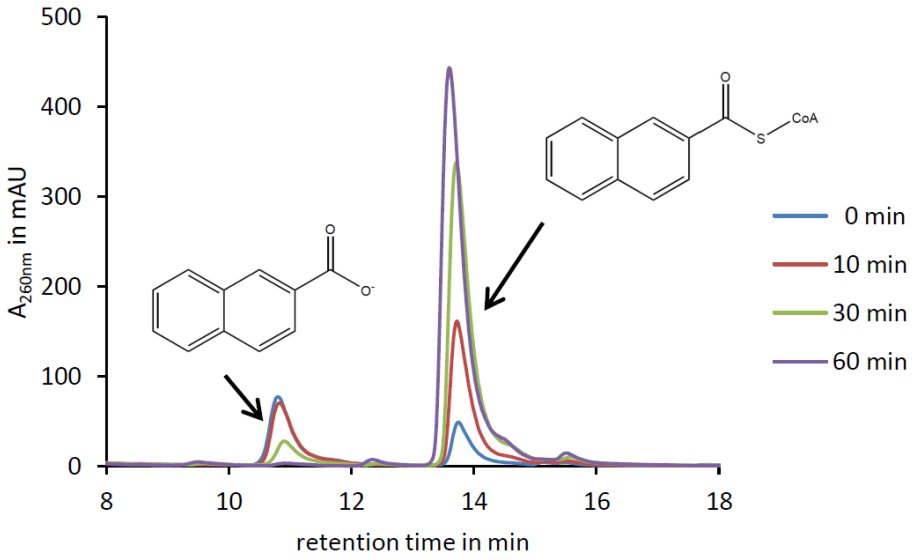


Figure 8 HPLC chromatograms depicting the time course of the 2-naphthoate:CoA ligase assay measured *in vitro* towards the natural substrate with cell free extract of *Deltaproteobacterium* NaphS2.

After 60 min of incubation, the initially added 1 mM of 2-naphthoate was completely converted to the respective CoA-thioester. The small amount of 2-naphthoyl-CoA detected in the sample taken at t = 0 min was introduced via the cell free extract which contained it as metabolite. In NaphS2-cfe, some ligase activity (ca. 20% of the activity measured in standard assays) was also observed without addition of ATP, probably because the cell free extract already contained some ATP. This activity without ATP addition was not observed in N47-cfe, but in contrast to NaphS2-cfe, this extract appeared to contain free coenzyme A since partial ligase activity was in this case observed even without addition of the latter co-factor.

Cell free extracts of both strains showed only slightly lower ligase activities when ATP was replaced by other nucleoside triphosphates (CTP, GTP, ITP or UTP) or by ADP.

However, the extracts possibly contained kinase activities enabling the regeneration of ATP from other NTPs or ADP, so the nucleoside-specificity of the ligases can only be determined in assays with purified enzymes.

2.04.02. Ligase assays with substrate analogues

Substituted analogues of 2-naphthoate or heteroaromatic compounds (quinoline-derivatives) were also converted to their CoA-thioesters in cell free extracts of the two naphthalene degraders N47 and NaphS2 as exemplarily shown in Figure 9 for the conversion of 1-fluoro-2-naphthoate in NaphS2-cfe.

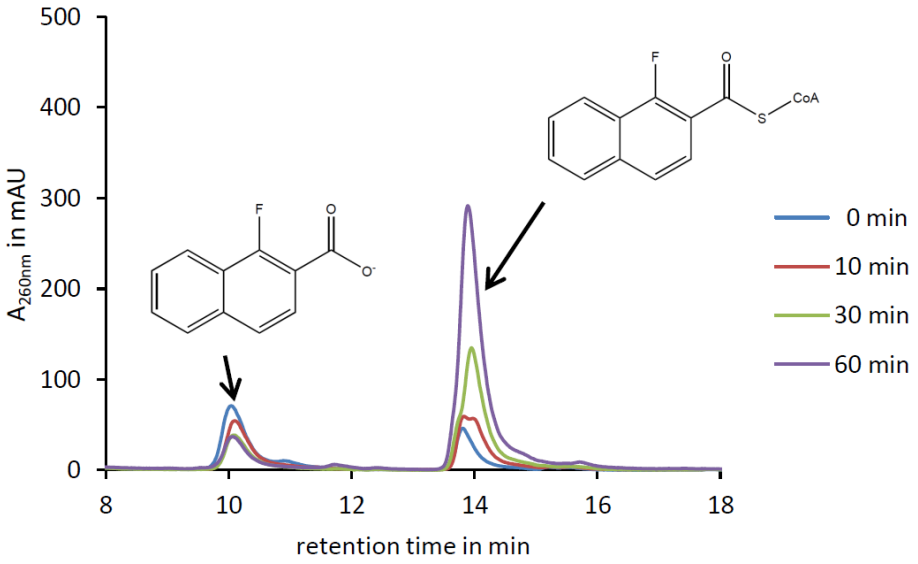


Figure 9 HPLC chromatograms showing the time course of the 2-naphthoate:CoA ligase assay measured *in vitro* towards the substrate analogue 1-fluoro-2-naphthoate with cell free extract of *Deltaproteobacterium* NaphS2.

The peak with an elution time of ca. 10 min represents the substrate analogue 1-fluoro-2-naphthoate and decreases during the incubation. The peak of the newly formed 1-fluoro-2-naphthoyl-CoA overlaps with the peak of 2-naphthoate introduced via the

cell free extract due to the very similar elution times. This effect can be observed best at the chromatogram of the sample taken after 10 min incubation (red line).

Unlike observed for the natural substrate 2-naphthoate, the 1 mM of this substrate analogue is only partially converted after an incubation time of 60 min. For a rough comparison of substrate specificities, the relative ligase activity of each substrate analogue was calculated as follows: The amount of CoA-ester formed from the substrate analogue after 60 min was divided by the amount of 2-naphthoyl-CoA formed from 2-naphthoate by the same extract within the same time. For NaphS2-cfe, the relative ligase activity towards 1-fluoro-2-naphthoate was 66%. In contrast, conversion of the same substrate analogue in cell free extract of N47 (N47-cfe) yielded the same amount of CoA-ester as the conversion of the natural substrate in this extract. Also the relative ligase activities towards other substituted or heteroaromatic substrates analogues were higher in N47-cfe than in NaphS2-cfe (Figure 10).

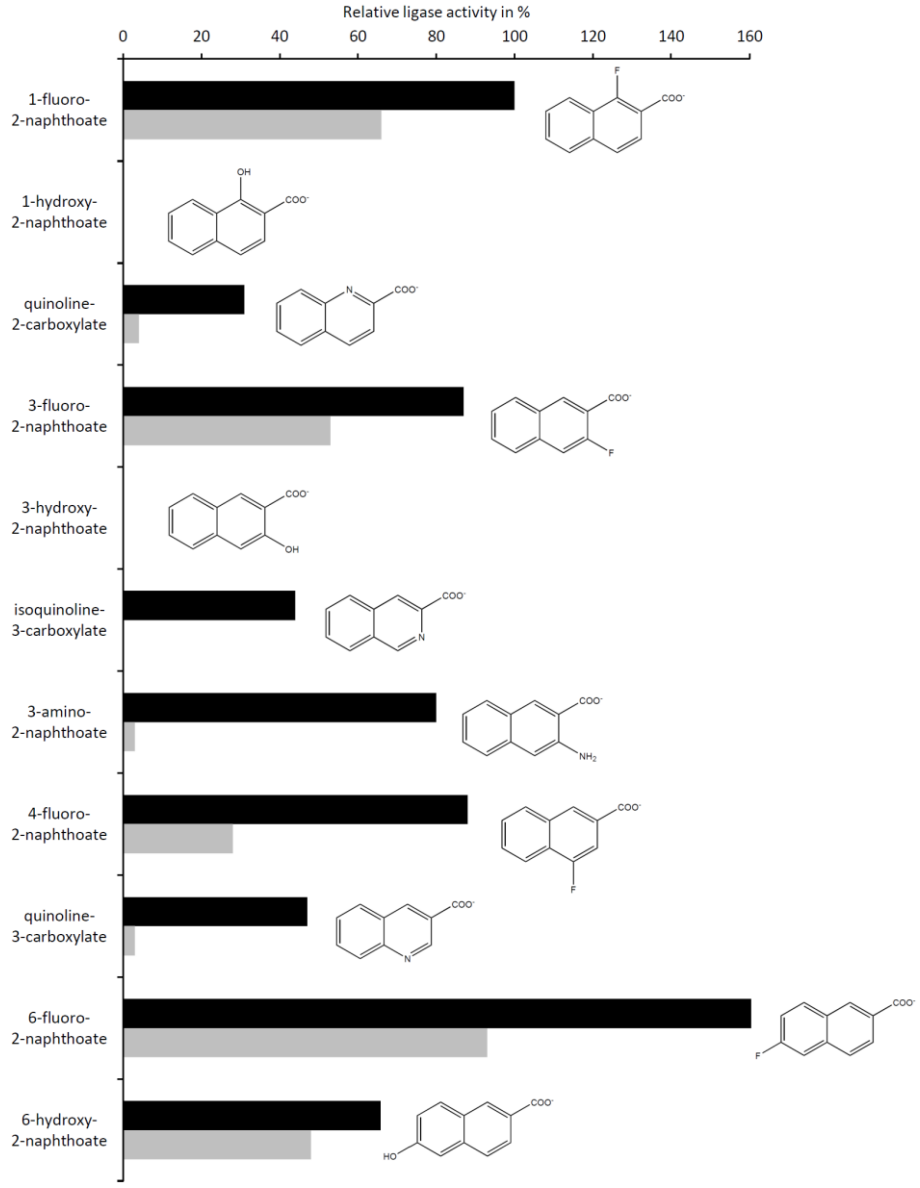


Figure 10 Relative CoA-ligase activity in cell free extracts towards substituted substrate analogues. Activity towards 2-naphthoate was used as reference. Black bars: N47; grey bars: NaphS2.

All substrate analogues with a fluorine atom at the carboxylated ring were converted with a relative ligase activity around 100% in N47-cfe. They were also converted in NaphS2-cfe, but with a significantly lower relative activity. The ligase activity in N47-cfe towards 6-fluoro-2-naphthoate, which has a fluorine-atom at the non-carboxylated ring, was even higher than towards the natural substrate (163%). In NaphS2-cfe, the activity for this analogue was similar to the activity for 2-naphthoate.

An explicit difference between the two extracts was observed for the relative ligase activities towards 3-amino-2-naphthoate, which was 80% in N47-cfe but only 3% in NaphS2-cfe. For the nitrogen containing heteroaromatic compounds quinoline-2-carboxylate, quinoline-3-carboxylate and isoquinoline-3-carboxylate the relative activities were 31 – 47% in N47-cfe and 0 – 4% in NaphS2-cfe.

Substitution with a hydroxyl group next to the carboxylated C2-position appeared to have the most detrimental effect on ligase activity. 1- and 3-hydroxy-2-naphthoate were not converted in either of the cell free extracts. In contrast, the C6-substituted 6-hydroxy-2-naphthoate was converted in N47-cfe as well as in NaphS2-cfe with a relative ligase activity of 66% and 48%, respectively.

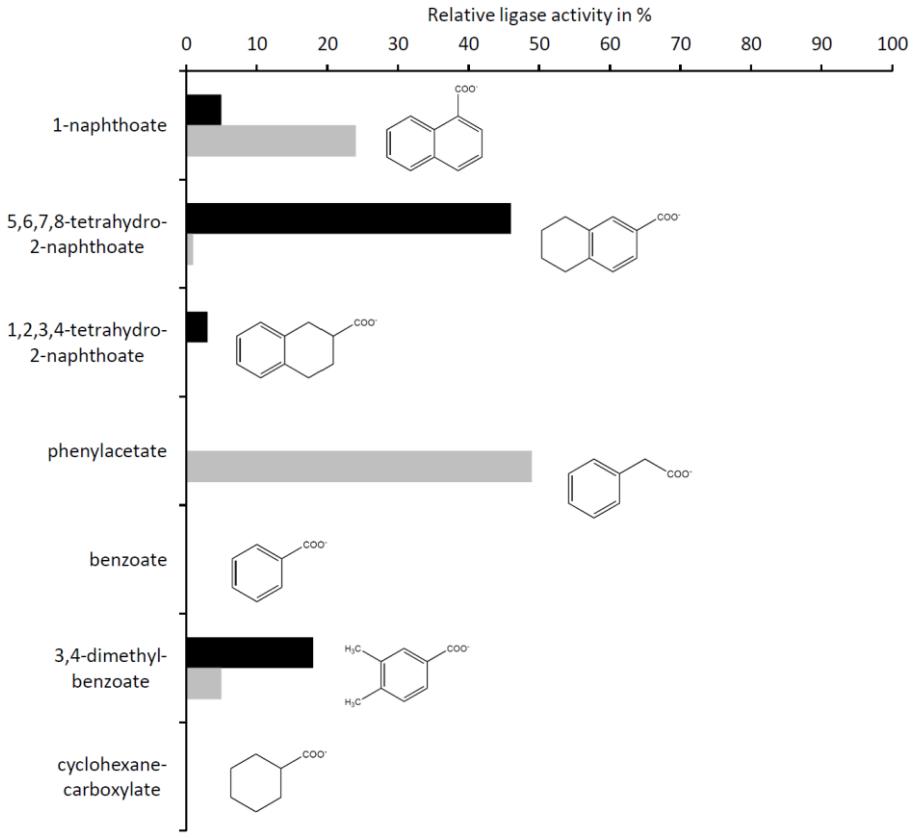


Figure 11 Relative CoA-ligase activity in cell free extracts towards substrates similar to 2-naphthoate. Activity towards 2-naphthoate was used as reference. Black bars: N47; grey bars: NaphS2.

The monocyclic compounds benzoate and cyclohexanecarboxylate were not converted, whereas 3,4-dimethylbenzoate was converted at least with a low relative ligase activity (18% and 5%, respectively) in N47-cfe and NaphS2-cfe. The two methyl groups of this compound mimic a second ring to some extent. Therefore, the observed ligase activity indicates that both 2-naphthoate:CoA ligases are specific for bicyclic compounds.

The partially reduced substrate analogues 1,2,3,4- and 5,6,7,8-tetrahydro-2-naphthoate were both substantially better converted in N47-cfe than in NaphS2-cfe. The relative ligase activities were 46% versus 1% for 5,6,7,8-tetrahydro-2-naphthoate, which resembles a derivative of 2-naphthoate that is reduced by four electrons at the non-carboxylated ring. In contrast, relative ligase activity towards 1,2,3,4-tetrahydro-2-naphthoate, a derivative reduced at the carboxylated ring, was only 3% in N47-cfe while no conversion could be observed in NaphS2-cfe.

The two substrate analogues in which the relative position of the carboxyl group is shifted in comparison to 2-naphthoate were both better converted in NaphS2-cfe. Towards 1-naphthoate, relative ligase activities were 5% in N47-cfe and 24% in NaphS2-cfe. For phenylacetate, no conversion could be detected in N47-cfe but a comparably high relative ligase activity of 49% was observed in naphS2-cfe.

2.04.03. Heterologous gene expression

The putative 2-naphthoate:CoA ligase from NaphS2, NPH_5477, and its homologues from N47, N47_I06840, were heterologously produced as His_6-tagged in *E. coli*. The production of N47_I06840 yielded an insoluble protein, even if reducing agents like (*S*)-cysteine, dithioerythritol (DTE) or dithiothreitol (DTT) were added during heterologous gene-expression. Contrariwise, NPH_5477 could be obtained as soluble enzyme when produced in presence of the reducing agent (*S*)-cystein. The subsequent purification via His_6-affinity chromatography yielded an almost pure enzyme in the elution fraction with 100 mM imidazole (Figure 12).

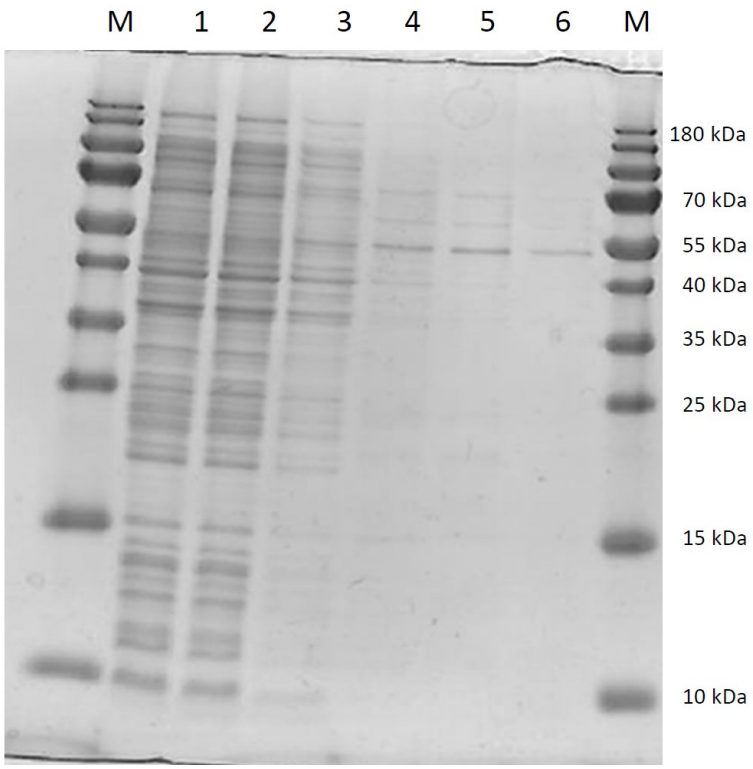


Figure 12 Analysis of the purification of NPH_5477 via SDS-PGAE. (M): PageRuler™ Prestained Protein Ladder (ThermoFisher Scientific). (1): cell free extract. (2): flow-through. (3): wash 1 with 25 mM imidazole. (4): wash 2 with 25 mM imidazole. (5): elution with 100 mM imidazole. (6): elution with 250 mM imidazole.

Derived from its migration in an SDS-PAGE, the molecular mass of the purified protein was estimated to be slightly below 55 kDa, which is in accordance with the calculated mass of 51,96 kDa for the His_6-tagged NPH_5477-enzyme.

2.04.04. Enzyme assays with purified NaphS2-ligase

The heterologously produced ligase from NaphS2 was tested for activity in ligase-assays analogously to the ones with cell free extracts. However, 2-naphthoate:CoA ligase activity was only detected when protein free extract of NaphS2 (2.03.03) was added to the assay mixture (Figure 13).

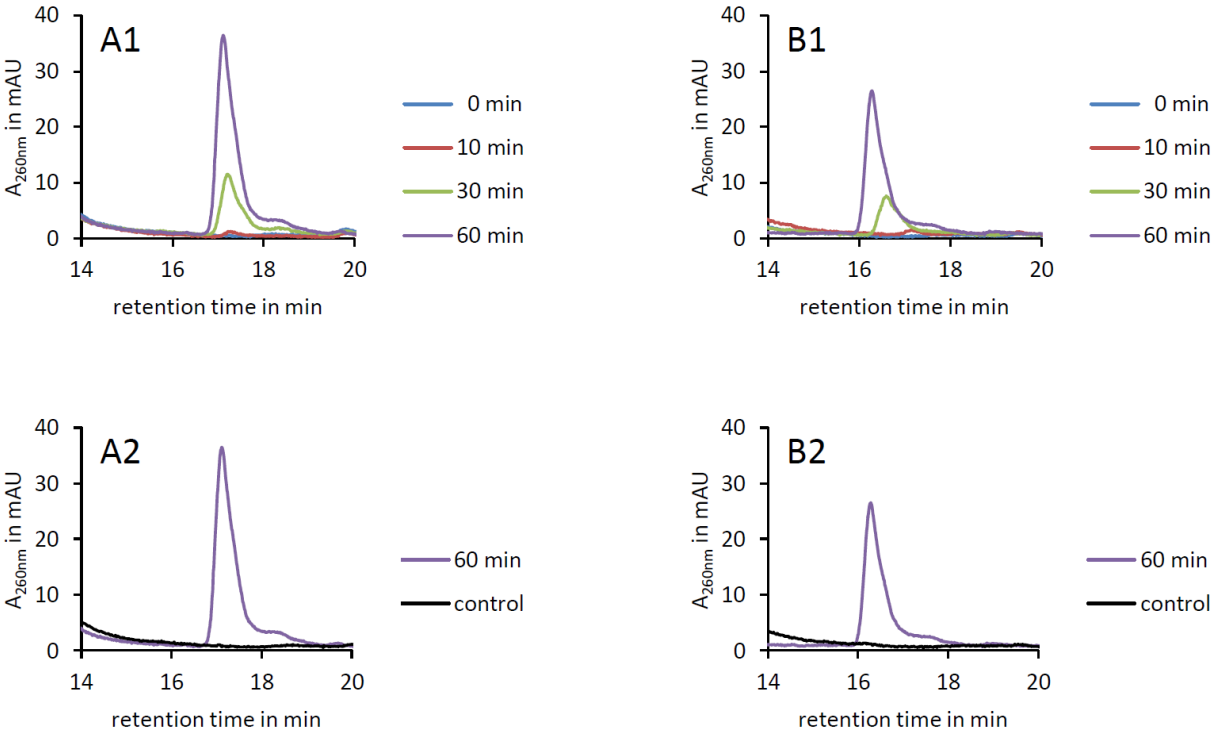


Figure 13 HPLC chromatograms of the 2-naphthoate:CoA ligase assay with heterologously produced putative ligase enzyme NPH_5477 and protein free extract of NaphS2. (A1): time-course of conversion in presence of ATP. (A2): comparison with ATP-containing control assay without enzyme. (B1): time-course of conversion in presence of ADP. (B2): comparison with ADP-containing control assay without enzyme.

In presence of ATP (A1) or ADP (B1), an increase of a peak representing 2-naphthoyl-CoA (elution time ca. 17 min) was observable during the incubation time. The difference in retention time compared to previously shown ligase-assays with cell free extracts was caused by a slightly altered HPLC-gradient. Comparison with control-assays without enzyme (A2 and B2) clearly indicated that the observed ligase-activities were caused by the heterologously produced NPH_5477 enzyme.

Up to now, it remains unclear which co-factor from the protein free extract of NaphS2 cells is necessary to support ligase activity of the purified enzyme. Neither the replacement of Mg^{2+} in the assays by Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+} or Zn^{2+} nor the addition of CTP, GTP, ITP or UTP instead of ATP resulted in activity when the protein free extract was omitted from the assay.

2.05. Discussion

An ATP-dependant 2-naphthoate:CoA ligase activity could be measured in cell free extracts of the sulphate-reducing naphthalene degraders N47 and NaphS2. This activity was insensitive towards oxygen. ATP could be replaced by other nucleoside triphosphates (NTPs) without significant loss of ligase activity. Nevertheless, it still needs to be proven with purified enzymes that the ligases indeed accept other NTPs as co-substrates and the observed activity is not only caused by kinases that regenerate ATP from ADP / AMP and NTPs. Previously studied benzoate:CoA ligases or phenyl-acetate:CoA ligases showed no activity when ATP was replaced by other NTPs (Geissler *et al.*, 1988; Auburger and Winter, 1992; El-Said Mohamed and Fuchs, 1993). However, those studies used a coupled enzyme assay with myokinase (formation of 2 ADP from ATP and AMP), pyruvate kinase (formation of ATP and pyruvate from ADP and phosphoenol-pyruvate) and lactate dehydrogenase (reduction of pyruvate with NADH, forming lactate and NAD^+) for the quantification of ligase-activity via the formation of NAD^+ (Geissler *et al.*, 1988; Ziegler *et al.*, 1989). This coupling system might not work for other NTPs and is certainly not suitable for testing for an ADP-mediated ligase-activity.

The ligase activities we measured in both cell extracts were specific for 2-naphthoate and a few other biaromatic carboxylic acids. The monoaromatic carboxylic acid benzoate or its aliphatic analogue cyclohexanecarboxylate did not serve as substrates. This indicates that there are no other CoA-ligases with specificity for cyclic substrates but the 2-naphthoate:CoA ligase active in the extract. It was therefore possible to estimate the substrate specificities of the latter by activity assays with cell free extracts. Nevertheless, these results should be verified by assays with purified enzymes once available.

The narrow substrate range of 2-naphthoate:CoA ligases is in agreement with previous observations for the substrate specificities of benzoate:CoA ligases (Geissler *et al.*, 1988; Auburger and Winter, 1992; Schuehle *et al.*, 2003). These enzymes reacted with lower affinity on substituted derivatives of benzoate and on heteroaromatic compounds but showed only very little or no activity towards cyclohexanecarboxylate or linear carboxylic acids. The closely related phenylacetate:CoA ligase was also found to be highly specific for its substrate and did not activate analogous compounds (El-Said Mohamed and Fuchs, 1993). Unlike in N47-cfe, phenylacetate was converted with a comparably

high relative ligase activity in NaphS2-cfe. Regarding the high sequence similarity of the postulated 2-naphthoate:CoA ligase enzyme from NaphS2 to phenylacetate-CoA ligases, this activity might be an evolutionary relict.

Regarding substituted substrate analogues, highest CoA-ligase activity was observed towards compounds with a fluorine functionality. Also previously characterised benzoate:CoA ligases showed almost the same activity with fluorobenzoates as with benzoate (Schennen *et al.*, 1985; Geissler *et al.*, 1988; Altenschmidt *et al.*, 1991; Auburger and Winter, 1992; Schuehle *et al.*, 2003; Lopez Barragan *et al.*, 2004). The *ortho*-substituted analogue 2-fluorobenzoate was even found to serve as alternative growth substrate for anaerobic benzoate degraders (Schennen *et al.*, 1985). Hence, anaerobic naphthalene degraders might also be able to grow on fluorinated analogues of 2-naphthoate. Growth experiments in our lab indicated that 3-fluoro-2-naphthoate can indeed serve as sole carbon source for *Deltaproteobacterium* NaphS2 (P. Weyrauch, unpublished results).

Conversion of hydroxylated substrate analogues was only observed for 6-hydroxy-2-naphthoate, which carries a hydroxyl group on the non-carboxylated ring. Analogues of 2-naphthoate with a hydroxyl group (1- and 3-hydroxy-2-naphthoate) next to the carboxyl group were not converted by the ligases from anaerobic naphthalene degraders. In accordance with this findings, most benzoate:CoA ligases are also not active towards hydroxybenzoates (Geissler *et al.*, 1988; Altenschmidt *et al.*, 1991; Laempe *et al.*, 2001). Some enzymes might be able to activate 3-hydroxy- or 4-hydroxybenzoate like the one from *Azoarcus* sp. strain CIB, but 2-hydroxybenzoate is typically not converted (Lopez Barragan *et al.*, 2004). The only exception is the enzyme from *R. palustris*, which converts 2-hydroxybenzoate (salicylate) with a low relative ligase activity of 5% (Geissler *et al.*, 1988). Bacteria that metabolise hydroxybenzoates have evolved specific CoA-ligases for the activation of 3-hydroxybenzoate (Altenschmidt *et al.*, 1993; Laempe *et al.*, 2001) or 4-hydroxybenzoate (Biegert *et al.*, 1993; Gibson *et al.*, 1994), respectively. These enzymes are likewise not active towards 2-hydroxybenzoate (Altenschmidt *et al.*, 1993; Biegert *et al.*, 1993) and there are no specific 2-hydroxybenzoate:CoA ligases known to date.

The amino-substituted 3-amino-2-naphthoate was converted with 80% relative ligase activity by 2-naphthoate:CoA ligase from N47 but with only little activity by the enzyme from NaphS2. Also benzoate:CoA ligases showed different specificities towards 2-amino-benzoate (anthranilate) in previous studies. While the enzymes from *A. evansii*, *Azoarcus* sp. strain CIB and *T. aromatica* had relative activities of 28%, 35% and 60%, respectively, towards this substrate analogue (Altenschmidt *et al.*, 1991; Schuehle *et al.*, 2003; Lopez Barragan *et al.*, 2004), the enzyme from *R. palustris* and a benzoate:CoA ligase isolated from an anaerobic benzoate degrading mixed culture showed very little or no activity with anthranilate (Geissler *et al.*, 1988; Auburger and Winter, 1992). For a specific activation of this substrate, anthranilate converting bacteria typically employ a separate anthranilate:CoA ligase (Altenschmidt *et al.*, 1991; Lochmeyer *et al.*, 1992; Coleman *et al.*, 2008).

Towards the nitrogen-containing heteroaromatic substrate analogues the ligase from N47 had once again a higher activity than the enzyme from NaphS2. Nevertheless, activity for these analogues was even in N47 significantly lower than for the natural substrate. In analogy, the monocyclic heteroaromatic compounds nicotinate and picolinate, which represent one-ringed analogues of 2-quinolinecarboxylate and 3-isoquinoline-carboxylate (nicotinate) or 3-quinolinecarboxylate (picolinate), respectively, were converted by benzoate:CoA ligases, but at a lower rate compared to the natural substrate (Geissler *et al.*, 1988; Auburger and Winter, 1992). This rate was significantly lower for nicotinate (heteroatom in *ortho*-position) than for picolinate (heteroatom in *meta*-position). Our data for the conversion of quinoline-derived substrate analogues of the 2-naphthoate:CoA ligases showed a similar trend.

While all tests for the substrate specificities of the 2-naphthoate:CoA ligases were performed with cell free extracts, we were also able to heterologously produce and purify the putative CoA-ligase NPH_5477 from *Deltaproteobacterium* strain NaphS2. For the first time we could demonstrate that this enzyme, whose corresponding gene is localised next to the genes coding for a homologue of the 2-naphthoyl-CoA reductase in the NaphS2 genome (Eberlein *et al.*, 2013b), indeed acts as a 2-naphthoate:CoA ligase. Activity was dependant on ATP or ADP, Mg^{2+} and a yet to be identified component present in protein free extracts of NaphS2. The ability to replace the co-factor ATP by

ADP offers an ATP-saving activation strategy. Since an enzyme homologous to NPH_5477 was found to be interacting with the ATP-dependant naphthalene carboxylase in N47 (Koelschbach, 2015), one might speculate that naphthalene carboxylase and 2-naphthoate:CoA share the energy delivered by one ATP for the initial activation of naphthalene forming 2-naphthoyl-CoA.

The nature of the necessary co-factor delivered by the protein free extract remains unclear. Alternative bivalent cations or nucleoside triphosphates other than ATP did not mediate activity of the heterologously produced enzyme. Hence, also a reaction mechanism completely different from the ones of known CoA-ligases has to be considered. An alternative, ATP-independent strategy for the formation of CoA-thioesters is known from the strict anaerobic iron-reducer *Geobacter metallireducens*, which employs a distinct succinyl-CoA:benzoate CoA-transferase for the synthesis of benzoyl-CoA (Oberender *et al.*, 2012). However, sequence similarities with known CoA-ligases clearly indicate the NPH_5477 should act as a CoA-ligase rather than as a CoA-transferase, which is also implied by its dependence on ATP or ADP.

Further studies will therefore focus on the purification of 2-naphthoate:CoA ligases from their native hosts (N47 and NaphS2) and on the identification of the so far unknown co-factor mediating their activity.

3. Identification of a gene cluster involved in the downstream pathway of anaerobic naphthalene degradation

Co-workers: Laura Henning, Janina Koelschbach, Simone Reichenbach, Victor Kang and Rainer Meckenstock

3.01. Abstract

Initial steps of anaerobic naphthalene degradation in sulphate-reducing bacteria proceed via carboxylation and ligation to naphthoyl-CoA followed by reduction of the naphthalene ring II to 5,6,7,8-tetrahydro-2-naphthoyl-CoA. Then, ring I is reduced via a dearomatising reductase similar to class I benzoyl-CoA reductases. The genes encoding this enzyme are surrounded by a cluster of genes coding for β-oxidation-like enzymes that were proposed to be involved in downstream degradation steps. Using a PCR-based operon mapping approach on mRNA of naphthalene-grown cells, we could show that all 22 genes within this cluster are co-transcribed and form an operon which we named the *thn*-operon. The previously reported up-regulation of some proteins encoded by the *thn*-operon during growth on naphthalene or 2-naphthoate indicates that this operon is involved in the downstream pathway of anaerobic naphthalene degradation. When heterologously expressed in *Escherichia coli* under oxic growth conditions, 15 of the 22 genes yielded soluble His_6-tagged proteins which could be purified by affinity chromatography. Activity assays were conducted with linear, cyclic, and branched substrate analogues and the activity of enoyl-CoA hydratases and a ring-cleaving β-oxoacyl-CoA hydrolase could be demonstrated. The fact that some of these enzymes were exclusively acting on cyclic substrates, whereas others were only converting linear substrates, implies their respective position in the pathway (before or after ring-opening). We propose a downstream pathway for anaerobic naphthalene degradation including ring fission via a hydrolase and subsequent β-oxidation-like reactions.

3.02. Introduction

Naphthalene is a sequent environmental pollutant (Preuss *et al.*, 2003) that can be eliminated by microorganisms. The aerobic degradation pathway proceeding via oxygenases has been studied in detail in the past (Gibson and Parales, 2000; Habe and Omori, 2003). In contrast, the current state of knowledge about naphthalene degradation in the absence of molecular oxygen is rather limited although most of the degradation in saturated sediments such as harbour sediments and contaminated aquifers is anaerobic (Meckenstock *et al.*, 2004; Meckenstock *et al.*, 2016). A better knowledge on microbial pollutant degraders and metabolic pathways could lead to the elucidation of specific metabolites, key enzymes or marker genes which in turn can be used to assess biodegradation processes at contaminated sites (von Netzer *et al.*, 2013; Porter and Young, 2014).

The anaerobic, sulphate-reducing enrichment culture N47 mainly consists of a *Desulfobacterium* which is able to use naphthalene as sole electron and carbon source (Meckenstock *et al.*, 2000). The initial activating reactions of this pathway have been recently elucidated: after carboxylation of naphthalene to 2-naphthoic acid (Mouttaki *et al.*, 2012), 2-naphthoyl-CoA is formed in a CoA-ligase reaction. The culture can also utilise 2-methylnaphthalene which is metabolised via fumarate-addition and subsequent β-oxidation-like reactions yielding 2-naphthoyl-CoA (Annweiler *et al.*, 2000; Safinowski and Meckenstock, 2004). The central intermediate 2-naphthoyl-CoA is then stepwise reduced to a hexahydro-2-naphthoyl-CoA with unknown conformation of the remaining double bonds (Eberlein *et al.*, 2013a; Estelmann *et al.*, 2015). The following metabolic steps are still unknown, but there is strong evidence for β-oxidation-like reactions leading to a CoA-ester of *cis*-2-(2-carboxymethyl)cyclohexane-1-carboxylic acid (Annweiler *et al.*, 2002).

A gene-cluster potentially coding for enzymes of the downstream pathway concluding the cleavage of ring I has been found to be expressed in 2-methylnaphthalene-grown cells of N47 in a proteome analysis (Selesi *et al.*, 2010). Some proteins encoded within this cluster were also found to appear in increased abundance in naphthalene-grown versus 2-methylnaphthalene-grown cells (Bergmann *et al.*, 2011b). In a differential expression analysis growing the sulphate-reducing naphthalene degrader *Deltaproteo-*

bacterium strain NaphS2 with 2-naphthoate versus benzoate or pyruvate, single genes from a homologous cluster were identified (DiDonato *et al.*, 2010).

The objective of this study was to elucidate the operon structure of the genes up-regulated during anaerobic naphthalene degradation and to analyse the potential function of the encoded enzymes.

3.03. Experimental procedures

3.03.01. Isolation of RNA from bacterial cells

RNA was isolated from 150 mL of a mid-exponential N47 culture grown on naphthalene (2.03.01) that was harvested as described in 2.03.02. Cells were resuspended in a 3:2 (v/v) mixture of 50 mM sodium acetate buffer pH 5.3 with 10 mM sodium EDTA and 200 mM sodium phosphate buffer pH 5.6, amended with 1% (w/v) sodium docecyl sulphate (Schmitt *et al.*, 1990; Lueders *et al.*, 2004) and opened by bead beating (Henckel *et al.*, 1999). Purification of nucleic acids was achieved by consecutive extraction with equal volumes of 25:24:1 (v/v/v) phenol/chloroform/isoamylalcohol and 24:1 (v/v) chloroform/isoamylalcohol and subsequent precipitation with two volumes of polyethyleneglycol (Griffiths *et al.*, 2000). The later additionally contained 2 µL of glycogen (Roche Deutschland GmbH, Mannheim, Germany). The total nucleic acid pellet was washed with 70% ethanol and resuspended in RNase-free water (Promega GmbH, Mannheim, Germany). Co-extracted DNA was removed by digestion with the RQ1 RNase-Free DNase (Promega) as described in the manufacturer's protocol. The RNA was visualised via agarose gel electrophoresis (Sambrook *et al.*, 1989) and analysed by using NanoDrop (Thermo Scientific, Wilmington, USA) to estimate the quality. Purified RNA was stored at -80 °C until further use.

3.03.02. Reverse transcription of mRNA into cDNA

The isolated RNA (3.03.01) was transcribed into cDNA (complementary DNA) via the GoScript™ Reverse Transcription System (Promega GmbH, Mannheim, Germany)

following the manufacturer's protocol and including the optional incubation step for 15 min at 70 °C to inactivate the reverse transcriptase after cDNA synthesis. Random hexamer primers were used for the PCR amplification. For a negative control testing for residual DNA contamination, an analogous reaction was set up omitting the reverse transcriptase.

3.03.03. PCR-based operon mapping

To find out whether two neighbouring genes are transcribed into the same polycistronic mRNA transcript, PCR primers covering the intergenic regions between two open reading frames were designed and ordered from Eurofins Genomics, Ebersberg, Germany. Typically, one primer was designed to anneal within the end region of one gene and another primer to anneal within the start region of the subsequent gene, together covering a region of 500 – 800 bp. Partly, primers originally designed for the amplification of target genes (see below) were used. In these cases the whole region from the start of the first open reading frame to the end of the second one was covered.

The operon mapping analysis focussed on a gene cluster around the genes coding for the four subunits of an enzyme with sequence homologies to benzoyl-CoA reductases (*N47_E41490 - N47_E41460*) that have been previously identified (Selesi *et al.*, 2010; Bergmann *et al.*, 2011b). For this study, the published annotation of open reading frames of the N47 genome (Bergmann *et al.*, 2011a) was corrected via the GLIMMER genome annotation tool (Delcher *et al.*, 1999). The primers used for the amplification of intergenic regions between the identified open reading frames are listed in Table 2.

Table 2 Primers used for PCR-based operon mapping. Primers marked with an asterisk were also used for the amplification of target genes for cloning purposes (see below).

Genome region	Primer name	Primer sequence (5' → 3')	fragment size (bp)
N47_E41520	E41520-for	TTCTGTTTGTGTGTACTGCAAATATCTG	425
	E41520-rev	ATATATTTCATCAAAACAAAACCTGTAATG	
N47_E41520 → ***N47_E41510***	4152end-for	GCCTTTTTCCATAAGTTGTTTCAATCG	845
	4151start-rev	AGCTGCCGATGCTGGTCAAGAG	
N47_E41510 → ***N47_E41500***	4151end-for	GCTGTATGATCTGTATTATAACAAAATC	520
	4150start-rev	CAACAAAAGCCTTCGATCCTGAG	
N47_E41500 → ***N47_E41490***	4150end-for	GGTCGTAAAGCCTGAAGAATTGATG	538
	4149start-rev	GTAATCATACCTTCCCTCCATTACC	
N47_E41490 → ***N47_E41480***	4149end-for	GACCCCCATGAAATCGATATTCC	623
	4148start-rev	CTGGAACCATTTGGCATGTGTGC	
N47_E41480 → ***N47_E41470***	4148end-for	GGTTCCATATATACTATCGGTCTGG	578
	4147start-rev	CCGACTCTTTCTGATCCAAACC	
N47_E41470 → ***N47_E41460***	4147end-for	CTCAATGGTAAGAAGAGTAGGC	707
	4146start-rev	TGAAATAGTAAGAAGATCGGAAAATACCTC	
N47_E41460 → ***N47_E41450***	4146end-for	TGAAAAAGGGAAAGTCGAATCTTTTCTG	559
	4145start-rev	GCACAACCGTTGCACTTATCAG	
N47_E41450 → ***N47_E41430***	4145end-for	GAAATATGCCTGTGCTCCCTGC	516
	4143start-rev	GCAGGACAGTTTTTCCTGCCTC	
N47_E41430 → ***N47_E41420***	4143end-for	GGCAGGCTCTTGACGAAGCATTAG	685
	4142start-rev	CTCCCCTTTTTTTCATATTTGTCCTCG	
N47_E41420 → ***N47_E41410***	4142end-for	CGATGAAGAATTTGCTAAAAAAACCCC	630
	4141start-rev	AATCACCAGGCCTTACAACCTTTAC	
N47_E41410 → ***N47_E41400***	4141end-for	TTTCTGAATTAATGAGCCGCGATTTCG	483
	4140start-rev	TTGGTTCCCGTTCCGTCTTGTCTTG	
N47_E41400 → ***N47_E41390***	4140end-for	AATACGGGTTGGCTTGCCTTTG	535
	4139start-rev	TTTCTTTTTAAGCGCCATGTCTTCAG	
N47_E41390 → ***N47_E41380***	E41390-for*	ATATCCATGGCTATAGAAAAAGTTGCCGTAATAG	1806
	E41380-rev*	ATATCTCGAGCCTTCCCTTCCATACGGGC	
N47_E41380 → ***N47_E41370***	E41380-for*	ATATCCATGGCTTATAAAAATATATTATACGAGATTG	1596
	E41370-rev*	ATATCTCGAGCATTCCGTTATATTTAGGCTTTCTTTTTTC	
N47_E41370 → ***N47_E41360***	E41370-for*	ATATCCATGGAAGGGAAGGTAGAGAAAAATATG	1807
	E41360-rev*	ATATCTCGAGGCTCGCCAGCCCCATAAAC	
N47_E41360 → ***N47_E41350***	4136end-for	ACCGGGTTCCCGTGGATCAATG	565
	4135start-rev	TTGCATATTAAGACAGCAGGCCAAAC	
N47_E41350 → ***N47_E41340***	4135end-for	CTGGTTGCAGGATCAGAAAAGGCCC	585
	4134start-rev	AGACGCTCCCTGAAAAAAAACGGTATCTG	
N47_E41340 → ***N47_E41330***	4134end-for	ATCCACTGCTAAAAAAGGAGAAATATCC	543
	4133start-rev	TTAACTCCTTAATAACCTTTGTTACTTCC	
N47_E41330 → ***N47_E41320***	E41330-for*	ATATTCATGAAAAAAGTAGTTATTGGTTCCGG	1761
	E41320-rev*	ATATCTCGAGGCACACACCGTATTGTTTTTTAATGC	
N47_E41320 → ***N47_E41310***	E41320-for*	ATATTCATGAAAAAGGTAAAGCTTACGGAATTGG	1586
	E41310-rev*	ATATCTCGAGTATACCAAGTTCTCTTGCCGCAG	
N47_E41310 → ***N47_E41300***	4131end-for	GTCTGTGGCGGGATGCTAAAATG	595
	4130start-rev	GTCGGAACATCCAAAGCATTCATAG	
N47_E41300 → ***N47_E41290***	4130end-for	TAGGTCTTATAGAAAAGGTTGTGCCTGC	655
	4129start-rev	ATGCCCCATATGCTCGACACCGC	

The operon mapping PCR was conducted with DreamTaq Green DNA Polymerase (Thermo Fisher Scientific) following the manufacturer's protocol. Optimal annealing temperatures for each primer pair were determined beforehand by a temperature gradient PCR with genomic DNA of N47 (2.03.04) as template. The final reaction mixture contained either cDNA (3.03.02), a negative control from the RT-PCR assay without reverse transcriptase or genomic DNA as positive control. Two genes were regarded to be co-transcribed, if:

i. the intergenic region could be amplified with the respective primer pair,

ii. the positive control showed an amplicon of the same size,

iii. the negative control showed no amplicon; therefore contamination of the cDNA with genomic DNA could be excluded.

3.03.04. Plasmid construction

Target genes were cloned into the pETM-13 vector (European Molecular Biology Laboratory, Heidelberg, Germany) via the NcoI and XhoI restriction sites as described previously (2.03.04). In some cases, the amplified inserts were digested with a different restriction endonuclease producing compatible ends, so the respective restriction sites were destroyed during the cloning procedure. All primer sequences and used restriction sites are listed in Table 3.

Table 3 Primers used for the amplification of genes from the *thn*-operon.

Gene name	Primer name	Primer sequence (5' → 3')	Restriction sites insert
thnA	E41500-for	ATATTCATGAAATATGAAAATATCATTTATGAGGTAAAG	BspHI
	E41500-rev	ATATCTCGAGTTTACCTGTAAAGACGGGTTTTCTTTTTTC	XhoI
thnF	E41450-for	ATATCCATGGGACTTGCCAATTATGGTTATAAGGATGG	NcoI
	E41450-rev	ATATCTCGAGCCAGGAATGATTAATCGCCCCTAAC	XhoI
thnG	E41430-for	ATATCCATGGCCGAGATTAGTCTGACAATAG	NcoI
	E41430-rev	ATATCTCGAGAAAGAGGTCATCAAGTTCGTCCATG	XhoI
thnH	E41420-for	ATATTCATGAAGGAAATCTTATATGATGATCTG	BspHI
	E41420-rev	ATATCTCGAGCTTAGGCCATATCGGACTTAG	XhoI
thnI	E41410-for	ATATTCATGAGTTGTTACAGAACTTTTGAAG	BspHI
	E41410-rev	ATATCTCGAGGTTATTTTTAGGGAACGCTGCC	XhoI
thnJ	E41400-for	ATGGCGGAAATGTTGGATTATCAGG	NcoI
	E41400-rev	ATATCTCGAGGATGATCTCATGTTTTTTCATTTTTTCC	XhoI
thnK	E41390-for	ATATCCATGGCTATAGAAAAAGTTGCCGTAATAG	NcoI
	E41390-rev	ATATCTCGAGCTTCTTTGAATAATCGTAAAATCCCTTGC	XhoI
thnL	E41380-for	ATATCCATGGCTTATAAAAATATATTATACGAGATTG	NcoI
	E41380-rev	ATATCTCGAGCCTTCCCTTCCATACGGGC	XhoI
thnM	E41370-for	ATATCCATGGAAGGGAAGGTAGAGAAAAATATG	NcoI
	E41370-rev	ATATCTCGAGCATTCCGTTATATTTAGGCTTTCTTTTTTC	XhoI
thnN	E41360-for	ATATCCATGGAAGATATTAAAGCAATAGATATAATG	NcoI
	E41360-rev	ATATCTCGAGGCTCGCCAGCCCCATAAAC	XhoI
thnO	E41350-for	ATATCCATGGATTTTCAATTAGAGCCTGAACTTG	NcoI
	E41350-rev	ATATCTCGAGCTTCCAACCTAACGCGTTGTTTG	XhoI
thnP	E41340-for	ATATTCATGAAATATGGAACTAAACCAAAAACAGG	BspHI
	E41340-rev	ATATCTCGAGACTGTTTATCTTATTGAAAGCACTTTTTAG	XhoI
thnQ	E41330-for	ATATTCATGAAAAAAGTAGTTATTGGTTCCGG	BspHI
	E41330-rev	ATATCTCGAGGCTCACCTCCATTAACTGACTTTC	XhoI
thnS	E41320-for	ATATTCATGAAAAAGGTAAAGCTTACGGAATTGG	BspHI
	E41320-rev	ATATCTCGAGGCACACACCGTATTGTTTTTTAATGC	XhoI
thnT	E41310-for	ATATCCATGGATTTTGAGCTTTCTGAAGAACAG	NcoI
	E41310-rev	ATATCTCGAGTATACCAAGTTCTCTTGCCGCAG	XhoI
thnU	E41300-for	ATATCCATGGAGTCACAACTGGTTCACTATG	NcoI
	E41300-rev	ATATCTCGAGTTTACATTTATATTCAGGTTTTCTTTTTTCG	XhoI
thnV	E41290-for	ATATTCATGATGGCAAAAGAGTTAAGAGATGTAG	BspHI
	E41290-rev	ATATCTCGAGTCTCTGCAAATTCTCAAATATCACG	XhoI

3.03.05. Heterologous production and purification of His_6-tagged proteins

Proteins were heterologously produced and purified as depicted in 2.03.05. Reducing agents like (*S*)-cysteine, 2-mercaptoethanol and DTT were added during the production and purification of thiolase-like enzymes (ThnJ, ThnP and ThnV) as described in 2.03.05 but omitted for the production of all other enzymes. During the production of acyl-CoA

dehydrogenases (ThnO and ThnT), 10 mM riboflavin were added to the auto-induction medium and all purification buffers.

3.03.06. Synthesis and purification of CoA-esters

CoA-esters were synthesised from the free acids via their succinimidyl ester (Gross and Zenk, 1966). For transesterification, 0.25% (w/v) of the succinimidyl ester and 0.25% (w/v) coenzyme A (CoA-SH) were dissolved in anoxic 100 mM sodium hydrogen carbonate. The conversion was monitored by measuring the concentration of free CoA-SH via the Elman's test (Ellman, 1959). After successful transesterification, the reaction mixture was titrated to pH 3 with formic acid and three times extracted with diethyl ether. The aqueous phase was afterwards frozen at -80 °C and lyophilised. If commercially available, acid anhydrides were used as starting material for the CoA-ester synthesis instead of the respective succinimidyl esters.

For further purification, the powder obtained after lyophilisation was dissolved in *MilliQ* water containing 0.1% (v/v) formic acid (buffer A) and loaded to a Supelclean™ ENVI™-8 SPE column (Sigma-Aldrich Chemie GmbH, Munich, Germany) or a HyperSep™ C18 SPE column (Thermo Fisher Scientific, Ulm, Germany) that had been activated and equilibrated according to the manufacturer's protocol. The column was washed with buffer A and compounds were eluted by applying a step gradient (5 – 10% steps) from 5 – 40% buffer B. The latter consisted of acetonitrile with 0.1 (v/v) formic acid. Samples from each elution fraction were analysed by HPLC (see below) to identify the fraction(s) containing the pure CoA-ester. The relevant fractions were frozen at -80 °C and lyophilised. CoA-esters were stored on silica gel at -20 °C.

3.03.07. Hydratase/hydrolase assays

The activity of potential hydratases or hydrolases was assayed in 50 mM potassium phosphate buffer. Reactions were started by the addition of 0.5 -1.0 mM of the following substrates: crotonyl-CoA, cyclohex-1-ene-1-carboxyl-CoA, acetoacetyl-CoA, and 2-oxo-cyclohexane-1-carboxyl-CoA. Additional hydratase assays were conducted with

3-methylcrotonyl-CoA and 2-cyclohexylideneacetyl-CoA as analogues for branched substrates. All assays were incubated at 30 °C and 900 rpm.

3.03.08. β-Hydroxyacyl-CoA dehydrogenase assays

Potential 3-hydroxyacyl-CoA dehydrogenases were assayed for activity both in forward and in reverse direction in 50 mM potassium phosphate buffer pH 8.0 at 30 °C and 900 rpm. Forward reactions mixtures contained 1 mM 3-hydroxybutyryl-CoA or 2-hydroxycyclohexane-1-carboxyl-CoA plus 5 mM NADH, whereas mixtures for reverse reactions contained 1 mM acetoacetyl-CoA or 2-oxocyclohexanecarboxyl-CoA plus 5 mM NAD^+.

3.03.09. Acyl-CoA dehydrogenase assays

The activity of potential acyl-CoA dehydrogenases was assayed both in forward and in reverse direction in 100 mM MOPS/KOH buffer pH 7.3 at 30 °C and 900 rpm. Assays for the forward reactions contained 0.5 mM butyryl-CoA, cyclohexanecarboxyl-CoA, 3-methylbutyryl-CoA or 2-cyclohexylacetyl-CoA plus 2 mM ferrocenium hexafluoro-phosphate as artificial electron acceptor (Lehman and Thorpe, 1990). Reverse reaction mixtures contained 0.5 mM crotonyl-CoA, cyclohex-1-ene-1-carboxyl-CoA, 3-methyl-crotonyl-CoA or 2-cyclohexylideneacetyl-CoA plus 2 mM sodium dithionite as artificial electron donor.

3.03.10. β-oxoacyl-CoA thiolase assays

Thiolyse activity was tested with the respective enzymes in an assay in 100 mM MOPS/KOH buffer pH 7.3 with 15 mM $MgCl_2$ and 2 mM DTT at 30 °C and 900 rpm. For the forward reaction, 1 mM acetoacetyl-CoA and 1 mM free coenzyme A (CoA-SH) were added. The reverse reaction was tested with 0.5 mM butyryl-CoA, cyclohexanecarboxyl-CoA or 2-cyclohexaneacetyl-CoA plus 1 mM acetyl-CoA.

3.03.11. HPLC analysis

All enzyme assays were run discontinuously and the conversion of CoA-esters was analysed by HPLC as described in 2.03.08. Samples from the assay mixture were taken at different time points and the reaction was stopped by the addition of either pure methanol or methanol containing 0.1% (v/v) formic acid as depicted earlier (2.03.07). HPLC eluents for the analysis of hydratase/hydrolase assays and 3-hydroxyacyl-CoA and acyl-CoA dehydrogenase assays were 20 mM potassium phosphate buffer pH 6.0 and 5 – 60% methanol over 11 min at a flow-rate of 0.75 mL/min. For thiolase assays, eluents were 10 mM potassium phosphate buffer pH 6.0 and 5 – 20% acetonitrile over 15 min with a flow-rate of 1 mL/min.

3.04. Results

3.04.01. Operon mapping for N47

In a PCR-based operon mapping approach, neighbouring genes from a gene-cluster (*N47_E41510 – N47_E41290*) of *Desulfobacterium* N47 were tested for co-transcription into the same polycistronic mRNA transcript. The cluster contains 22 genes that were shown to be expressed in N47 cells growing on 2-methylnaphthalene (Selesi *et al.*, 2010). The genes that were referred to as *ORF27 – ORF48* in the cited publication have meanwhile been annotated as *N47_E41510 – N47_E41290* (Bergmann *et al.*, 2011a).

The PCR-based co-expression study showed that the intergenic regions between all these genes could be amplified from cDNA indicating that all 22 genes form an operon and are co-transcribed (exemplarily result shown in Figure 14.

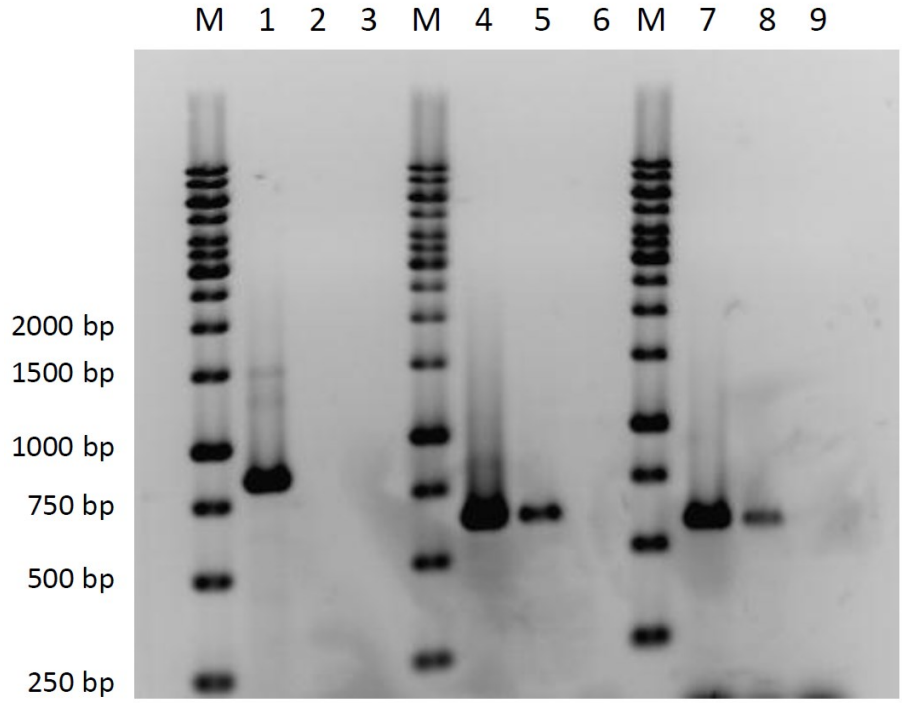


Figure 14 Exemplarily result of an operon mapping assay. Primers were designed for the PCR-amplification of intergenic regions on cDNA of *Desulfobacterium* N47 and the PCR products were analysed via agarose-gelelectrophoresis. (M): GeneRuler™ 1 kb DNA Ladder (ThermoFisher Scientific). (1) – (3): Intergenic region between *N47_E41520* and *N47_E41510* → gDNA (positive control), cDNA, negative control. (4) – (6): Intergenic region between *N47_E41490* and *N47_E41480* → gDNA, cDNA, negative control. (7) – (9): Intergenic region between *N47_E41480* and *N47_E41470* → gDNA, cDNA, negative control.

The gene *N47_E41520*, which is located directly upstream of *N47_E41510*, is not part of the operon. The intergenic region between *N47_E41520* and *N47_E41510* could only be amplified from genomic DNA (gDNA) but not from cDNA that was derived from mRNA of naphthalene-grown cells (Figure 14). In contrast, the intergenic regions between *N47_E41490* and *N47_E41480* as well as between *N47_E41480* and *N47_E41470* could be amplified both from gDNA and from cDNA indicating co-transcription of the respective genes. The same held true for all other genes of the cluster *N47_E41510 – N47_E41290*.

The protein encoded by the four genes *N47_E41490 – N47*_E41460 has previously been identified as potential 2-naphthoyl-CoA reductase, designated as NcrABCD (Selesi *et al.*, 2010; Bergmann *et al.*, 2011b). Since recent studies discovered two reductases from the

old yellow enzyme (OYE) family to be the 2-napthoyl-CoA and 5,6-dihydro-2-naphthoyl-CoA reductase (Eberlein *et al.*, 2013b; Estelmann *et al.*, 2015), this protein should now rather be regarded as the 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase. This is in accordance with sequence homologies of the enzyme to benzoyl-CoA reductases of the *Azoarcus*-type class I aryl-CoA reductases (Lopez Barragan *et al.*, 2004; Song and Ward, 2005). Because of its apparent involvement in the degradation pathway of 5,6,7,8-**t**etra**h**ydro-2-**n**aphthoyl-CoA, we named the newly identified operon the *thn*-operon and the included genes *thnA* – *thnV* (Figure 15).

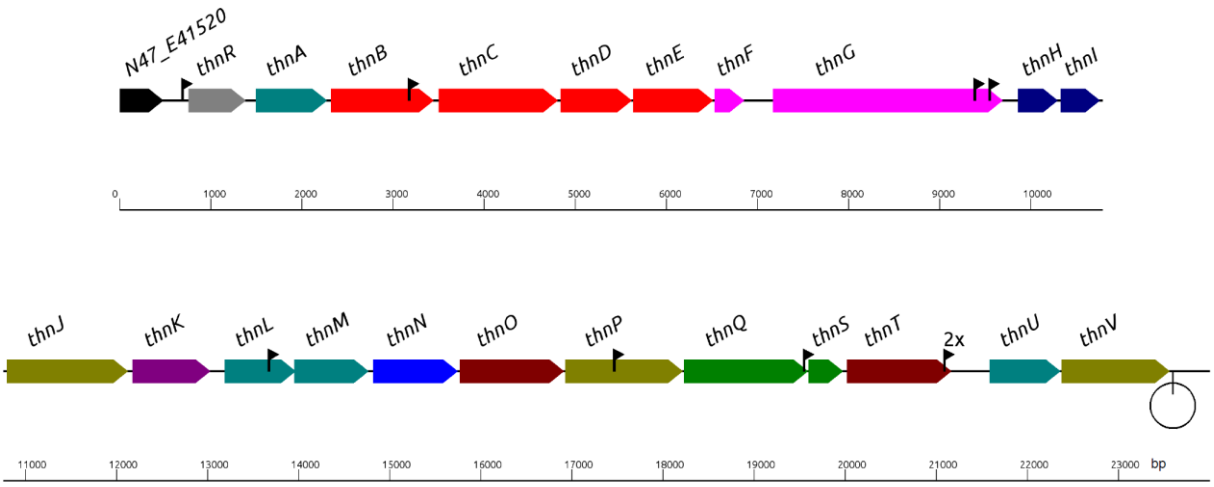


Figure 15 Organisation of the *thn*-operon and adjecent genes. Picture was created via the Genome2D tool (Baerends *et al.*, 2004). Colours indicate the types of enzymes putatively encoded by the respective genes. Black: gene outside the operon. Grey: transcriptional regulator. Teal: enoyl-CoA hydratase/hydrolase/ isomerase. Red: subunit oh the THNCoA reductase. Fuchsia: ferredoxin/oxidoreductase. Navy: MaoC-like *R*-specific hydratase. Olive: CoA-transferase/β-oxoacyl-CoA thiolase. Purple: β-hydroxyacyl-CoA dehdrogenase. Blue: metallo-dependant hydrolase. Maroon: acyl-CoA dehydrogenase. Small arrows indicate potential promoter sites deduced from the DNA-sequence, the open circle a potential transcription termination site. Scale bar indicates length in bp. PePPER was used for the prediction of promoter elements (de Jong *et al.*, 2012) and ARNold for the prediction of Rho-independent transcription terminators (Macke *et al.*, 2001; Naville *et al.*, 2011).

A BLAST-analysis (Altschul *et al.*, 1997) of the gene-product of *N47_E41510* revealed similarities to transcriptional regulators of the TetR-family (Ramos *et al.*, 2005). Thus,

the encoded protein is assumed as the regulator of the *thn*-operon and the corresponding gene was named *thnR*.

Regarding the remarkable size of the identified operon of almost 23 kb, it is very unlikely that transcription starts from one single promoter. An exploration via the PePPER tool (de Jong *et al.*, 2012) revealed nine potential promoters within the operon (Figure 15). One of these potential promoter sites is located directly upstream of *thnR*, the first gene of the *thn*-operon. A potential transcription terminator could be formed by a hairpin-structure that was identified via the RNAmotif algorithm (Macke *et al.*, 2001). This structure is located 29 bp downstream of *thnV*, the last gene of the operon.

Besides the 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase ThnBCDE, the *thn*-operon encodes for gene products which were classified according to their similarity to crotonase-like enoyl-CoA hydratases/hydrolases (ThnA, ThnL, ThnM and ThnU), MaoC-like *R*-specific hydratases (ThnH and ThnI), a metallo-dependent hydratase (ThnN), a β-hydroxyacyl-CoA dehydrogenase ThnK, acyl-CoA dehydrogenases (ThnO and ThnT), β-oxoacyl-CoA thiolases (ThnJ and ThnV), and a CoA-transferase (ThnP). All these enzymes might be involved in β-oxidation-like reactions following the ring-dearomatisation. Downstream of the reductase genes, a ferredoxin ThnF and an oxidoreductase ThnG are encoded. These proteins could provide electrons at a very low redox potential for the reductase.

3.04.02. Heterologous gene expression

Genes from the *thn*-operon were heterologously expressed in *E. coli* under aerobic growth conditions to yield His_6-tagged proteins that were afterwards purified by affinity chromatography. The result of an exemplarily purification of the crotonase-like enzymes ThnL and ThnM is depicted in Figure 16.

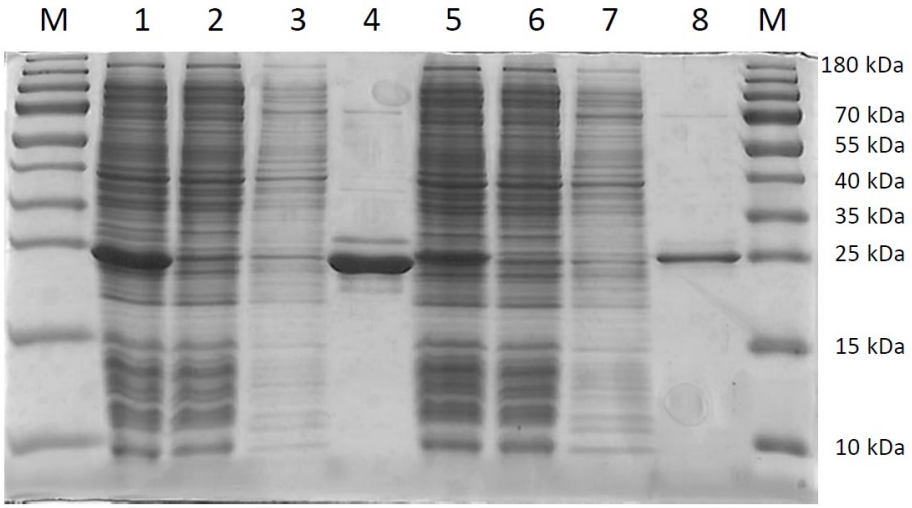


Figure 16 Analysis of a typical protein purification via SDS-PAGE. (M): PageRuler™ Prestained Protein Ladder (ThermoFisher Scientific). (1) – (4): Purification of ThnL → cell free extract, flow-through, wash fraction 20 mM imidazole, elution fraction 100 mM imidazole. (5) – (8): Purification of ThnM → cell free extract, flow-through, wash fraction 20 mM imidazole, elution fraction 100 mM imidazole.

Out of 17 expressed genes, 15 led to the production of soluble proteins. The transcriptional regulator ThnR and the 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase ThnBCDE were not included in the expression attempts. ThnN and ThnQ were insoluble in various standard expression systems tested.

3.04.03. Hydrolase/hydratase assays

Potential hydratases/hydrolases from the *thn*-operon (ThnA, ThnH, ThnI, ThnL, ThnM and ThnU) were heterologously produced and tested for activity towards several substrate analogues. Activity assays were conducted with linear (crotonyl-CoA and acetoacetyl-CoA) and with cyclic (cyclohex-1-ene-1-carboxyl-CoA and 2-oxocyclohexane-1-carboxyl-CoA) compounds representing analogues of potential substrates.

ThnU acted as enoyl-CoA hydratase on crotonyl-CoA which we chose as general analogue for linear α,β-unsaturated acyl-CoA compounds (Figure 17).

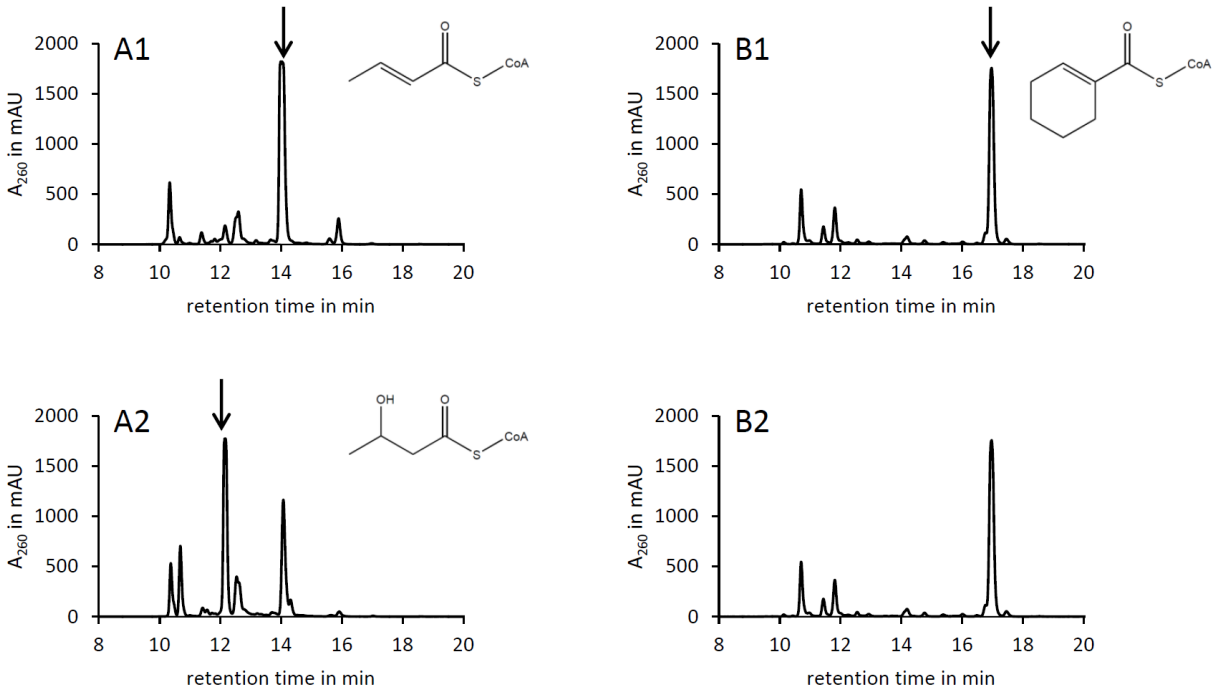


Figure 17 HPLC chromatograms of hydratase assays with ThnU after 60 min of incubation. (A1): enzyme-free control with crotonyl-CoA. (A2): crotonyl-CoA + ThnU. (B1): enzyme-free control with cyclohex-1-ene-1-carboxyl-CoA. (B2): ThnU + cyclohex-1-ene-1-carboxyl-CoA.

Conversion of crotonyl-CoA by the hydratase yielded 3-hydroxybutyryl-CoA. The enzyme did not convert the cyclic substrate cyclohex-1-ene-1-carboxyl-CoA. However, ThnU also converted the linear substrate analogue for β-oxoacyl-CoA hydrolases, acetoacetyl-CoA (data not shown). It was thus identified as bifunctional hydratase and hydrolase with specificity for linear substrates.

In contrast, ThnL was only active as hydrolase on the cyclic substrate 2-oxocyclohexane-1-carboxyl-CoA but did not convert acetoacetyl-CoA (Figure 18).

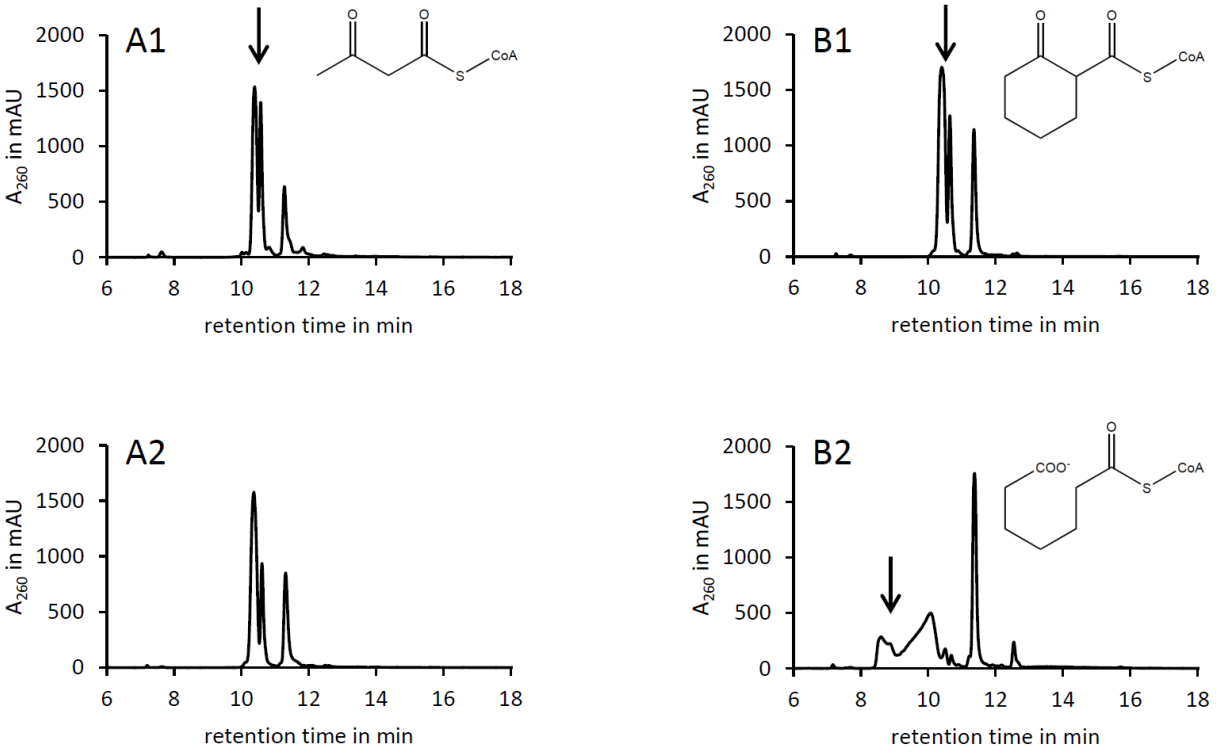


Figure 18 HPLC chromatograms of hydrolse assays with ThnL after 60 min of incubation. (A1): enzyme-free control with acetoacetyl-CoA. (A2): acetoacetyl-CoA + ThnL. (B1): enzyme-free control with 2-oxo-cyclohexane-1-carboxyl-CoA. (B2): 2-oxocyclohexane-1-carboxyl-CoA + ThnL.

Hydratase substrates were not converted by ThnL. Therefore, ThnL appears to be a ring-cleaving hydrolase. The cleavage product, pimeloyl-CoA, was rather instable in the enzyme assays because of its dicarboxylic nature probably enabling the formation of an intra-molecular anhydride with consequent cleavage of the thioester-bond to CoA.

A remarkable characteristic was observed for the MaoC-like enoyl-CoA hydratases ThnH and ThnI: Hydratase activity towards crotonyl-CoA was only observed in assays that contained both enzymes but not in assays with ThnH or ThnI alone (Figure 19).

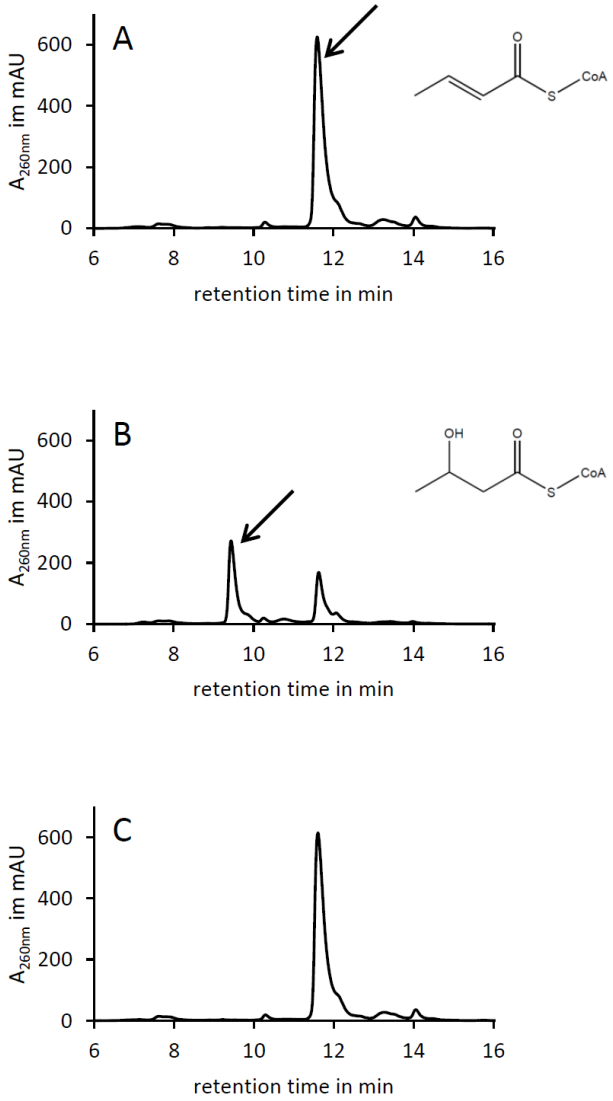
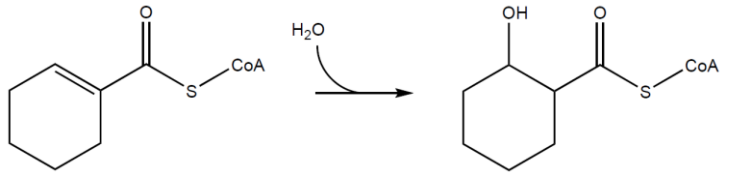
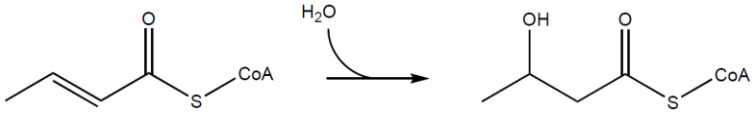
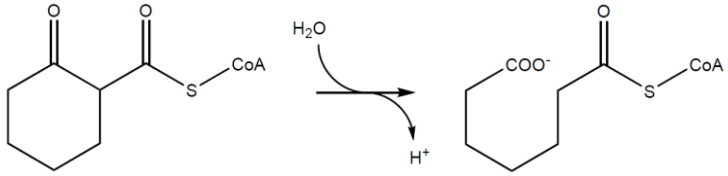
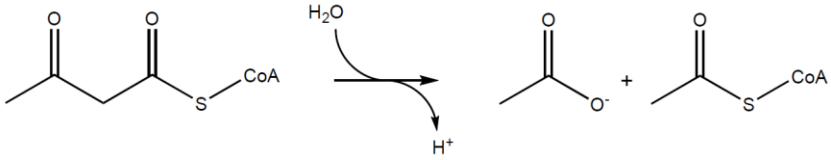
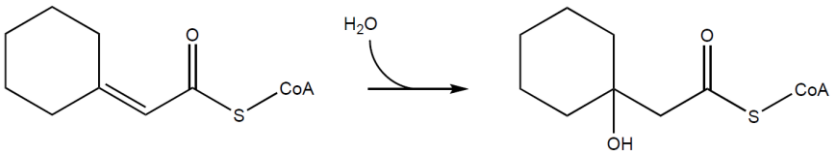
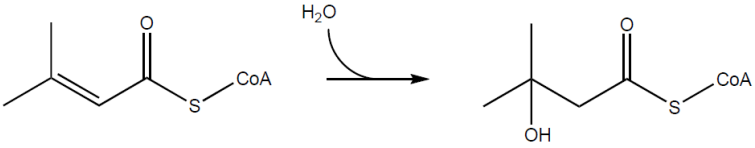


Figure 19 HPLC chromatograms of hydratase assays with ThnH and ThnI after 60 min of incubation. (A): crotonyl-CoA + ThnH alone. (B): crotonyl-CoA + ThnH + ThnI. (C): crotonyl-CoA + ThnI alone.

Thus, ThnHI was characterised as a heteromeric enoyl-CoA hydratase. The cyclic substrate analogue was not converted by ThnHI, indicating specificity for linear substrates.

Since branched intermediates are expected to occur during the downstream pathway of anaerobic naphthalene degradation, also the branched substrate analogues 3-methyl-crotonyl-CoA (linear) and 2-cyclohexylideneacetyl-CoA (cyclic) were tested for conversion by the potential hydratases (Table 4).

Table 4 Summary of conversion tests with potential hydratases / hydrolases and substrate analogues.

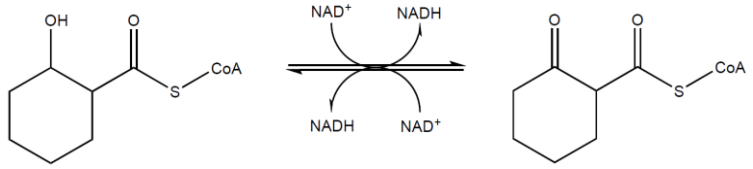
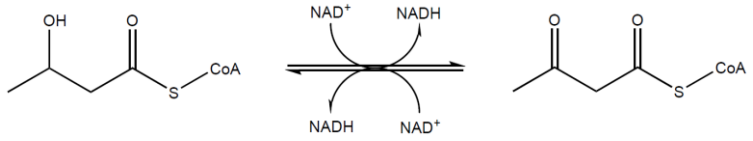
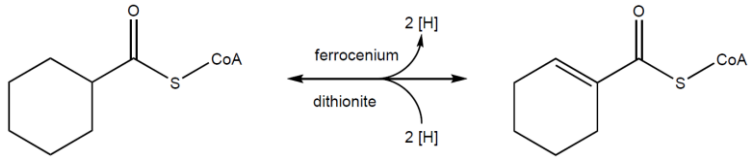
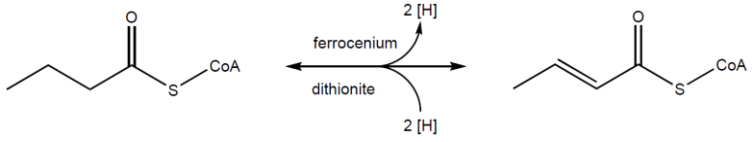
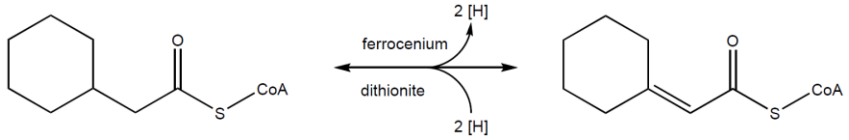
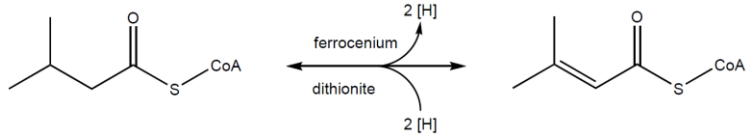
Enzyme reaction	Reaction scheme	Catalysed by:
hydratase reaction on cyclic substrate		n.d.
hydratase reaction on linear substrate		ThnHI ThnU
ring-opening hydrolase		ThnL
hydrolase reaction on linear substrate		ThnU
hydratase reaction on branched, cyclic substrate		n.d.
hydratase reaction on branched, linear substrate		n.d.

None of the tested enzymes showed enoyl-CoA hydratase activity towards the branched substrate analogues. Also activity towards the cyclic substrate cyclohex-1-ene-1-carboxyl-CoA was not detected amongst the tested enzymes

3.04.04. Other enzyme assays

In addition to the hydratases/hydrolases, other enzymes from the *thn*-operon were tested for activity towards substrate analogues. The activity of the potential β-hydroxyacyl-CoA dehydrogenase ThnK was assayed in forward and in reverse direction with cyclic and linear substrate-couples (Table 5).

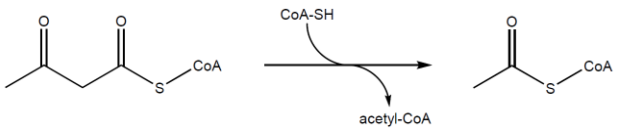
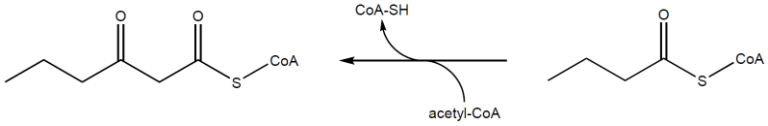
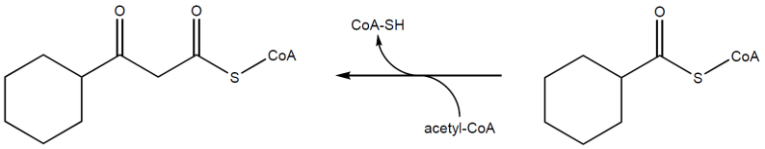
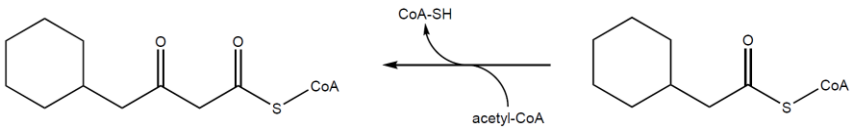
Table 5 Overview of β-hydroxyacyl-CoA and acyl-CoA dehydrogenase reactions with substrate analogues.

Substrate forward reaction	Reaction scheme	Substrate reverse reaction
2-hydroxy-cyclo-hexane-1-carboxyl-CoA		2-oxo-cyclohexane-1-carboxyl-CoA
3-hydroxy-butyryl-CoA		aceto-acetyl-CoA
cyclo-hexane-carboxyl-CoA		cyclohex-1-ene-1-carboxyl-CoA
butyryl-CoA		crotonyl-CoA
2-cyclo-hexylacetyl-CoA		2-cyclo-hexylidene-acetyl-CoA
3-methyl-butyryl-CoA		3-methyl-crotonyl-CoA

The forward reaction, the oxidation of a β-hydroxyacyl-CoA substrate, was driven by the addition of NAD^+ as electron acceptor and the reverse reaction, the reduction of a β-oxoacyl-CoA substrate, by the addition of NADH as electron donor. Likewise, the putative acyl-CoA dehydrogenases ThnO and ThnT were assayed towards couples of cyclic, linear, cyclic-branched and linear-branched substrates (Table 5). In these cases, ferrocenium served as electron acceptor for the forward reaction and dithionite as electron donor for the reverse reaction. However, no activity could be detected towards any of the tested substrates.

Also the presumable β-oxoacyl-CoA thiolases ThnJ and ThnV were tested for the conversion of different substrate analogues (Table 6).

Table 6 Overview of β-oxoacyl-CoA thiolase reactions (forward/reverse) with substrate analogues.

Substrate forward reaction	Reaction scheme	Substrate reverse reaction
acetoacetyl-CoA		acetyl-CoA
		butyryl-CoA
		cyclohexane-carboxyl-CoA
		cyclohexyl-aetyl-CoA

For the substrate couple acetoacetyl-CoA/acetyl-CoA, both the forward reaction (addition of free coenzyme A) and the reverse reaction (addition of acetyl-CoA) were assayed. The reaction on other substrates was only tested in the reverse direction (Table 6). But like the dehydrogenases, also the putative β-oxoacyl-CoA thiolases appeared to be inactive towards all tested substrates.

3.05. Discussion

3.05.01. The *thn*-operon

The genes that encode for the reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) are surrounded by a cluster of genes coding for hydratases, dehydrogenases, hydrolases and thiolases. Using a PCR-based operon mapping approach for mRNA from an N47 culture grown on naphthalene, we could show that all 22 genes within this cluster are co-transcribed simultaneously. This indicates that the whole gene cluster forms one operon that is involved in the downstream pathway of anaerobic naphthalene degradation. All genes of this operon were also shown to be expressed in N47 cells growing on 2-methylnaphthalene in a non-differential approach (Selesi *et al.*, 2010). The operon was termed *thn*-operon in reference to the first substrate of the degradation pathway, 5,6,7,8-**t**etra**h**ydro-2-**n**aphthoyl-CoA.

An almost identical cluster of genes homologous to the *thn*-genes was also found in the genome sequence of *Deltaproteobacterium* NaphS2, a marine anaerobic naphthalene degrader (DiDonato *et al.*, 2010) as depicted in Table 7.

Table 7 Open reading frames of the *thn*-operon in N47 and NaphS2 and the hypothetical functions of the encoded enzymes as predicted via BLAST analyses (Altschul *et al.*, 1997).

Protein	hypothetical protein function	ORF in N47	ORF in NaphS2
ThnR	TetR-family transcriptional regulator	*N47_E41510*	*NPH_5886*
ThnA	enoyl-CoA hydratase/hydrolase/isomerase	*N47_E41500*	*NPH_5887*
ThnB	5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase	*N47_E41490*	*NPH_5888*
ThnC	5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase	*N47_E41480*	*NPH_5889*
ThnD	5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase	*N47_E41470*	*NPH_5890*
ThnE	5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase	*N47_E41460*	*NPH_5891*
ThnF	ferredoxin	*N47_E41450*	*NPH_5892*
ThnG	oxidoreductase	*N47_E41430*	n.p.
ThnH	*R*-β-hydroxyacl-CoA dehydratase MaoC-like	*N47_E41420*	*NPH_5893*
ThnI	*R*-β-hydroxyacl-CoA dehydratase MaoC-like	*N47_E41410*	*NPH_5894*
ThnJ	β-oxoacyl-CoA thiolase	*N47_E41400*	*NPH_5895*
ThnK	β-hydroxyacyl-CoA dehydrogenase	*N47_E41390*	*NPH_5896*
ThnL	enoyl-CoA hydratase/hydrolase/isomerase	*N47_E41380*	*NPH_5897*
ThnM	enoyl-CoA hydratase/hydrolase/isomerase	*N47_E41370*	*NPH_5898*
ThnN	metallo-dependant hydrolase (TIM barrel fold)	*N47_E41360*	*NPH_5899*
ThnO	acyl-CoA dehydrogenase	*N47_E41350*	*NPH_5900*
ThnW	β-oxoacyl-ACP reductase	n.p.	*NPH_5901*
ThnP	CoA-transferase / acetyl-CoA hydrolase	*N47_E41340*	*NPH_5902*
ThnQ	acyl-CoA:acetate-lyase AtuA-like	*N47_E41330*	*NPH_5903*
ThnS	acyl-CoA:acetate-lyase AtuA-like	*N47_E41320*	*NPH_5904*
ThnX	β-oxoacyl-ACP reductase	n.p.	*NPH_5905*
ThnY	β-hydroxyacyl-CoA dehydrogenase	n.p.	*NPH_5906*
ThnT	acyl-CoA dehydrogenase	*N47_E41310*	*NPH_5907*
ThnU	enoyl-CoA hydratase/hydrolase/isomerase	*N47_E41300*	*NPH_5908*
ThnV	β-oxoacyl-CoA thiolase	*N47_E41290*	*NPH_5909*

n.p.: Respective gene not present in the gene cluster.

The gene coding for a putative oxidoreductase ThnG is missing in the gene-cluster of NaphS2. On the other hand, this cluster contains genes coding for β-oxoacyl-ACP reductases (ThnW and ThnX) that are not present in theN47-gene cluster as well as a gene encoding an additional β-hydroxyacyl-CoA dehydrogenase (ThnY).

The assumption that proteins encoded by the *thn*-operon are involved in the naphthalene degradation pathway is additionally supported by previous findings showing that ThnA and the THNCoA reductase subunits ThnC and ThnE were up-regulated in N47 during growth on naphthalene (Bergmann *et al.*, 2011b). Furthermore, ThnM and ThnQ

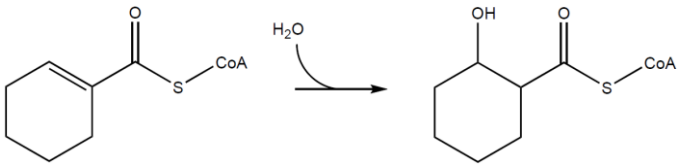
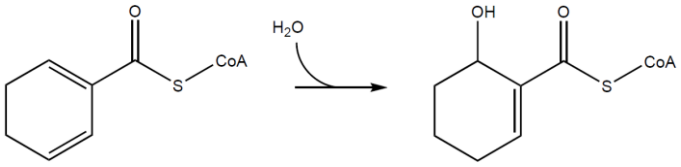
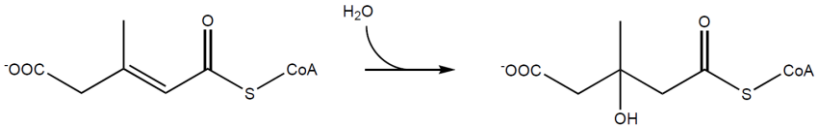
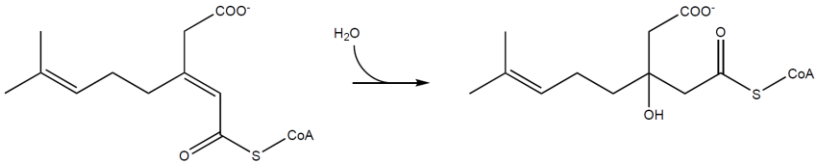
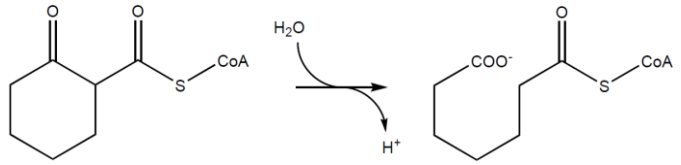
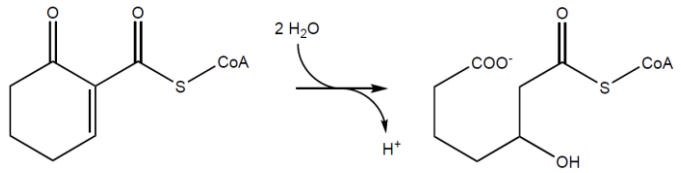
were up-regulated on the transcriptional level in the marine naphthalene-degrading strain NaphS2 grown with 2-naphthoate versus benzoate or pyruvate (DiDonato *et al.*, 2010). Finally, also the predicted functions of several encoded enzymes (hydratases, dehydrogenases, hydrolases, and thiolases) are in accordance with an involvement in the downstream degradation pathway, which is expected to proceed via β-oxidation-like reactions (Annweiler *et al.*, 2002).

3.05.02. Enoyl-CoA hydratases/hydrolases

Four genes coding for enzymes from the crotonase superfamily (Holden *et al.*, 2001) are included in the *thn*-operon. This superfamily consists of various enzymes with diverse functions, hydratases and hydrolases being just two examples. A common mechanistic feature of all these enzymes is the stabilisation of an enolate-anion (Hamed *et al.*, 2008). Via enzyme assays with substrate analogues, we could show that ThnL acts as a ring-cleaving hydrolase whereas ThnU is a bifunctional hydratase/hydrolase with specificity for linear substrates. The latter enzyme is therefore apparently involved in the down-stream degradation pathway after ring fission.

In the well-studied anaerobic degradation pathways of benzoyl-CoA, crotonase-like enzymes catalyse the water addition to cyclic mono- or dienoyl-CoA compounds and the posterior ring-opening hydrolysis. In *Rhodopseudomonas palustris*, initial dearomatisation of benzoyl-CoA produces the monoene compound cyclohex-1-enecarboxyl-CoA (Perrotta and Harwood, 1994). Therefore, *R. palustris* employs a cyclohex-1-enecarboxyl-CoA hydratase BadK and a 6-oxocyclohexane-1-carboxyl-CoA hydrolase BadI (Egland *et al.*, 1997; Pelletier and Harwood, 1998) for the further degradation pathway (Table 8).

Table 8 Crotonase-like hydratases/hydrolases involved in other metabolic pathways

Enzyme reaction	Reaction scheme	Known enzymes
cyclohex-1-ene-1-carboxyl-CoA hydratase		BadK
cyclohexa-1,5-diene-1-carboxyl-CoA hydratase		Dch,Dch-2*, BamR, BzdW, MbdW*
3-methyl-glutaconyl-CoA hydratase		LiuE
3-iso-hexenyl-glutaconyl-CoA hydratase		AtuE
6-oxocyclo-hexane-1-carboxyl-CoA hydrolase		BadI
6-oxocyclo-hex-1-ene-1-carboxyl-CoA hydrolase		Oah, Oah-2* BamA, BzdY, MbdY*

*: Enzymes marked with an asterisk catalyse equivalent reactions on methylated substrate-analogues.

Contrary to the pathway observed in *R. palustris*, anaerobic benzoate degradation in *Thauera aromatica* proceeds via the diene-intermediate cyclohexa-1,5-diene-1-carboxyl-CoA (Boll and Fuchs, 1995; Laempe *et al.*, 1998). Hence, the further conversion in *T. aromatica* is catalysed by a particular diene-specific cyclohexa-1,5-diene-1-carboxyl-CoA hydratase Dch that adds water in 1,4-position (Laempe *et al.*, 1998). The subse-

quent hydrolysis is mediated by the 6-oxocyclohex-1-ene-1-carboxyl-CoA hydrolase Oah (Laempe *et al.*, 1999). This enzyme acts both as a hydratase and as a hydrolase on its substrate yielding 3-hydroxypimeloyl-CoA. In comparison to other crotonase-like hydratases/hydrolases, Oah exhibits a large C-terminal extension as unique structural feature (Kuntze *et al.*, 2008).

The degradation route via homologues of Dch and Oah occurs in a variety of anaerobic benzoate-degraders like *Aromatoleum aromaticum* EbN1 (Kuhner *et al.*, 2005; Rabus *et al.*, 2005), *Azoarcus sp.* CIB (Lopez Barragan *et al.*, 2004; Juarez *et al.*, 2013; Martin-Moldes *et al.*, 2015), *Geobacter metallireducens* (Wischgoll *et al.*, 2005; Aklujkar *et al.*, 2009), and *Magnetospirillum sp.* pMbN1 (Lahme *et al.*, 2012). *A. aromaticum* and *Azoarcus sp.* CIP additionally possess homologues of BadK and BadI for cyclohexanecarboxylate degradation that proceeds via cyclohex-1-ene-1-carboxyl-CoA (Rabus *et al.*, 2005; Martin-Moldes *et al.*, 2015).

Methylated benzoate-derivatives can also be metabolised via slightly modified pathways. *Azoarcus sp.* CIB degrades 3-methylbenzoate via the hydratase MbdW and hydrolase MbdY (Juarez *et al.*, 2013) and *Magnetospirillum sp.* pMbN1 metabolises 4-methylbenzoate via the crotonase-like enzymes Dch-2 and Oah-2 (Lahme *et al.*, 2012). These pathways might be comparable to the degradation of 5,6,7,8-tetrahydro-2-naphthoyl-CoA since this compound can be considered as derivative of benzoyl-CoA with alkyl-side-chains in C3- and C4-position.

However, crotonase-like enzymes from the *thn*-operon might as well convert branched intermediates that are expected to occur during later steps of the anaerobic naphthalene degradation pathway. Comparable enzymes are for example the 3-methylglutaconyl-CoA hydratases from *Pseudomonas aeruginosa* PAO1 and *Acinetobacter sp.* DSM 11652 (Aguilar *et al.*, 2006; Mack *et al.*, 2006). A homologue enzyme has been proposed to be involved in the aforementioned anaerobic degradation pathway of 4-methylbenzoate in *Magnetospirillum sp.* pMbN1 (Lahme *et al.*, 2012). Besides, also the acyclic terpene utilisation pathway proceeds via hydratases with specificity for branched substrates, namely the 3-isohexenylglutaconyl-CoA hydratases AtuE from *P. aeruginosa* PAO1 and *Pseudomonas citronellolis* (Aguilar *et al.*, 2006; Poudel *et al.*, 2015).

A phylogenetic analysis of the crotonase-like enzymes encoded within the *thn*-operon aligned with known hydratases/hydrolases revealed that ThnA, ThnM, and ThnU are related to monoenoyl-CoA hydratases (Figure 20).

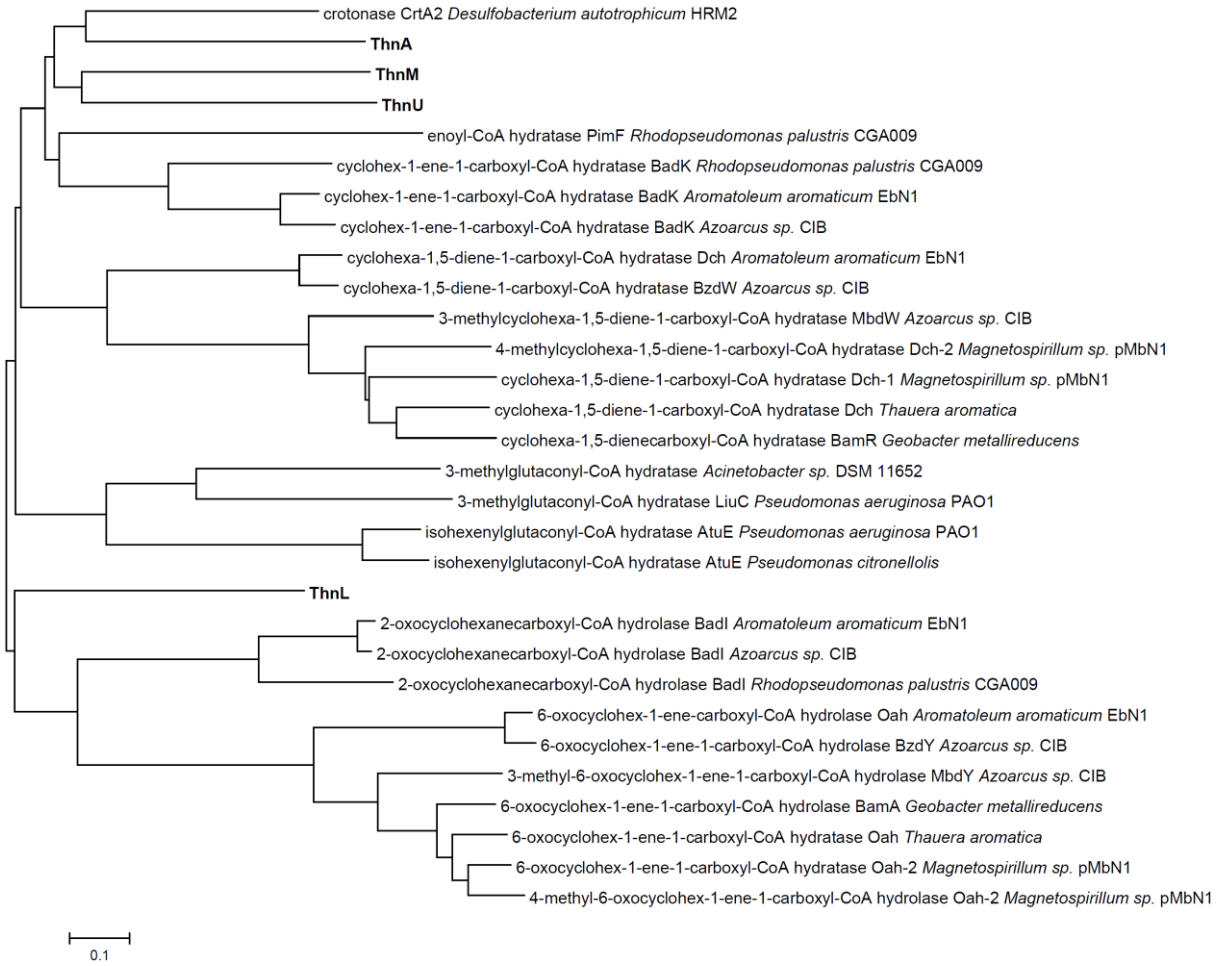


Figure 20 Phylogenetic tree (Neighbor-Joining) of crotonase-like proteins ThnA, ThnL, ThnM and ThnU encoded within the *thn*-operon aligned with crotonase-like hydratases and hydrolases from comparable anaerobic degradation pathways. Alignment was performed with the MEGA6 software (Tamura *et al.*, 2013) using the MUSCLE algorithm (Edgar, 2004). The 3-hydroxybutyryl-CoA dehydratase CrtA from *Desulfobacterium autotrophicum* HRM2 (Strittmatter *et al.*, 2009) served as outgroup representing an ordinary crotonase from a closely related strain and the enoyl-CoA hydratase PimF from the pimelate degradation pathway of *Rhodopseudomonas palustris* CGA009 (Larimer *et al.*, 2004; Harrison and Harwood, 2005) represented a crotonase-like enzyme specific for linear dicarboxylic compounds. Scale-bar represents 10% sequence divergence.

Thus, these three enzymes most likely hydratise linear or cyclic monoenes that occur during anaerobic naphthalene degradation. Hydratases with specificity for dienoyl-CoAs or branched monoenes are apparently not encoded within the *thn*-operon.

We could demonstrate that the remaining crotonase-like enzyme ThnL catalyses the hydrolytic ring-cleavage at the substrate analogue 2-oxocyclohexanecarboxyl-CoA. On a phylogenetic level, ThnL does not cluster with the known ring-cleaving hydrolases but forms an own branch (Figure 20). The distinct sequence of this enzyme might allow discriminating ring-cleaving hydrolases that act on polycyclic substrates from other hydrolases based on their amino acid sequence. In an analogous manner, the bifunctional 6-oxocyclohex-1-ene-1-carboxyl-CoA hydratase/hydrolase serves as indicator for obligate anaerobes degrading monoaromatic compounds and the corresponding gene can be used as gene-marker (Kuntze *et al.*, 2008).

In contrast to the aforementioned crotonase-like enzymes, ThnHI belongs to the family of MaoC-like *R*-specific hydratases (Engeland and Kindl, 1991; Fukui *et al.*, 1998). Enzymes of this family share a common structural feature, the so-called HotDog fold (Dillon and Bateman, 2004). The observed heteromeric structure of ThnHI is in accordance with the properties of related proteins that usually contain two HotDog domains. The two domains can either be part of a fusion protein or be encoded by adjacent genes of one operon (Dillon and Bateman, 2004). The latter seems to be the case for ThnHI.

3.05.03. Other enzymes encoded by the *thn*-operon

Sequence analyses of other enzymes encoded within the *thn*-operon identified candidate enzymes that may catalyse further β-oxidation-like reactions. ThnK and ThnY (the latter only present in the gene cluster of NaphS2) share sequence homologies with β-hydroxyacyl-CoA dehydrogenases (Lehninger and Greville, 1953; Hillmer and Gottschalk, 1974; Birktoft *et al.*, 1987). These enzymes oxidise a β-hydroxyacyl-CoA in a NAD^+-dependant reaction yielding a β-oxo-intermediate and can typically also catalise the reverse reaction with NADH as co-substrate. ThnK most likely reacts on a bicyclic β-hydroxy-intermediate in analogy to reactions occurring in anaerobic benzoyl-CoA degradation pathways (Laempe *et al.*, 1999; Pelletier and Harwood, 2000).

ThnO and ThnT belong to the acyl-CoA dehydrogenase (ACAD) family (Shaw and Engel, 1984; Kim *et al.*, 1993). They are assumedly responsible for the unsaturation of acyl side-chains after first ring-fission or of linear intermediates in the later catabolic pathway. ACAD-like enzymes are also known from other anaerobic degradation pathways, e.g. the cyclohexanecarboxyl-CoA dehydrogenase involved in the anaerobic catabolism of cyclohexanecarboxylate (Kung *et al.*, 2013; Kung *et al.*, 2014) or the benzylsuccinyl-CoA dehydrogenase involved in anaerobic toluene degradation (Leutwein and Heider, 2002).

The proposed reactions of other enzymes encoded by the *thn*-operon directly involve CoA-residues. ThnJ and ThnV are related to β-oxoacyl-CoA thiolases (Goldman, 1954; Mathieu *et al.*, 1997) and are therefore assumed to catalyse the thiolytic release of an acetyl-CoA unit from a β-oxoacyl-CoA intermediate, which is the final step of one β-oxidation cycle.

ThnP is affiliated with CoA-transferases catalysing an inter- or intramolecular transfer of CoA between organic acids (Vanderwinkel *et al.*, 1968; Scherf and Buckel, 1991; Jacob *et al.*, 1997). After the first ring-opening, the anaerobic naphthalene degradation pathway proceeds via dicarboxylic compounds (Annweiler *et al.*, 2002). Later metabolic steps might require the transfer of CoA between the two acyl-side-chains catalysed by ThnP.

ThnQ and ThnS seem to form a heterodimer ThnQS which is related to the 3-hydroxy-3-isohexenylglutaryl-CoA:acetate-lyase AtuA from the acyclic terpene utilisation pathway (Foerster-Fromme *et al.*, 2006; Foerster-Fromme and Jendrossek, 2010). This enzyme acts as an acetate-lyase on a branched intermediate. A similar reaction might also occur in anaerobic naphthalene catabolism since the β-oxidation-like degradation of bicyclic compounds inevitably produces branched intermediates.

The function of ThnN in the pathway remains unclear. This enzyme belongs to the metallo-dependent hydratase (amidohydrolase) superfamily (Holm and Sander, 1997), a superfamily of enzymes with versatile functions. Enzymes of this family share a common three-dimensional structure, the so-called TIM barrel fold (Wierenga, 2001) and an

active-site architecture that enables the deprotonation of water via a metal ion for a subsequent nucleophilic attack on the substrate. A similar protein is encoded by *bamU* from a gene clusters involved in anaerobic benzoate degradation of *Geobacter metallireducens*, but the function of this protein is as well unknown (Wischgoll *et al.*, 2005).

ThnW and ThnX are exclusively encoded in the NaphS2 cluster and are predicted as β-oxoacyl-ACP reductases (Toomey and Wakil, 1966). Enzymes of this type are usually involved in the synthesis of fatty acids, which is the reverse pathway of β-oxidation. Considering the absence of homologous genes in the *thn*-operon of N47, *thnW* and *thnX* most likely do not encode for enzymes involved in the downstream pathway of anaerobic naphthalene degradation.

While some of the heterologously produced crotonase-like enzymes encoded by the *thn*-operon were active towards general substrate analogues in enzyme assays, other enzymes did not convert any of the general substrate analogues although heterologous expression yielded soluble proteins. One reason might be that the His_6-tag introduced during the heterologous production interferes with oligomerisation of the proteins. Crotonase-like enzymes often occur as trimers or as dimers of trimers (Hamed *et al.*, 2008) and also ACAD-like enzymes usually oligomerise in a homoterameric structure (Kim and Miura, 2004). Future attempts will therefore try to remove the His_6-tag after purification or to purify the enzymes from the native hosts.

3.05.04. Proposed ring cleavage pathway

The product of the reductive dearomatisation of 5,6,7,8-tetrahydronaphthoyl-CoA observed *in vitro* was a hexahydro-2-naphthoyl-CoA of unknown conformation (Eberlein *et al.*, 2013a). A β-oxidation-like transformation of this intermediate would be initiated by a dienoyl-CoA hydratase. Subsequent oxidation by a β-hydroxyacyl-CoA dehydrogenase would yield a β-oxooctahydro-2-naphtoyl-CoA (Figure 21 panel A).

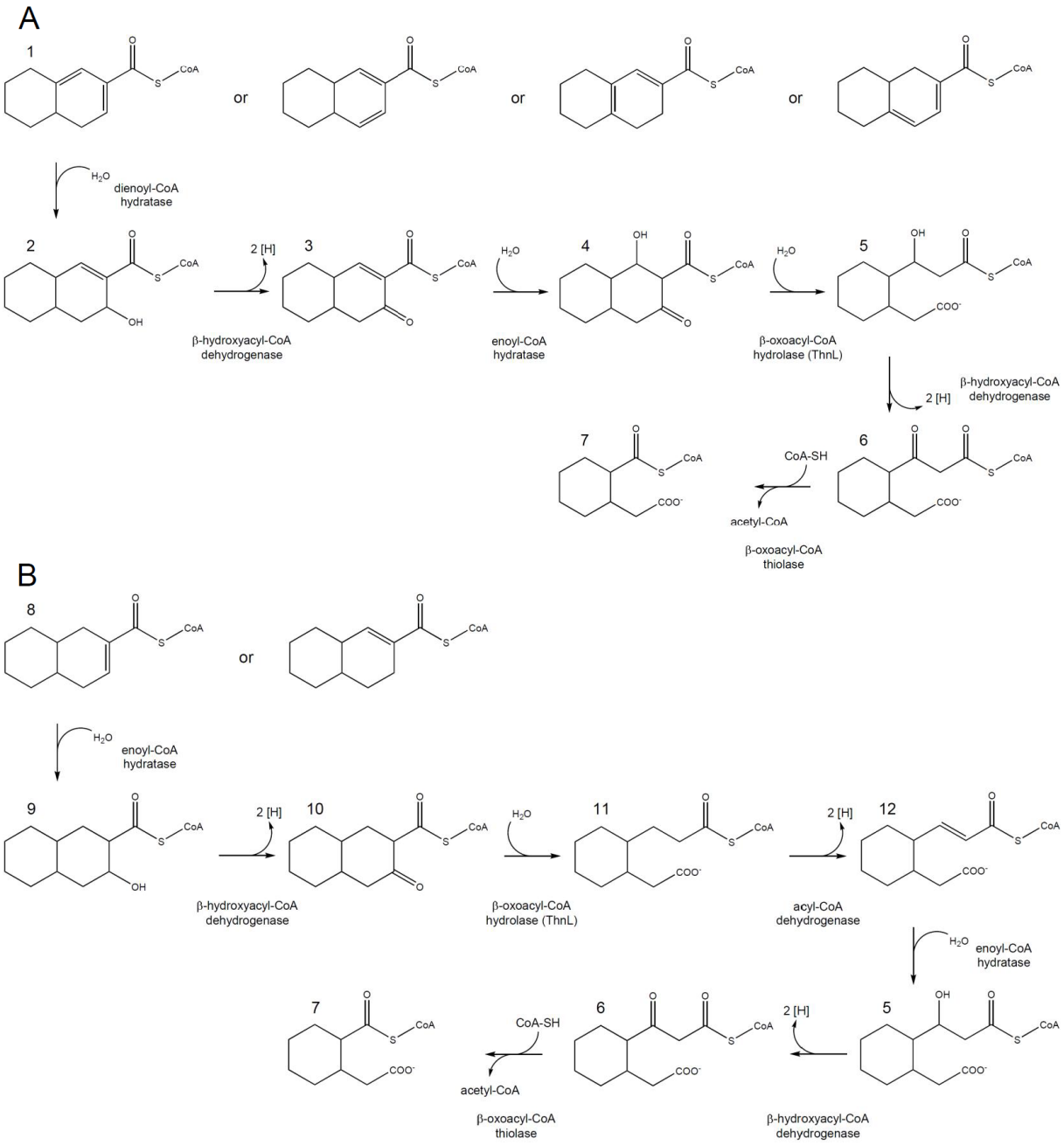


Figure 21 Proposed β-oxidation-like downstream degradation pathways starting from one possible isomer each of hexa- and octahydro-2-naphthoyl-CoA. (1): 4,4a,5,6,7,8-hexahydro-2-naphthoyl-CoA. (2): 3-hydroxy-3,4,4a,5,6,7,8,8a-octahydro-2-naphthoyl-CoA. (3): 3-oxo-3,4,4a,5,6,7,8,8a-octahydro-2-naphthoyl-CoA. (4): 1-hydroxy-3-oxodecahydro-2-naphthoyl-CoA. (5): 3-(2-(carboxymethyl)cyclohexyl)3-hydroxypropionyl-CoA. (6): 3-(2-(carboxymethyl)cyclohexyl)-3-oxopropionyl-CoA. (7): 2-(carboxymethyl)cyclohexane-1-carboxyl-CoA. (8): 1,4,4a,45,6,7,8,8a-octahydro-2-naphthoyl-CoA. (9): 3-hydroxydecahydro-2-naphthoyl-CoA. (10): 3-oxodecahydro-2-naphthoyl-CoA. (11): 3-(2-(carboxymethyl)-cyclohexyl)propionyl-CoA. (12): 3-(2-(carboxymethyl)cyclohexyl)acrylyl-CoA.

The latter represents a two-ringed analogue of the substrate of the bifunctional 6-oxocyclohex-1-ene-1-carboxyl-CoA hydratase/hydrolase known from obligate anaerobic benzoate degraders (Laempe *et al.*, 1999; Kuntze *et al.*, 2008). However, the genomes of N47 and NaphS2 do not include genes coding for a homologue enzyme. Conversion of this intermediate might therefore be achieved via two separate enzymes, an enoyl-CoA hydratase (for example ThnM) and the subsequently acting ring-cleaving hydrolase ThnL. Nevertheless, ThnL was only tested for conversion of β-oxo-cyclohexanecarboxyl-CoA and it remains unclear whether it is able to hydrolyse a β'-hydroxy-β-oxo intermediate with an additional hydroxyl group.

In the proposed pathway, ring-cleavage occurs between C2 and C3 of β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA, but depending on the conformation of hexahydro-2-naphthoyl-CoA, it might also occur between C1 and C2 leading to a slightly different cleavage product. In any case, ring-cleavage produces a dicarboxylic cyclohexane-derivative that can be further metabolised via dehydrogenation and subsequent thiolytic release of an acetyl-CoA unit yielding a CoA-thioester of 2-(carboxymethyl)cyclohexane-1-carboxylic acid (Figure 21). The *cis*-isomer of this compound was previously detected in culture extracts of N47 (Annweiler *et al.*, 2002). Depending on the position of the first ring-cleavage, the CoA-thioester would either be located at the carboymethyl-residue (cleavage between C1 and C2) or at the carboxyl-residue (cleavage between C2 and C3).

Since our phylogenetic analyses revealed that only enzymes similar to monoenoyl-CoA hydratases but not to dienoyl-CoA hydratases are encoded by the *thn*-operon, the reductive dearomatisation of THNCoA might also produce the monoene intermediate octahydro-2-naphthoyl-CoA. In fact, the *in vitro* reduction of THNCoA stopped at the hexahydro intermediate. However, in case this was indeed the final reduction product, a further conversion by a hydratase should be observable, since the activity of the respective enzymes from the closely related benzoyl-CoA pathway, benzoyl-CoA reductase and cyclohexa-1,5-diene-1-carboxyl-CoA hydratase, could be measured in one assay (Boll *et al.*, 2000b). A further indication for octahydro-2-naphthoyl-CoA as THNCoA reductase product is the fact that in primary studies with the enrichment culture N47 octahydro-2-naphthoic acid but not hexahydro-2-naphthoic acid was observed as metabolite in cell extracts (Meckenstock *et al.*, 2000).

Assuming the reduction to proceed to an octahydro intermediate, the latter could be hydratised via one of the enoyl-CoA hydratases encoded by the *thn*-operon (for example ThnA). Subsequent dehydrogenation of this hydratase product would yield β-oxodecahydro-2-naphthoyl-CoA, a two-ringed analogue of β-oxocyclohexane-carboxyl-CoA (Figure 21, panel B). ThnL was tested positive for conversion of this substrate analogue, so it should also be able to hydrolyse β-oxodecahydro-2-naphthoyl-CoA. Further metabolisation would continue similarly to the pathway starting from hexahydro-2-naphthoyl-CoA as depicted above, but include an additional acyl-CoA dehydrogenase step. The two possible pathways converge at the monocyclic intermediate 4-(2-carboxycyclohexyl)-3-hydroxybutyryl-CoA.

The nature of the enzymes encoded within the newly identified *thn*-operon indicates a degradation of THNCoA via the octahydro- rather than via the hexahydro-intermediate. However, it cannot be ruled out that the pathway proceeds via hexahydro-2-naphthoyl-CoA and involves unusual enzymes. Future studies therefore need to test the hydratases identified in this work for conversion of all possible hexahydro- and octahydro-intermediates in order to identify their natural substrate.

4. Identification of the product of the 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase

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4.01. Abstract

Dearomatising ring-reductases play an important role in anaerobic degradation pathways of aromatic compounds. Naphthalene degradation by sulphate-reducing bacteria is known to proceed via 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA), which gets reduced to a hexahydro-2-naphthoyl-CoA (HHNCoA) with unknown conformation by an enzyme similar to class I benzoyl-CoA reductases. In previous studies, the respective reaction was measured with NADH as electron donor, but neither could a complete conversion of the added THNCoA be achieved, nor could further metabolites be detected. When analysing analogously performed assays via LC-MS scanning for expected metabolites in single ion mode, we could show that small amounts of the product of a HHNCoA hydratase are formed in the assays, but the downstream conversion by an NAD^+-dependant β-hydroxyacyl-CoA dehydrogenase is inhibited by the excess of NADH present in these assays. Experiments with alternative electron donors indicated that 2-oxoglutarate is the natural electron donor of the THNCoA reducing system and low-potential electrons are delivered in the form of reduced ferredoxin via a 2-oxoglutarate:ferredoxin oxidoreductase. With 2-oxoglutarate as electron donor, a complete conversion of THNCoA was observed and further metabolites could be detected. These metabolites indicate a β-oxidation-like downstream pathway with water addition to HHNCoA and a first ring-fission via a hydrolase acting on a β'-hydroxy-β-oxo-decahydro-2-naphthoyl-CoA intermediate. Furthermore, we observed a spontaneous abiotic reduction of HHNCoA to two isomers of octahydro-2-naphthoyl-CoA. A comparison of the retention times of these two isomers with the one of the CoA ester of a chemically synthesised reference compound

gave a hint on the conformation of the HHNCoA produced by THNCoA reductase which we propose to be 3,4,4a,5,6,7,8-HHNCoA.

4.02. Introduction

4.02.01. Dearomatising reactions during the anaerobic degradation of aromatic compounds

Aromatic compounds ubiquitously occur in biomass for example in the form of aromatic amino acids, quinones and flavonoids. In addition, the lignin contained in plant biomass, one of the most frequent organic polymers on earth, consists of aromatic building blocks (Adler, 1977). Lignin can be broken down into its monomers by fungi and bacteria in the presence of oxygen via extracellular peroxidase and laccase enzymes (Kirk and Farrell, 1987; Bugg *et al.*, 2011). Under oxic conditions, the degradation pathways of a variety of aromatic monomers, also including polycyclic compounds like naphthalene, converge at the central intermediates catechol (1,2-dihydroxybenzene) or protocatechuate (3,4-dihydroxybenzoate) and further degradation typically proceeds via the β-ketoadipate pathway (Stainer and Ornston, 1973; Harwood and Parales, 1996). Initial reactions leading to catechol or protocatechuate usually involve mono- or dioxygenases which facilitate an attack on the aromatic system with molecular oxygen as co-substrate (Dagley *et al.*, 1960; Dagley *et al.*, 1964).

In contrast, anaerobic bacteria, which cannot rely on oxygen as co-substrate, channel monoaromatic substrates to the central intermediate benzoyl-CoA (Harwood *et al.*, 1999) and break the aromatic system in a reductive manner via dearomatising aryl-CoA reductases (Boll *et al.*, 2002; Gibson and Harwood, 2002). These enzymes work via a so-called Birch-like mechanism that includes alternate electron and proton transfer steps and produces a radical anion intermediate (Boll, 2005a). It is therefore comparable to the Birch reduction known in organic chemistry, which uses solvated electrons generated by dissolving alkali metals in liquid ammonia for the reduction of aromatic compounds (Birch and Smith, 1958; Birch *et al.*, 1980).

In natural systems, three different strategies enabling this kind of reaction are known to date: Class I benzoyl-CoA reductases consist of four different subunits and their catalytic activities are ATP-dependant and oxygen sensitive (Boll and Fuchs, 1995). They are usually found in facultative anaerobic bacteria like *Thauera aromatica*, formerly known as *Pseudomonas* strain K172 (Koch and Fuchs, 1992; Koch *et al.*, 1993; Boll and Fuchs, 1995), *Azoarcus* strain CIB (Lopez Barragan *et al.*, 2004) or *Rhodospeudomonas palustris* (Egland *et al.*, 1997). Regarding their amino acid sequence, benzoyl-CoA reductases of class I can be subdivided into two specific types: The *Thauera* type, found for example in *T. aromatica*, *R. palustris* and *Magnetospirillum magnetotacticum* versus the *Azoarcus* type, found in several *Azoarcus* species and in *Aromatoleum aromaticum* EbN1 (Lopez Barragan *et al.*, 2004; Boll, 2005a; Rabus *et al.*, 2005; Song and Ward, 2005).

All class I benzoyl-CoA reductases hydrolyse one ATP to ADP + P_i per electron transferred and use ferredoxin as natural electron donor (Boll and Fuchs, 1998). Electron transfer is mediated by [4Fe-4S] clusters (Boll *et al.*, 1997; Boll *et al.*, 2000a). During the catalytic cycle, a phosphorylated enzyme intermediate is formed which gets dephosphorylated and oxidised upon electron transfer to the substrate (Unciuleac and Boll, 2001). Subsequently, ferredoxin reduces the enzyme to enable another cycle (Unciuleac and Boll, 2001). ATP is not only required for the aforementioned formation of a phosphorylated enzyme intermediate but nucleotide binding also induces a conformational change that facilitates internal electron transfer within the reductase complex (Moebitz *et al.*, 2004). 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 (Egland *et al.*, 1997; Breese *et al.*, 1998; Lopez Barragan *et al.*, 2004). In *T. aromatica*, ferredoxin is reduced via a 2-oxoglutarate:ferredoxin oxidoreductase (Doerner and Boll, 2002), whereas *A. evansii* uses a combination of a $NADP^+$-dependant 2-oxoglutarate dehydrogenase and a NADPH:ferredoxin oxidoreductase (Ebenau-Jehle *et al.*, 2003). In both cases, the oxidation of the end product of the benzoyl-CoA pathway, acetyl-CoA, via the citric acid cycle is coupled to the reduction of benzoyl-CoA since 2-oxoglutarate, an intermediate of the citric acid cycle, indirectly serves as electron donor.

Class II benzoyl-CoA reductases are also sensitive towards oxygen and, in contrast to the class I reductases, ATP-independent (Boll *et al.*, 2014). The vast majority of strict anaerobes that can utilise aromatic compounds employ a class II reductase (Loeffler *et al.*, 2011). These enzymes were for example studied in the sulphate-reducer *Desulfococcus multivorans* (Peters *et al.*, 2004), the iron-reducer *Geobacter metallireducens* (Wischgoll *et al.*, 2005; Kung *et al.*, 2009) or the fermenting bacterium *Syntrophus aciditrophicus* (McInerney *et al.*, 2007). Class II reductases are huge enzyme complexes that are proposed to have a modular composition of eight different subunits (Boll *et al.*, 2014). ATP-independent reduction of the aromatic system is most likely achieved via a so-called electron bifurcation mechanism (Buckel and Thauer, 2013) in which per one low-potential electron from ferredoxin transferred to the substrate another one is transferred from ferredoxin to the high-potential electron acceptor $NAD(P)^+$ making the overall process exergonic (Boll *et al.*, 2014; Boll *et al.*, 2016).

4.02.02. Aryl-CoA reductases involved in the anaerobic microbial degradation of naphthalene

A third type of aryl-CoA reductases was recently discovered in sulphate-reducing naphthalene degraders (Eberlein *et al.*, 2013b). These class III reductases belong to the old yellow enzyme (OYE) family and are ATP-independent and insensitive towards oxygen. In the anaerobic degradation pathway of naphthalene two distinct OYE-like enzymes catalyse the reduction of 2-naphthoyl-CoA and 5,6-dihydro-2-naphthoyl-CoA, respectively (Estelmann *et al.*, 2015).

The subsequent reduction of 5,6,7,8-tetrahydro-2-napthoyl-CoA (THNCoA) in this pathway is probably catalysed by enzymes with similarity to the class I benzoyl-CoA reductases of the *Azoarcus*-type that were identified in the anaerobic naphthalene degrading strains *Deltaproteobacterium* NaphS2 and *Desulfobacterium* N47 (DiDonato *et al.*, 2010; Bergmann *et al.*, 2011a). THNCoA can be regarded as a 3,4-alkylated derivative of benzoyl-CoA. The identification of a 4-methylbenzoyl-CoA reductase that shares sequence similarities with *Azoarcus*-type class I reductases indicates that these enzymes are in principal capable of reducing alkylated aromatic rings (Lahme *et al.*, 2012). In cell

free extracts of N47, THNCoA was reduced to a hexahydro-2-naphthoyl-CoA of yet unknown conformation (Eberlein *et al.*, 2013a). This reaction was dependant on ATP and an electron donor with preference for NADH. 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 NaphS2 and N47 (DiDonato *et al.*, 2010; Bergmann *et al.*, 2011a). These enzymes might be involved in NAD(P)H-dependant electron transfer to THNCoA via ferredoxin in analogy to the electron generating systems of benzoyl-CoA reduction described above.

It still remains an open question whether the hexahydro-2-naphthoyl-CoA (HHNCoA) observed in THNCoA reductase assays (Eberlein *et al.*, 2013a) is the final reductase product or if the reduction proceeds to octahydro-2-naphthoyl-CoA (OHNCoA). The latter would be analogous to cyclohex-1-enecarboxyl-CoA, which is known as intermediate of the benzoyl-CoA pathway in *R. palustris* (Egland *et al.*, 1997). The aforementioned similarity of the identified THNCoA reductase to benzoyl-CoA reductases of the *Azoarcus*-type might be an indication for HHNCoA as reductase product since known reductases of this type most likely transfer two electrons to their substrate (Lopez Barragan *et al.*, 2004; Song and Ward, 2005). However, the number of electrons transferred by the reductase cannot be unequivocally deduced from sequence homologies alone since also the benzoyl-CoA reductases from *T. aromatica* and *R. palustris* are highly similar on protein sequence level (Song and Ward, 2005) but the enzyme from *T. aromatica* transfers two electrons (Boll *et al.*, 2000b) while the one from *R. palustris* is assumed to transfer a total of four electrons (Harwood *et al.*, 1999).

The objective of this work was to identify the final product of THNCoA reduction in the anaerobic naphthalene catabolism and to identify the natural electron source of this process. Unlike the aerobic degradation pathways, metabolic pathways of anaerobic degradation of mono- and polycyclic compounds do not converge at the same central intermediate. The anaerobic catabolism of naphthalene, which can serve as model compound of polycyclic aromatic hydrocarbons, does not proceed via benzoyl-CoA but via metabolites with a cyclohexane skeleton (Annweiler *et al.*, 2002). A further study on the THNCoA reductase is expected to be useful for deciphering this so far widely

unknown pathway since the nature of the reductase product presumably implies subsequent metabolic steps.

4.03. Experimental procedures

4.03.01. Growth of bacterial cells

The enrichment culture N47 and *Deltaproteobacterium* strain NaphS2 were grown as described in 2.03.01. A freshly harvested 1.6 L culture was used for each set of assays.

4.03.02. Anaerobic preparation of cell free extracts

The preparation of anaerobic cell free extracts was similar to the procedure described in 2.03.02, but the following steps were conducted in an anaerobic LABstar Glove Box Workstation (M. Braun, Garching, Germany) containing a N_2-atmosphere: Transfer of cultures into air-tight centrifuge beakers, 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.

4.03.03. Synthesis of reduced derivatives of 2-naphthoic acid

An unspecific mixture of hexahydro-2-naphthoic acids was synthesised from 5,6,7,8-tetrahydro-2-naphthoic acid by the group of Prof. Dr. Carsten Schmuck (Chair for Organic Chemistry, University of Duisburg-Essen, Germany) following a literature procedure originally developed for the synthesis of cyclohexa-1,5-diene-1-carboxylic acid from benzoic acid (Reynolds *et al.*, 1992; Laempe *et al.*, 1998).

Isomeric pure 1,4,4a,5,6,7,8,8a-octahydro- and 3,4,4a,5,6,7,8,8a-octahydro-2-naphthoic acid were synthesised from decalon via the respective octahydro-2-carbonitrile by Masih Sadeghi in the group of Prof. Dr. Bernard Golding, (School of Chemistry, University of Newcastle, UK) as described previously (Eberlein *et al.*, 2013a).

4.03.04. Synthesis of potential downstream metabolites

Enantiomeric mixtures of *cis*-2-((*E*)-3-carboxyallyl)cyclohexane-1-carboxylic acid and *cis*-(*E*)-3-(2-(carboxymethyl)cyclohexyl)acrylic acid were synthesised by Dr. Andrey Zaytsev in the group of Prof. Dr. Bernard Golding, University of Newcastle, UK.

4.03.05. Synthesis and purification of CoA-esters

5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) was synthesised from the free acid via its succinimidyl ester as described in 3.03.06. A Strata® Phenyl SPE column (Phenomenex, Aschaffenburg, Germany) was used for purification.

4.03.06. Discontinuous reductase assays

THNCoA reductase activity was assayed under strict anaerobic conditions in 100 mM MOPS/KOH buffer pH 7.3 with 15 mM $MgCl_2$ as described previously (Eberlein *et al.*, 2013a). The 200 µL reaction mixture typically contained 60 – 100 µL anaerobic cell free extract of enrichment culture N47 or *Deltaproteobacterium* NaphS2 (4.03.02), 5 mM ATP, 5 mM electron donor (NADH, NADPH, sodium dithionite or 2-oxoglutarate) and 50 µM THNCoA. Assays with 2-oxoglutarat as electron donor were conducted with and without addition of 1 mM CoA-SH. Some assays additionally contained 1 – 5 mM electron acceptors like NAD^+ or $NADP^+$, or a combination of two electron donors. 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 (2.03.07).

4.03.07. Ligase assays with hexa- and octahydro-2-naphthoic acids

Ligase assays with reduced analogues of 5,6,7,8-tetrahydro-2-naphthoate were conducted as depicted previously (2.03.07). Instead of 2-naphthoate, the reaction mixtures contained either a mixture of hexahydro-2-naphthoate isomers or specific isomers of octahydro-2-naphthoate, namely 1,4,4a,5,6,7,8,8a-octahydro-2-naphthoate or 3,4,4a,5,6,7,8,8a-octahydro-2-naphthoate.

4.03.08. Assays with potential downstream metabolites

The CoA-esters of the chemically synthesised 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 N47 (Annweiler *et al.*, 2002), were tested for conversion by enoyl-CoA hydratases in cell free extracts of N47 and NaphS2 as described previously (3.03.07). Due to the two carboxyl groups present in these compounds, a mixture of two isomers with differing in the attachment sites of the CoA-thioester was obtained upon synthesis of the respective CoA-esters. The complete mixture was used as 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, are expected to be converted in a β-oxidation-like manner.

4.03.09. HPLC analysis

Samples were analysed like described in 2.03.08, but a shorter acetonitrile gradient from 10 – 25% over 15 min was applied.

4.03.10. LC-MS analysis

LC-MS analyses (liquid-chromatography coupled to mass-spectrometry) were performed with a LC-2040C system coupled to a LCMS-2020 single quadrupole mass-spectrometer (Shimadzu Deutschland, Duisburg, Germany). Samples were separated via a Nucleodur C18 Gravity-SB column, 100 × 3 mm, 5 µm particle size (Macherey-Nagel, Dueren, Germany). The column oven was set to 35 °C. Eluent A was water with 0.1% (w/v) ammonium acetate, eluent B 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, the temperature to 350 °C. Nebulising gas flow was 1.5 L/min, drying gas flow

12 L/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 9) were detected in single ion mode with ionisation conditions as optimised for 5,6,7,8-tetrahydro-2-naphthoyl-CoA (see below).

Table 9 Mass-to-charge ratios of metabolites expected in 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase assays. Depending on the final reductase product – hexahydro-2-naphthoyl-CoA (HHNCoA) or octahydro-2-naphthoyl-CoA (OHNCoA) – and on the position of the first ring-opening – between C1 and C2 or between C2 and C3 – different downstream metabolites should emerge.

m/z	Metabolite of HHNCoA pathway	Metabolite of 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		octahydro-2-naphthoyl-CoA
946	β-hydroxyoctahydro-2-naphthoyl-CoA	β-oxodecahydro-2-naphthoyl-CoA
944	β-oxooctahydro-2-naphthoyl-CoA	
948		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-(2carboxycyclohexyl)-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)cyclohexane-carboxyl-CoA 2-(2-carboxycyclohexyl)-acetyl-CoA	2-(carboxymethyl)cyclohexane-carboxyl-CoA 2-(2-carboxycyclohexyl)-acetyl-CoA

For the detection of all these ions, the desolvation line was operated at 0 V and 250 °C. Settings for the Qarray™ ion guide were as follows: DC voltage 0 V, RF voltage 117 V.

4.04. Results

4.04.01. THNCoA reductase reaction in cell free extract of N47

The reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) to hexahydro-2-naphthoyl-CoA (HHNCoA) could be measured with NADH as electron donor as reported previously (Eberlein *et al.*, 2013a). This reaction appeared to be inhibited when the assay mixture was additionally amended with sodium dithionite or NAD^+ (data not shown). However, when the latter co-substrates were added to the assay mixture after a pre-incubation time of 30 min, further conversion of the previously formed HHNCoA could be detected (Figure 22).

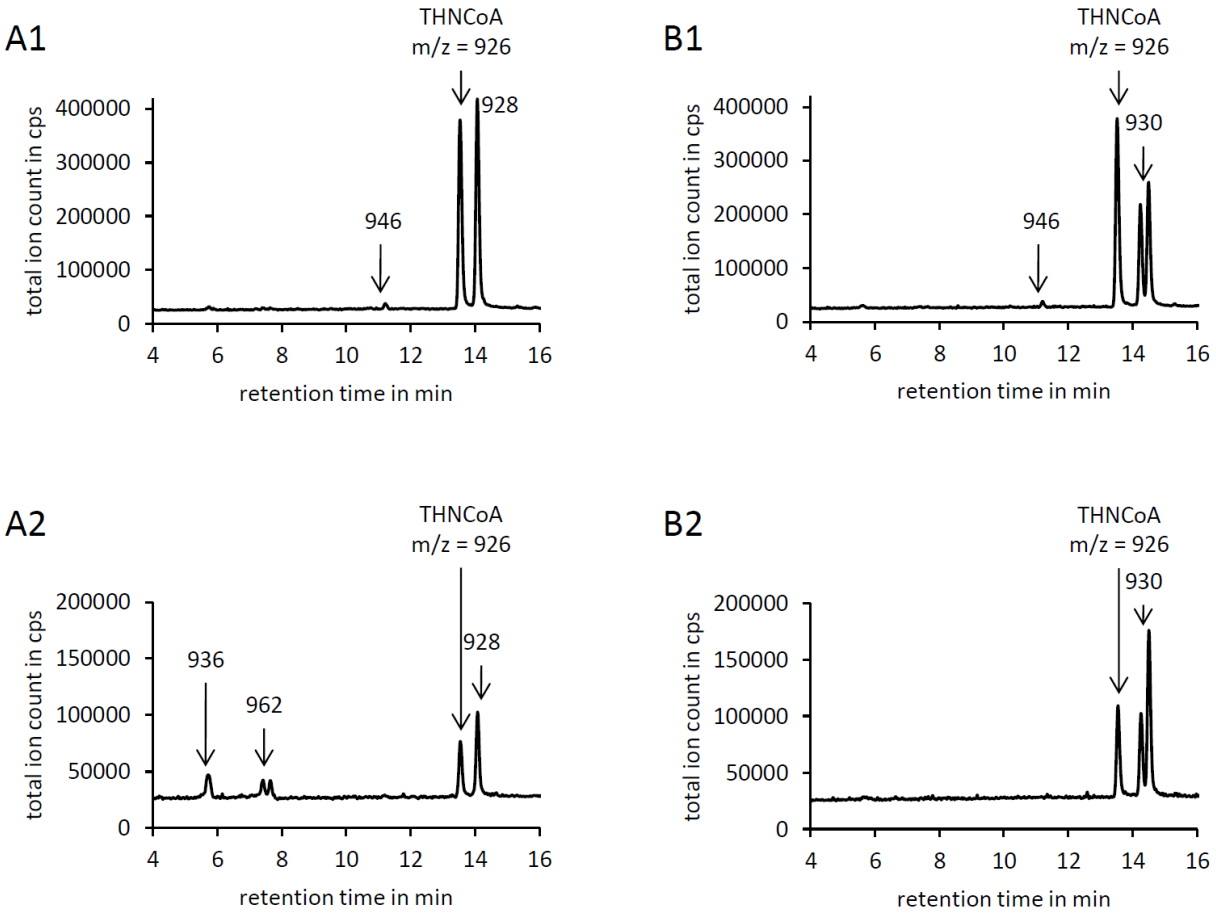


Figure 22 LC-MS chromatograms of reductase assays with 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) in cell free extracts of N47. All assays contained 50 µM THNCoA, 5 mM ATP and 5 mM NADH. Sodium dithionite or NAD^+ (5 mM each) were added after a pre-incubation time of 30 min. (A1): assay with NAD^+ directly after addition of the latter (30 min). (A2): assay with NAD^+ 60 min after addition. (B1): assay with sodium dithionite directly after addition of the latter (30 min). (B2): assay with sodium dithionite 60 min after addition. Please note different y-scales between panels A1/B1 and A2/B2. All samples were analysed via LC-MS in single ion mode scanning for expected metabolites.

In both samples taken after 15 min, ca. 50% of the initially added THNCoA (m/z = 926, retention time 13.6 min) were converted and a peak representing HHNCoA (m/z = 928, retention time 14.1 min) was formed (Figure 22 panel A1). However, when dithionite was added to the assay mixture, HHNCoA appeared to be immediately converted to two compounds with m/z = 930 (retention time 14.3 and 14.5 min) that were most likely isomers of OHNCoA (Figure 22 panel B1). Later experiments indicated that this conversion is due to a spontaneous chemical reaction since it also occurred when the enzymes

of the cell free extract were inactivated by addition of 66% methanol prior to dithionite addition (data not shown). No further metabolites were formed in the assay after addition of dithionite. The peaks of the previously formed metabolites only decreased, probably due to the unspecific activity of thioesterases (Figure 22 panel B2).

A new peak with m/z = 946 (retention time 11.3 min) appeared in both assays after 15 min. This one could represent either the product of a hexahydro-2-naphthoyl-CoA reductase, β-hydroxyoctahydro-2-naphthoyl-CoA 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 addition of NAD^+ two new peaks arose (Figure 22 panel A2). A compound with m/z = 962 appeared as double-peak in the LC-MS chromatogram when the standard buffer system (pH 7.0) was used, but appeared as one single peak when the chromatography with ammonium acetate buffer at pH 5.5 (data not shown). This mass-to-charge ration might represent either the substrate of a ring-opening hydrolase in the HHNCoA pathway, β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA, or the product of the respective hydrolase from the OHNCoA pathway which could be 3-(2-(carboxymethyl)cyclohexyl)acrylyl-CoA or 4-(2-carboxycyclohexyl)crotonyl-CoA. However, the pH-dependant formation of a double-peak in the LC-MS chromatogram points at a compound that can undergo keto-enol tautomerism and is therefore indicative for β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA, the only possible metabolite with a keto group and m/z = 962. The other peak arising after NAD^+ addition (retention time 5.8 min) had a mass-to-charge ratio of 936 and was therefore most likely the CoA-ester of 2-(2-carboxycyclohexyl)acetic acid, an earlier reported metabolite of anaerobic naphthalene degradation (Annweiler *et al.*, 2002). NAD^+ seems to be an essential co-substrate for an enzyme involved in the conversion steps downstream of the THNCoA, most likely a β-hydroxyacyl-CoA dehydrogenase.

In reductase assays with N47-cfe that contained only ATP and NADH as co-substrates, the reduction of THNCoA to HHNCoA typically stopped at a conversion rate of ca. 60% and the aforementioned metabolite with m/z = 946 was also detected in these assays. (Figure 23 panels A1 – A3).

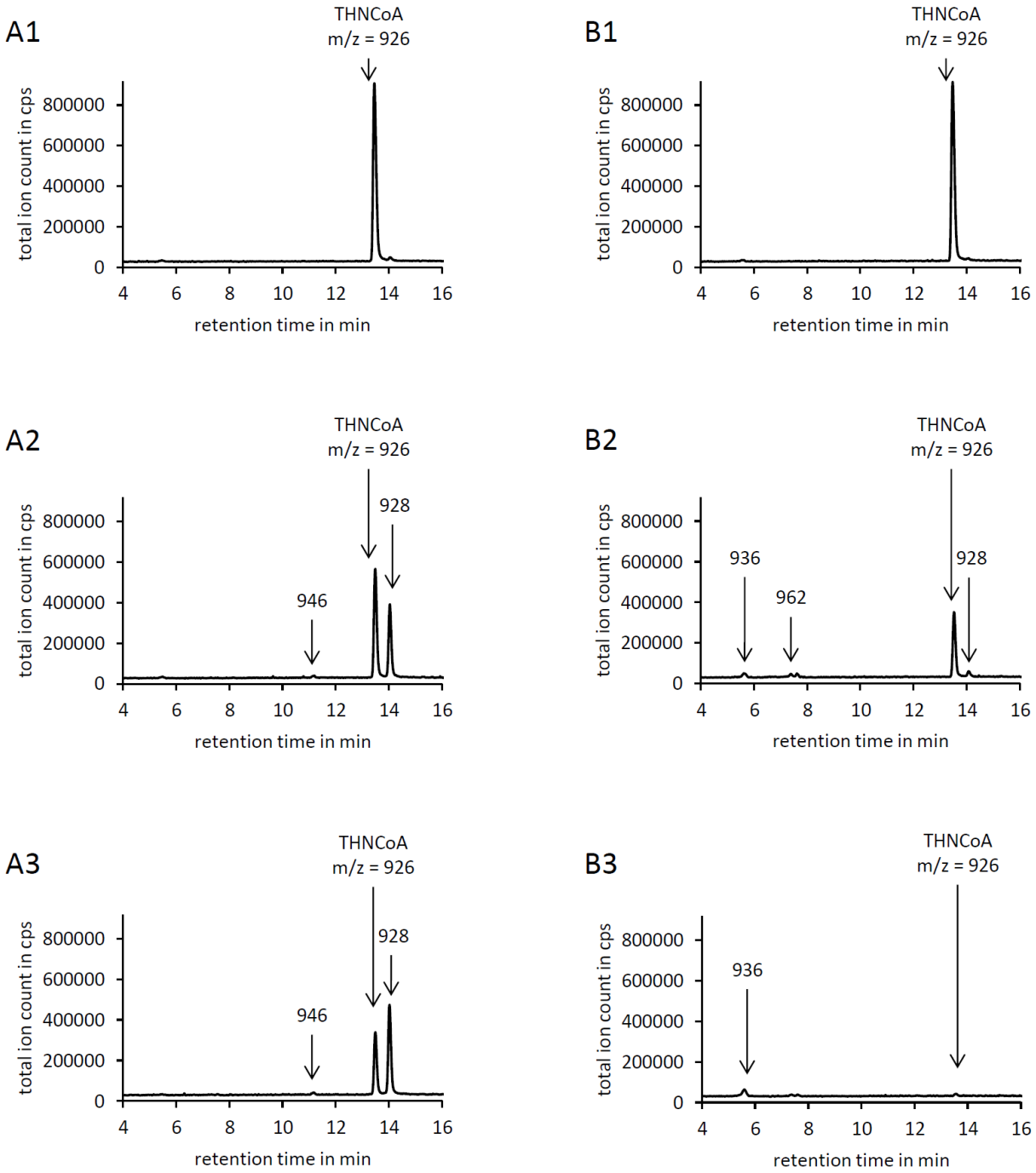


Figure 23 LC-MS chromatograms of reductase assays with 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) in cell free extracts of N47. All assays contained 50 µM THNCoA and 5 mM ATP. As electron donor, assay A contained 5 mM NADH and assay B contained 5 mM 2-oxoglutarate. (A1)/(B1): 0 min incubation time. (A2)/(B2): 15 min incubation time. (A3)/(B3): 90 min incubation time. All samples were analysed via LC-MS in single ion mode scanning for expected metabolites.

Subsequent experiments with an increased concentration of THNCoA indicated that the reduction of the latter was actually not limited to a certain conversion rate but to a fix amount of THNCoA that could be reduced under the given assay conditions, so most likely a co-substrate necessary for the reduction was depleted.

When 2-oxoglutarate was added as electron donor instead of NADH, a complete conversion of THNCoA was observed (Figure 23 panels B1 – B3). HHNCoA was only transiently detected and was obviously further converted 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 pre-incubation time of 30 min (see above).

These observations indicate that 2-oxoglutarate serves as electron donor for the THNCoA reductase, most likely by regenerating reduced ferredoxin via a 2-oxoglutarate:ferredoxin oxidoreductase comparable to the ferredoxin reducing system reported previously for *T. aromatica* (Doerner and Boll, 2002). However, 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). Cell free extracts of N47 harvested during the exponential growth phase typically contain some free coenzyme A as observed in previous experiments with the naphthoate:CoA ligases (2.04.01). This amount of CoA-SH might be sufficient to feed the 2-oxoglutarate:ferredoxin oxidoreductase reaction. Additionally, CoA-SH might be regenerated via unspecific thioesterases hydrolysing acyl-CoA compounds in the assay mixture. Further experiments showed that the addition of 1 mM CoA-SH even had an inhibitory effect, probably due to ATP-dependant coenzyme A ligases present in the extract which depleted the ATP required for the THNCoA reductase reaction.

4.04.02. THNCoA reductase reaction in cell free extract of NaphS2

In contrast to N47-cfe, which showed a clear preference for NADH over NADPH as electron donor for the THNCoA reducing system, NaphS2-cfe produced almost the same THNCoA conversion rates with either of these two co-substrates. The maximum conver-

sion rate was however only ca. 30% which is significantly lower than the rate obtained with N47-cfe (Figure 24 panel A).

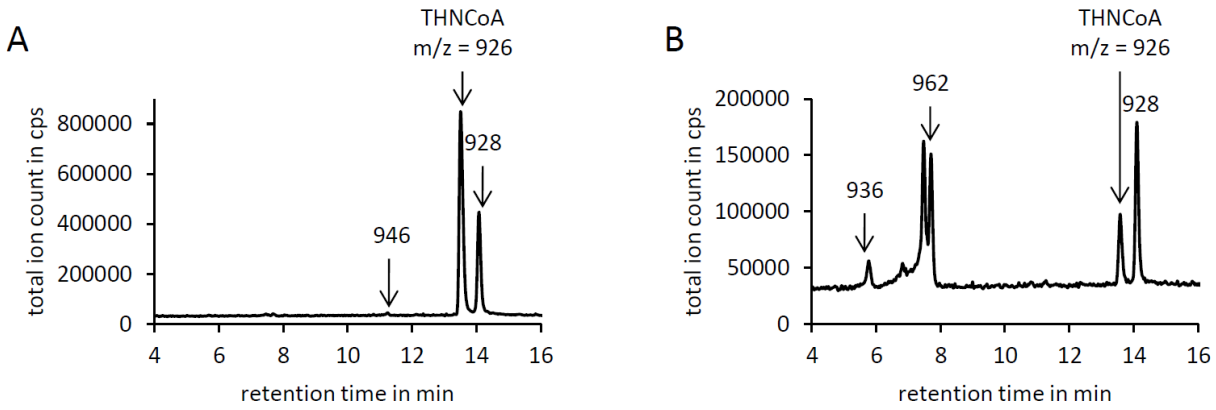


Figure 24 LC-MS chromatograms of reductase assays with 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) in cell free extracts of NaphS2 after 90 min incubation. Both assays (A and B) contained 50 µM THNCoA, 5 mM ATP and 5 mM NADPH. Assay B additionally contained 5 mM NAD^+. Please note different y-scales between panels A and B. Samples were analysed via LC-MS in single ion mode scanning for expected metabolites.

Again a metabolite with m/z = 946 and a retention time of 11.3 min appeared which had already been detected in THNCoA reductase assays with N47-cfe. Since the reductase reaction with NADPH was not inhibited by the presence of NAD^+, those two co-substrates 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 (Figure 24 panel B) which had the same retention times as the respective compounds formed in assays with N47-cfe. Hence the downstream pathways in N47 and NaphS2 most likely proceed via the same intermediates.

Unlike described for N47-cfe, where the NADH-dependant reduction of THNCoA seemed to be inhibited by the presence of dithionite, the latter could even serve as artificial electron donor for the reduction with NaphS2-cfe and conversion stopped at the same

OHNCoA isomers that were observed when the HHNCoA formed in reductase assays with N47-cfe was abiotically further reduced to OHNCoA by the addition of dithionite.

Best THNCoA conversion with NaphS2-cfe was obtained when 2-oxoglutarate was added as electron donor matching the results obtained with N47-cfe, albeit with the difference that the 2-oxoglutarate dependant reduction in NaphS2-cfe indeed required CoA-SH as additional co-factor as it is expected for a 2-oxoglutarate:ferredoxin oxidoreductase (Figure 25 panels A and B).

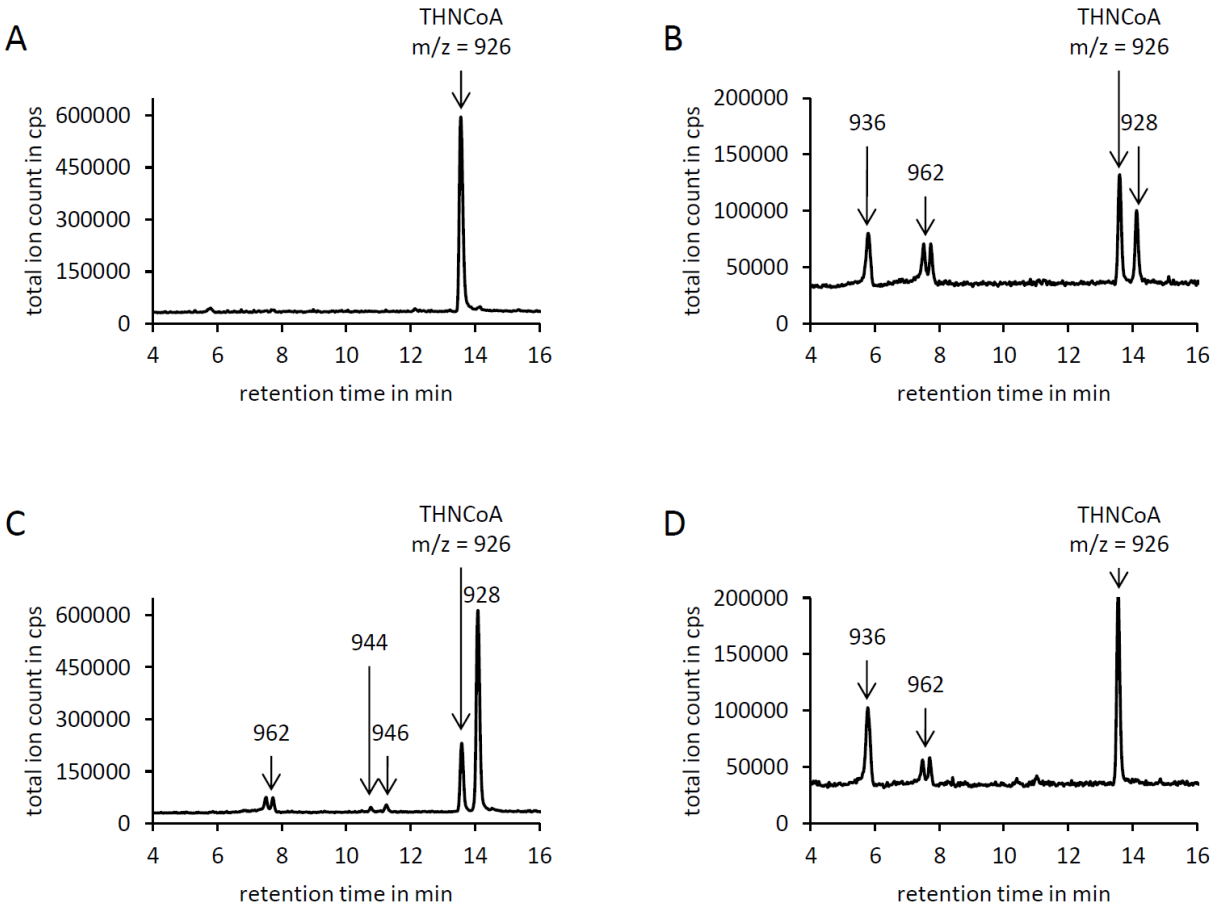


Figure 25 LC-MS chromatograms of reductase assays with 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) in cell free extracts of NaphS2 after 90 min incubation. All assays (A – D) contained 50 µM THNCoA, 5 mM ATP and 5 mM 2-oxoglutarate. Additionally, assays B – D contained the following components: (B): 1 mM CoA-SH. (C): 1 mM CoA-SH + 5 mM NADH. (D): 1 mM CoA-SH + 5 mM NAD^+. Please note different y-scales between panels A/C and B/D. Samples were analysed via LC-MS in single ion mode scanning for expected metabolites.

Without addition of CoA-SH, no significant conversion of THNCoA took place (panel A). Only if both 2-oxoglutarate (5 mM) and coenzyme A (1 mM) were added to the standard assay mixture, THNCoA drastically degreased and HHNCoA as well as the metabolites with m/z = 962 and m/z = 936 appeared (panel B). A similar picture was observed if the assays were additionally amended with NAD^+, but in this case no HHNCoA but only residual THNCoA and the downstream metabolites were detectable after 90 min incubation (panel D). In contrast, addition of NADH to assays containing 2-oxoglutarate and CoA-SH caused an accumulation of HHNCoA and seemed to inhibit further conver-

sion of this intermediate (panel C). 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^+ for this reaction. In presence of excess of NADH, the equilibrium of this reaction shifts towards the reduced substrate which prevents further conversion and leads to the accumulation of upstream metabolites.

In assays that contained 2-oxoglutarate as electron donor and were additionally amended with CoA-SH and NADH, a novel intermediate with m/z = 944 (retention time 10.8 min) was detected (Figure 25 panel C). The mass-to-charge ratio points at a β-oxo-octahydro-2-naphthoyl-CoA metabolite which serves as distinct indicator for a pathway proceeding via HHNCoA since no metabolites with equal mass are expected to be formed in a pathway via OHNCoA (see Table 9).

4.04.03. Conversion of hexa- and octahydro-2-naphthoic acids

A mixture of isomers of hexahydro-2-naphthoate as well as the isomeric pure compounds 1,4,4a,5,6,7,8,8a-octahydro-2-naphthoate 3,4,4a,5,6,7,8,8a-octahydro-2-naphthoate were tested for conversion to their CoA-thioester via a side reaction of the 2-naphthoate:CoA ligase in cell free extracts of N47. If this reaction formed an HHNCoA or OHNCoA isomer which was identical to the product of the THNCoA reductase, a further conversion by enzymes acting subsequent to the THNCoA reductase in the anaerobic naphthalene degradation pathway should be observable.

The hexahydro-2-naphthoate isomers turned out to be rather unstable and prone to undergo a disproportionation reaction forming tetrahydro-2-naphthoate and octahydro-2-naphthoate. Hence the CoA-ligase reaction on this substrate mixture mainly yielded THNCoA and a smaller share of OHNCoA as confirmed by LC-MS analysis (data not shown). HHNCoA was only formed in very little amounts that did not allow for the identification of further conversion products.

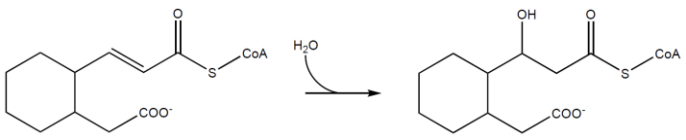
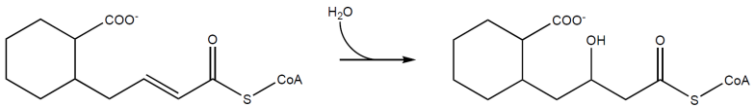
For 1,4,4a,5,6,7,8,8a-octahydro-2-naphthoate, no conversion to the respective CoA-ester occurred so the 2-naphthoate:CoA ligase did most likely not accept it as a substrate. In contrast, 3,4,4a,5,6,7,8,8a-octahydro-2-naphthoate was converted to an OHNCoA with

m/z = 930 and a retention time of 14.9 min. Regarding the retention time, this 3,4,4a,5,6,7,8,8a-OHNCoA was different from the OHNCoA isomers formed from the THNCoA reductase product (HHNCoA) upon addition of dithionite (see above). Although significant amounts of 3,4,4a,5,6,7,8,8a-OHNCoA were formed, no further metabolites could be detected. This finding most likely allows eliminating 3,4,4a,5,6,7,8,8a-OHNCoA from the list of possible products of the THNCoA reductase.

4.04.04. Assays with potential downstream metabolites

The two possible upstream metabolites *cis*-(*E*)-3-(2-(carboxymethyl)cyclohexyl)acrylyl-CoA and *cis*-4-(2-carboxycyclohexyl)crotonyl-CoA were tested for conversion in cell free extracts of naphthalene-/2-naphthoate-grown N47 or NaphS2 cultures. The naturally occurring metabolite should be converted by an enoyl-CoA hydratase which is assumed not to require any co-substrate but water (Table 10).

Table 10 Expected conversion of potential downstream metabolites in cell free extracts.

Substrate	Reaction scheme	Product
***cis-(E)*-3-(2-(carboxy-methyl)cyclo-hexyl)-acrylyl-CoA**		*cis*-3-(2-(carboxy-methyl)cyclo-hexyl)-3-hydroxy-propionyl-CoA
***cis*-4-(2-carboxy-cyclohexyl)-crotonyl-CoA**		*cis*-4-(2-carboxy-cyclohexyl)-3-hydroxy-butyryl-CoA

HPLC-analysis of the assay mixtures revealed no change for any of the two compounds during incubation in cell free extracts (data not shown). This indicates that neither

cis-(*E*)-3-(2-(carboxymethyl)cyclohexyl)acrylyl-CoA nor *cis*-4-(2-carboxycyclohexyl)-crotonyl-CoA can be metabolised by sulphate-reducing naphthalene degraders.

4.05. Discussion

4.05.01. The product of THNCoA reductase

The reduction of 5,6,7,8,-tetrahydro-2-naphthoyl-CoA (THNCoA) to a hexahydro-2-naphthoyl-CoA (HHNCoA) via an enzyme similar to the class I benzoyl-CoA reductases was previously demonstrated with cell free extracts of the N47 enrichment culture amended with NADH as best working electron donor (Eberlein *et al.*, 2013a). However, no complete conversion of THNCoA could be achieved in these assays and no further metabolites downstream of HHNCoA were detected. Our results clearly indicate that 2-oxoglutarate rather than NADH serves as electron donor in the natural THNCoA reducing system. *Desulfobacterium* N47 operates a modified citric acid cycle in which the ordinary 2-oxoglutarate dehydrogenase (Sanadi *et al.*, 1952; Ochoa, 2006) is replaced by a 2-oxoglutarate:ferredoxin oxioreductase (Bergmann *et al.*, 2011a). This enzyme can generate reduced ferredoxin reductase upon conversion of 2-oxoglutarate and CoA-SH to succinyl-CoA and CO_2 (Doerner and Boll, 2002). Reduced ferredoxin then serves as low-potential electrons delivering co-factor for the THNCoA reductase in analogy to the well-studied mechanism of benzoyl-CoA reductases (Boll and Fuchs, 1998).

The aforementioned NADH-driven reduction was supposedly an experimental artefact that was only observed because of the high excess of NADH (5 mM) present in the assay, enabling the generation of reduced ferredoxin. The reduction of ferredoxin by NADH should not occur at standard conditions regarding the standard redox potentials of -320 mV for NADH and around -430 mV for ferredoxin (Voet *et al.*, 2010). However, it might be thermodynamically favoured by a high NADH/NAD^+ ratio. According to the Nernst equation ($E_{red} = E^0_{red} - RT/zF \times \ln(c_{red}/c_{ox})$, whereas RT/F is 26.124 mV at 30 °C), a doubling of the NADH/NAD^+ ratio lowers the reduction potential by about 13 mV, so a ca. 500-fold excess of NADH over NAD^+ could make an electron transfer from NADH to ferredoxin thermodynamically feasible (-320 mV – 13 mV ×ln(512) = 437 mM). This

electron transfer might be mediated by an oxidoreductase but does most likely not take place under natural conditions, since this ratio is far above the one expected inside a living cell. Model studies with *Escherichia coli* found an NADH/NAD^+ ratio of 1/30 in glucose-fed aerobic cultures (Bennett *et al.*, 2009). Under anaerobic growth conditions, a significantly higher ratio was observed albeit it was still < 1 (de Graef *et al.*, 1999). Therefore, the only options to achieve ferredoxin reduction by NADH are either an energy-dependant electron transfer, which is rather unlikely to happen in energy-limited strict anaerobic bacteria, or an electron bifurcating process which couples the transfer of one electron to a low-potential acceptor to the transfer of another electron to an energetically favourable acceptor (Buckel and Thauer, 2013). However, the observed complete conversion of THNCoA in reductase assays with 2-oxoglutarate and CoA-SH as electron generating system in combination with the identification of genes coding for a ferredoxin and an oxidoreductase which directly neighbour the genes coding for the THNCoA reductase subunits (3.05.01) palpably point at 2-oxoglutarate rather than NADH as electron donor for THNCoA reduction. The reason why the reduction with NADH as co-substrate stopped before complete THNCoA conversion might be that upon usage of NADH as electron donor the NADH/NAD^+ ratio was lowered so the electron transfer from NADH to ferredoxin became thermodynamically unfavourable.

On the other hand, the high excess of NADH in those assays presumably inhibited an NAD^+-dependant β-hydroxyacyl-CoA dehydrogenase involved in the downstream pathway, which lead to an accumulation of upstream metabolites. In assays with other electron donors (2-oxoglutarate or NADPH), a better conversion of THNCoA and the formation of further metabolites was observed. An even better conversion of THNCoA was achieved by the addition of NAD^+, the supposed dehydrogenase co-substrate. We were able to identify two novel downstream metabolites with m/z = 946 and with m/z = 944. While a metabolite with m/z = 946 might occur both in a pathway via HHNCoA as well as in a pathway via octahydro-2-naphthoyl-CoA (OHNCoA) and could be either a β-hydroxyoctahydro-2-naphthoyl-CoA, resulting from water addition to HHNCoA, or a β-oxodecahydro-2-naphthoyl-CoA, resulting from water addition to OHNCoA and a subsequent dehydrogenase reaction, the latter mass-to-charge ratio of 944 is specific for a β-oxooctahydro-2-naphthoyl-CoA, a metabolite that only occurs in a

downstream pathway starting from HHNCoA (see Figure 21). We assume a downstream pathway involving a hydratase reaction on HHNCoA and a subsequent β-hydroxyacyl-CoA dehydrogenase which act in analogy to cyclohexa-1,5-diene-1-carboxyl-CoA hydratase (Laempe *et al.*, 1998) and 6-hydroxycyclohex-1-ene-1-carboxyl-CoA dehydrogenase (Laempe *et al.*, 1999) from the benzoyl-CoA pathway in *T. aromatica*.

Earlier studies with the THNCoA reductase could not detect any metabolites downstream of HHNCoA which was surprising since 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 (Boll *et al.*, 2000b). 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 LC-MS in single ion mode and was overlooked in previous studies that used HPLC for analysing the *in vitro* assays (Eberlein *et al.*, 2013a). Our results indicate that if the subsequent β-hydroxyacyl-CoA dehydrogenase is inhibited, mainly HHNCoA rather than its hydratase product accumulated as upstream metabolite. Since the co-substrate of a hydratase – water – is present in excess in aqueous assays, the equilibrium of such a reaction is typically expected to be in favour for the product. However, equilibrium concentrations of substrate and product were reported to be almost equal for cyclohexa-1,5-diene-1-carboxyl-CoA hydratase (Laempe *et al.*, 1998), so the very low concentration of HHNCoA hydratase product observed in our assays is actually not unrealistic. Assuming that water addition occurs in β,γ'-position in analogy to cyclohexa-1,5-diene-1-carboxyl-CoA hydratases, the HHNCoA hydratase could additionally be handicapped by the second ring as one of the ring junctions might be in γ'-position, depending on the conformation of HHNCoA.

Further novel metabolites identified in our assays (m/z = 962 and m/z = 936) assumably represented the CoA-esters of compounds that were earlier identified in culture extracts of the N47 enrichment culture (Annweiler *et al.*, 2002). While m/z = 936 matches the mass of 2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-ester, the other metabolite with m/z = 962 is most likely not a CoA-ester of the earlier postulated 3-(2-(carboxymethyl)cyclo-hexyl)acrylic acid or 4-(2-carboxycyclohexyl)crotonic acid but rather a β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA. This was deduced from the

chromatographic behaviour of this metabolite which indicated a structure that can undergo keto-enol tautomerism. In the benzoyl-CoA pathway, the analogous β'-hydroxy-β-oxo-intermediate is normally not detected as free metabolite since the Oah enzyme, which acts on 6-oxocyclohex-1-ene-1-carboxyl-CoA in this pahthway, has both enoyl-CoA hydratase and ring hydrolysing activity (Laempe *et al.*, 1999; Kuntze *et al.*, 2008). 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 (3.05.02). We assume that β-oxo-octahydro-2-naphthoyl-CoA hydratase and β'-hydroxy-β-oxo-decahydro-2-naphthoyl-CoA hydrolase are two separate enzymes in this pathway which is in accordance with the detection of β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA as free metabolite.

The artificial electron donor sodium dithionite, which had earlier been used for the benzoyl-CoA reductase (Boll and Fuchs, 1995), rather seemed to have an inhibitory effect on the THNCoA reducing system in cell free extract of N47 but worked well as electron donor for THNCoA reduction with NaphS2-cfe. In both cases, assays amended with dithionite did not form HHNCoA but two OHNCoA isomers as reduction products. This is in accordance with the earlier observed formation of dead-end products in assays with the related enzymes benzoyl-CoA reductase and cyclohexa-1,5-diene-1-carboxyl-CoA hydratase via artificial isomerisation and hydration reactions after addition of dithionite (Boll *et al.*, 2000b). Interestingly, we could show that the reduction of HHNCoA to OHNCoA by dithionite is a spontaneous abiotic reaction since it also occurred after inactivation of the cell free extract. This spontaneous HHNCoA reduction in presence of a strong reducing agent might also be an explanation for the previously reported occurrence of octahydro-2-naphthoic acid in cell extracts of the sulphate-reducing enrichment culture N47 (Annweiler *et al.*, 2002). The high amount of sulphide present in those extracts might be sufficient to reduce HHNCoA to OHNCoA.

Furthermore, the observed abiotic reduction of HHNCoA might also allow for a new interpretation of the benzoyl-CoA pathway in *R. palustris*: It is known that in this bacterium the β-oxidation-like degradation steps following the dearomatising ring-reduction start from a cyclic monoene intermediate rather than from a cyclohexadiene intermediate as reported for *T. aromatica* and a variety of other bacteria (Egland *et al.*,

1997; Harwood *et al.*, 1999). However, it is still unclear whether the diene intermediate is further reduced via the reverse reaction of an ordinary acyl-CoA dehydrogenase subsequently to the benzoyl-CoA reductase or if the dearomatising reductase itself yields cyclohex-1-enecarboxyl-CoA by transferring in total four electrons to benzoyl-CoA. To our knowledge, neither of these two assumptions could be verified or proven wrong to date. Based on our discovery of spontaneous abiotic reduction of the cyclic diene HHNCoA in assays with cell free extracts, one might speculate that a similar process is also possible under physiological conditions in *R. palustris*, albeit further experiments are needed to elucidate which kind of electron donors can mediate this spontaneous reduction. Following this new hypothesis, *R. palustris* might not need to employ an enzyme for the active reduction of the cyclohexadiene intermediate. Alternatively, a yet to be discovered mechanism could direct the spontaneously occurring reduction to the right position to obtain a suitable cyclohexenecarboxyl-CoA isomer.

Going back to the anaerobic naphthalene degradation pathway, the experiments with dithionite also give a hint to the conformation of the occurring HHNCoA isomer: Both OHNCoA isomers formed upon dithionite-mediated reduction of HHNCoA are different from 3,4,4a,5,6,7,8,8a-OHNCoA produced from 3,4,4a,5,6,7,8,8a-octahydro-2-napthoic acid by a side-reaction of the 2-naphthoate:CoA ligase. In the latter OHNCoA, the remaining double-bond is located between C1 and C2. Assuming that the spontaneous chemical reduction happens unspecifically at either of the two double bonds without further isomersiation, the resulting two OHNCoA isomers should exhibit the remaining double-bond in one of the two positions where it was located in HHNCoA. Therefore, the absence of an OHNCoA isomer with a double-bond between C1 and C2 indicates that also the HHNCoA produced by the THNCoA reductase has no double-bond in this position. This would allow to reduce the list of possible HHNCoA isomers depicted in Figure 5 (namely 4,4a,5,6,7,8-HHNCoA, 4a,5,6,7,8,8a-HHNCoA, 3,4,5,6,7,8-HHNCoA or 1,5,6,7,8,8a-HHNCoA) to only two remaining isomers, 4,4a,5,6,7,8-HHNCoA or 1,8a,5,6,7,8-HHNCoA. Since we have strong indications for a downstream degradation sequence similar to the one described for the benzoyl-CoA pathway in *T. aromatica*, which would not work for 1,8a,5,6,7,8-HHNCoA due to the unsuitable arrangement of its

double-bonds, we propose a pathway via 4,4a,5,6,7,8-HHNCoA involving β-like steps with ring-cleavage between C2 and C3 (Figure 26).

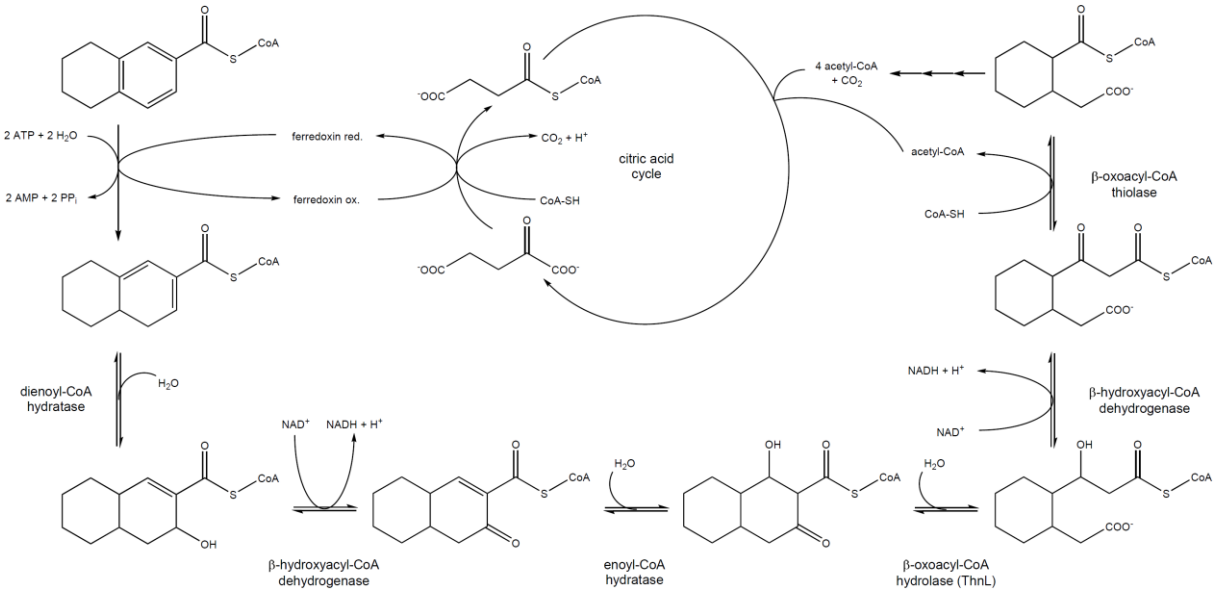


Figure 26 Proposed degradation sequence of 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) assuming the product of the THNCoA reductase to be 4,4a,5,6,7,8-hexahydro-2-naphthoyl-CoA. The subsequent β-oxidation-like pathway via 2-(carboxymethyl)cyclohexane-1-carboxyl-CoA feeds up to five acetyl-CoA to the citric acid cycle which in turn generates reduced ferredoxin as co-factor for the THNCoA reductase via a 2-oxoglutarate:ferredoxin oxidoreductase. The latter enzyme converts 2-oxoglutarate and CoA-SH to succinyl-CoA and CO_2 whereupon two electrons are transferred to ferredoxin.

In this pathway up to five acetyl-CoA formed via β-oxidation-like reactions can be fed into a modified citric acid cycle. The cycle in return provides low-potential electrons for the THNCoA reductase via a 2-oxoglutarate:ferredoxin oxidoreductase which correlates to the electron generating system of the benzoyl-CoA reductase in *T. aromatica* (Doerner and Boll, 2002). Therefore, the anaerobic naphthalene degradation pathway seems to feature a similar coupling of the dearomatising reductase to the oxidation of the end-product of the pathway, acetyl-CoA, to CO_2.

It has to be mentioned that the prior deduction of the position of the double-bonds in HHNCoA from the positions observed in the OHNCoA isomers after abiotic reduction of

HHNCoA holds some uncertainties since previous experiments showed that a cyclic diene may undergo isomerising reactions upon dithionite addition to cell free extracts (Boll *et al.*, 2000b). But unlike described in this work, our experiments showed that the formation of the same OHNCoA isomers from HHNCoA also occurred abiotically which excludes enzymatic isomerisation, albeit chemical isomerisation remains possible. Hence our results provide a strong hint to the conformation of HHNCoA but require further confirmation, for example by NMR-analysis. Nevertheless, a reduction of THNCoA in C3- and C4a-position seems to be the most reasonable option, as the related benzoyl-CoA reductases transfer electrons and protons to the carbon atom opposite of the carboxyl-CoA residue and to one of its neighbouring carbon atoms (Koch *et al.*, 1993; Buckel and Keese, 1995). In THNCoA, C4a is opposite of the carboxyl group and its neighbouring atoms inside the aromatic ring are C3 and C8a, so the two HHNCoA isomers 4,4a,5,6,7,8-HHNCoA and 4a,5,6,7,8,8a-HHNCoA are comparable to the product of benzoyl-CoA reductase, cyclohexa-1,5-diene-1-carboxyl-CoA. Comparing the positions C3 and C8a, the latter is presumably more difficult to access by the enzyme due to its connection to the saturated ring. Thus also mechanistic considerations imply that the transfer of electrons and protons to HHNCoA most likely occurs at C3 and C4a, leading to 4,4a,5,6,7,8-HHNCoA.

4.05.02. 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 ratio in GC-MS analyses (Annweiler *et al.*, 2002). Since the further downstream metabolite 2-(carboxy-methyl)cyclohexane-1-carboxylic acid was found to occur only as *cis*-isomer (Annweiler *et al.*, 2002) and β-oxidation-like reactions usually proceed via *E*-unsaturated CoA-ester intermediates (Schulz, 1991), we tested the chemically synthesised CoA-esters of the *cis*-(*E*)- isomers of the two compounds for conversion in cell free extracts of N47 and NaphS2. However, none of the tested CoA-esters seemed to be metabolised. Albeit 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 in cell free extracts of sulphate-reducing naphthalene degraders casts doubt on the previous interpretation of the identified mass.

In the referred work, metabolites were extracted as free acids from N47 cultures and the acids were derivatised by trimethylsulfonium hydroxide, which acts as a methylating agent on carboxyl groups (Yamauchi *et al.*, 1979). High-resolution GC-MS could confirm the elemental composition of the respective metabolite as $C_{11}H_{16}O_4$ (non-methylated 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 (Figure 27).

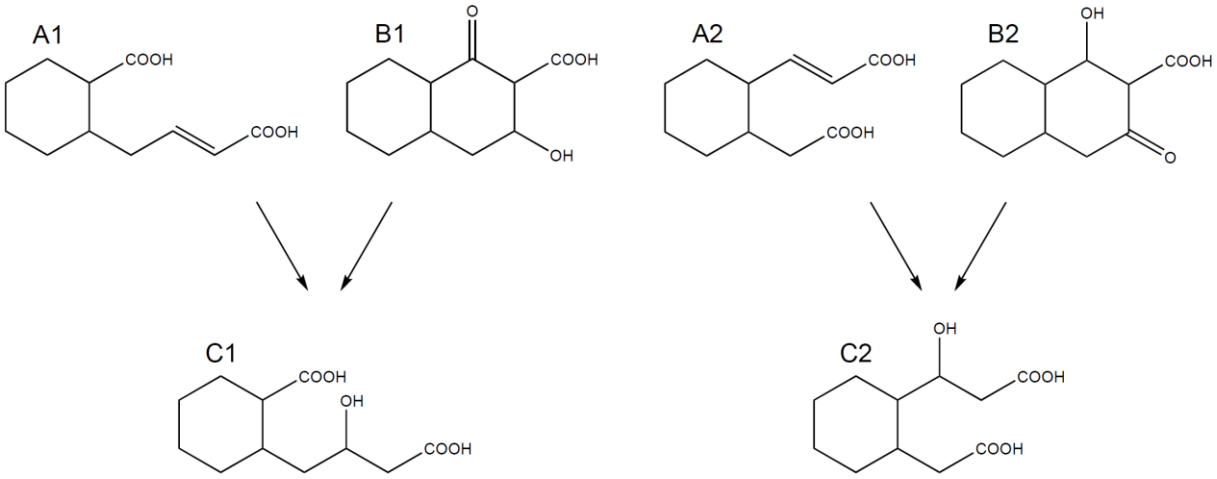


Figure 27 Potential intermediates of anaerobic napthalene degradation correlating with the previously observed elemental composition $C_{11}H_{16}O_4$ (A and B) and their downstream metabolites (C). (A1): 4-(2-carboxycyclohexyl)but-2-enoic acid. (A2): 3-(2-(carboxymethyl)cyclohexyl)acrylic acid. (B1): 3-hydroxy-1-oxodecahydro-2-naphthoyl-CoA. (B2): 1-hydroxy-3-oxodecahydro-2-naphthoyl-CoA. (C1): 4-(2-carboxycyclohexyl)-3-hydroxybutyric acid. (C2): 3-(2-(carboxymethyl)cyclohexyl)-3-hydroxyacrylic acid.

Assuming hexahydro-2-naphthoyl-CoA as the final product of reductive dearomatisation (Eberlein *et al.*, 2013a), 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 (Figure 21). This would represent a

reaction scheme similar to the 6-oxocyclohex-1-enecarboxyl-CoA hydratase/hydrolase from anaerobic benzoate degrading bacteria (Kuntze *et al.*, 2008). 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 (Figure 27). Regarding the apparent absence of an enoyl-CoA hydratase activity towards cyclohexane-derivatives with unsaturated C3- or C4-side-chains, we postulate β'-hydroxy-β-oxo-decahydro-2-naphthoyl-CoA as true intermediate of anaerobic naphthalene degradation rather than 3-(2-(carboxymethyl)cyclohexyl)acrylyl-CoA or 4-(2-carboxycyclohexyl)-crotonyl-CoA as proposed previously.

This is in perfect agreement with our analysis of downstream metabolites in THNCoA reductase assays (see above), so the postulated pathway via HHNCoA and β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA (Figure 26) is supported by the results of two independent experimental approaches.

5. Conversion of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-ester in the downstream pathway of anaerobic naphthalene degradation

Co-workers: Andrey Zaytsev, Bernard Golding, Susanne Stephan, Oliver Schmitz, Nicole Nightingale, Rainer Meckenstock

5.01. Abstract

The cyclohexane-derivative *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid has previously been identified as metabolite in the pathway of anaerobic degradation of naphthalene. We tested the corresponding CoA-esters of isomers and analogues of this compound for conversion in cell free extracts of the anaerobic naphthalene degraders N47 and NaphS2. Conversion was only observed for the *cis*-isomer, verifying that this is a true intermediate and no dead-end product. As confirmed by mass-spectrometric analyses, conversion is achieved via an acyl-CoA dehydrogenase and a subsequent hydratase yielding an intermediate with a tertiary hydroxyl group. Enzymes from the recently identified *thn*-operon could be associated with these reactions based on their sequence homologies to enzymes from related pathways. We propose a novel kind of ring-opening lyase, ThnQS, to be involved in the subsequent cleavage of the cyclohexane ring. We could also prove conversion of pimeloyl-CoA and the potential downstream metabolite glutaryl-CoA, which allows proposing a link to the central metabolism via a non-decarboxylating glutaryl-CoA dehydrogenase and a subsequent glutaconyl-CoA decarboxylase.

5.02. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a major class of environmental pollutants with carcinogenic and toxic effects on humans and environmental organisms (Preuss *et al.*, 2003; Boehm and Page, 2007). PAHs occur naturally in coal tar and crude oil and are

produced during combustion processes (Wilkes and Schwarzbauer, 2010). Due to the human exploitation of these resources and correlated spills, ground- and surface-waters as well as sediments are frequently contaminated with PAHs (Harkins *et al.*, 1988; Luthy *et al.*, 1994). Environmental remediation of PAH-contaminated sites is mainly achieved via microbial degradation (Johnsen *et al.*, 2005; Peng *et al.*, 2008; Gupta *et al.*, 2015). Since this biodegradation is usually limited by the depletion of oxygen, the most favourable electron acceptor (Christensen *et al.*, 1994; Wiedemeier, 1999; Meckenstock *et al.*, 2015), anaerobic degradation pathways are of high ecological importance.

Microbial pathways for anaerobic PAH degradation have mostly been studied with the bicyclic aromatic hydrocarbon naphthalene as model compound (Meckenstock *et al.*, 2004; Meckenstock *et al.*, 2016). The ability to achieve anaerobic naphthalene degradation has been demonstrated for the deltaproteobacterial strains N47 and NaphS2 with sulphate as electron acceptor (Galushko *et al.*, 1999; Meckenstock *et al.*, 2000). Initial activation of naphthalene is achieved through carboxylation to 2-naphthoate (Mouttaki *et al.*, 2012) followed by formation of the corresponding CoA-ester. Unlike the anaerobic degradation of monoaromatic hydrocarbons, the further degradation of naphthalene and other bicyclic aromatic hydrocarbons does not proceed via benzoyl-CoA as shown by metabolomic (Annweiler *et al.*, 2002) and transcriptomic (DiDonato *et al.*, 2010) approaches.

The further metabolisation of 2-naphthoyl-CoA proceeds via reductive dearomatisation with a first reduction taking place at the non-substituted ring of 2-naphthoyl-CoA yielding 5,6,7,8-tetrahydro-2-naphthoyl-CoA (Eberlein *et al.*, 2013b; Eberlein *et al.*, 2013a). The latter is reduced stepwise to a hexahydro-2-naphthoyl-CoA with unknown disposition of the remaining double bonds (Eberlein *et al.*, 2013a; Estelmann *et al.*, 2015). As deduced from gas chromatography–mass spectrometry (GC-MS) analyses of metabolites extracted from N47 cultures, β-oxidation-like reactions are expected for the downstream pathway (Annweiler *et al.*, 2002). After the first ring-opening reaction, the pathway proceeds via metabolites with a cyclohexane skeleton. The last identified metabolite of the downstream pathway was *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid (Annweiler *et al.*, 2002).

The present study investigates the fate of the intermediate *cis*-2-(carboxymethyl)-cyclohexane-1-carboxylic acid coenzyme A ester during the anaerobic degradation of naphthalene. The reaction sequence downstream of this intermediate is of special interest since it is assumed to be a pre-requisite for the second ring-cleavage.

5.03. Experimental procedures

5.03.01. Growth of bacterial cells and preparation of cell free extracts

The enrichment culture N47 and *Deltaproteobacterium* NaphS2 were grown in big cultures as delineated in 2.03.01. Cell free extracts were prepared aerobically as described in 2.03.02. Storage of the cell pellets for several months at -80 °C did not affect the measured enzyme activity.

5.03.02. Chemical synthesis of potential metabolites

The *cis*- and *trans*-isomer of 2-(carboxymethyl)cyclohexane-1-carboxylic acid as well as 2-(carboxymethyl)cyclohex-1-ene-1-carboxylic acid were synthesised by Dr. Andrey Zaytsev in the group of Prof. Dr. Bernard Golding, University of Newcastle, UK.

5.03.03. Synthesis and purification of CoA-esters

CoA-esters were synthesised via their and purified as depicted earlier (4.03.05). Glutaryl-CoA was synthesised from the commercially available glutaric anhydride in lieu of the respective succinimidyl-ester. Supelclean™ ENVI™-8 SPE columns (Sigma-Aldrich Chemie GmbH, Munich, Germany) were generally used for purification.

Due to the possible intramolecular formation of an anhydride that eliminates the CoA-thioester, all CoA-esters derived from dicarboxylic compounds were unstable in aqueous solutions with an estimated half-life of a few hours. Therefore, substrates or conversion products had to be stored as dry compounds.

5.03.04. Discontinuous assays with CoA-esters

MOPS/KOH buffer (100 mM, pH 7.3) containing 15 mM $MgCl_2$ was used as standard enzyme test buffer. The assays contained 30 – 50% (v/v) cell free extracts of N47 or NaphS2 (2.03.02) and were incubated at 30 °C and shaking with 900 rpm. All assays were conducted discontinuously (2.03.07) and subsequently analysed by HPLC (see below). Some assays were also performed with pure enzymes that were heterologously produced previously (2.03.05) replacing the cell free extracts.

Dehydrogenase assays for the conversion of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-esters, *trans*-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-esters, cyclohexanecarboxyl-CoA, cyclohexylacetyl-CoA, pimeloyl-CoA and glutaryl-CoA contained 1 mM of the respective substrate and 2 mM ferrocenium hexafluorophosphate as artificial electron acceptor (Lehman and Thorpe, 1990). Where indicated, assays for conversion of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-ester additionally contained 5 mM NAD^+ or 1 mM CoA-SH. Hydratase assays with 2-(carboxymethyl)cyclohex-1-ene-1-carboxylic acid CoA-esters contained 1 mM of the respective substrate without further additives.

Cis-2-(carboxymethyl)cyclohexane-1-carboxylic CoA-ester was also tested for conversion by a reverse thiolase reaction in assays containing 1 mM of the substrate, 2 mM acetyl-CoA, 2 mM DTT and 15 mM $MgCl_2$. To facilitate this reverse reaction and the reversal of further upstream reaction steps, NADH and/or sodium dithionite were added to some of the assays at a concentration of 5 mM each.

5.03.05. HPLC analysis

Samples were analysed by HPLC like described in 2.03.08, but a shorter gradient from 5 – 20% acetonitrile over 15 min was applied.

5.03.06. GC-MS analysis of extracted products

Single CoA-esters that were isolated during the HPLC run were hydrolysed and analysed via GC-MS as described previously (Eberlein *et al.*, 2013a). The hydrolysed metabolites were derivatised with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) converting all carboxylic acid groups to trimethylsilyl-esters (Stalling *et al.*, 1968). For the derivatisation reaction, water-free samples were amended with 20% (v/v) BSTFA and incubated at 60 °C for 30 min.

5.03.07. High-resolution MS analysis

LC-coupled high-resolution mass-spectrometric (HR-MS/MS) analysis was conducted by Susanne Stephan in the group of Prof. Dr. Oliver Schmitz (chair for Applied Analytical Chemistry, University of Duisburg Essen, Germany).

5.04. Results

5.04.01. Conversion of potential metabolites in cell free extract

To test for conversion in cell free extracts of N47 and NaphS2, CoA-thioesters of the known metabolite *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid were chemically synthesised as defined mixtures of isomers. The synthesis of the free acid afforded a racemic mixture (i.e. enantiomers, (1*R*,2*R*)- and (1*S*,2*S*)-2-(carboxymethyl)cyclohexane-1-carboxylic acid). The subsequent CoA-ester synthesis resulted in a total of four different *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic CoA-ester isomers as the dicarboxylic compounds had two possible carboxyl sites for the CoA-thioester formation. The mixtures obtained were used for conversion assays without further separation.

To test for possible enzyme reactions, a mixture of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-esters was added to cell free extracts of N47 or NaphS2. Due to the two possible attachment sites for the CoA-ester, the substrate mixture produced a double-peak in the HPLC-chromatogram representing the respective isomers which eluted around 12 min (Figure 28).

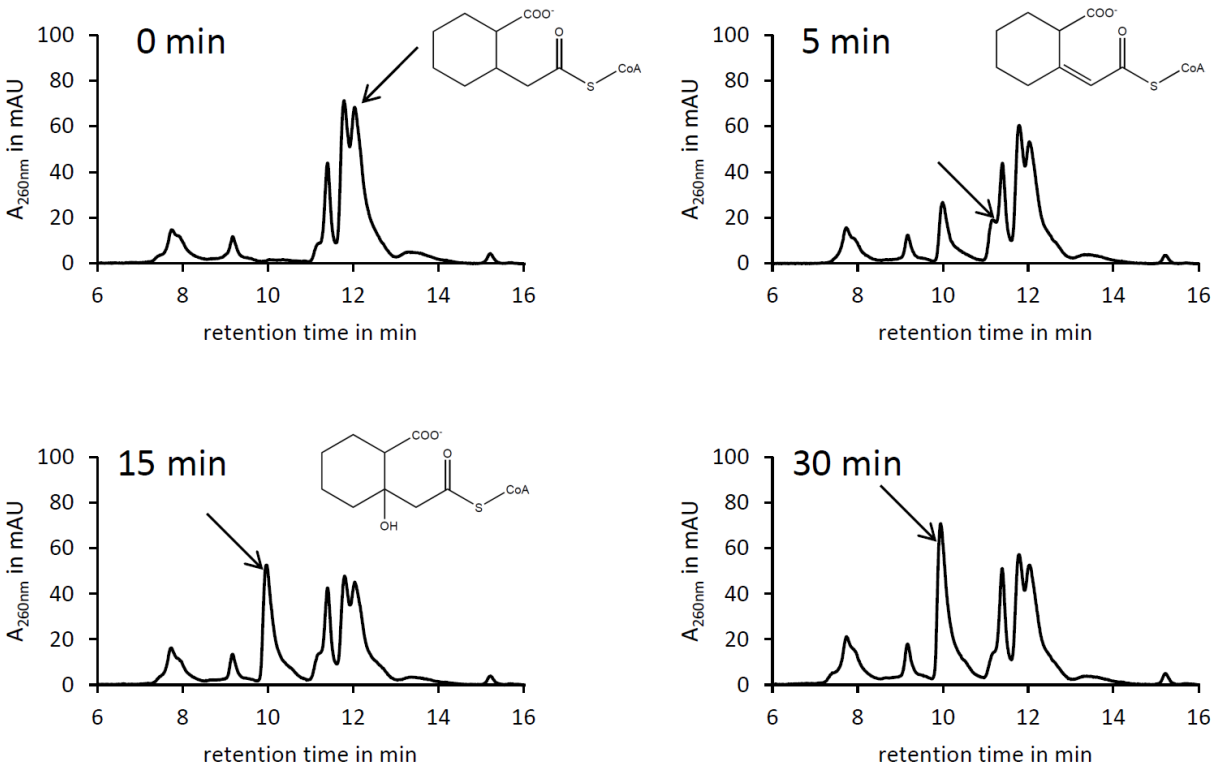


Figure 28 HPLC chromatograms showing the time-course of the conversion of CoA-esters of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid (four isomers) in cell free extract of strain NaphS2 with addition of 2 mM ferrocenium hexafluorophosphate as artificial electron acceptor. The figure shows a representative example out of several replicates with cell free extracts of N47 or NaphS2.

After 5 min of incubation with cell free extract, a new compound eluting at 11 min appeared. Since this conversion only occurred in the presence of the artificial electron acceptor ferrocenium hexafluorophosphate, it was most likely caused by an acyl-CoA dehydrogenase reaction introducing a C=C double-bond. This compound was immediately further converted giving rise to another product peak, which eluted after 10 min and presumably resulted from a water-addition to the double bond. While the first product peak decreased during further incubation, the second one increased, reaching its maximum after 30 min of incubation. The double-peak representing the substrate-isomers decreased simultaneously which might be caused by the activity of a CoA-transferase keeping the isomers in equilibrium.

Similar assays were conducted with a mixture of the four possible CoA-esters of *trans*-2-(carboxymethyl)cyclohexane-1-carboxylic acid and with a mixture of the two CoA-esters of 2-(carboxymethyl)cyclohex-1-ene-1-carboxylic acid (Figure 29).

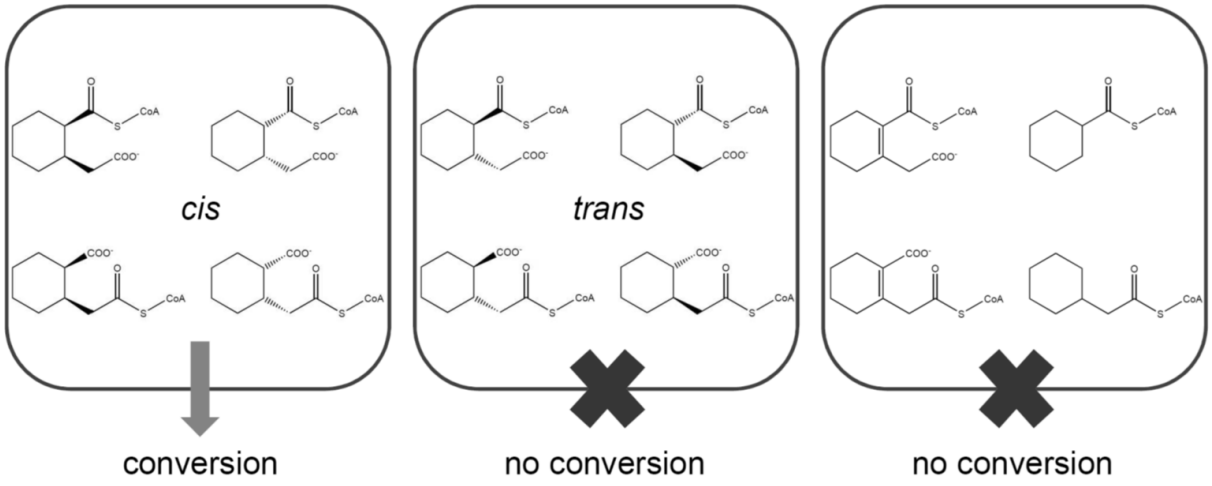


Figure 29 Schematic view of the results of enzyme assays testing different isomers and analogues of 2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-esters for conversion in cell free extracts of the napthalene degraders N47 and NaphS2.

In contrast to the *cis*-isomers, neither the *trans*-isomers nor the two possible CoA-esters of 2-(carboxymethyl)cyclohex-1-ene-1-carboxylic acid were converted by cell free extracts of N47 or NaphS2. Substrate analogues with only one side chain at the cyclohexane ring, namely cyclohexanecarboxyl-CoA and 2-cyclohexylacetyl-CoA were also not transformed (Figure 29). Therefore, the observed reaction specifically occurred on dicarboxylic compounds with a *cis*-configuration of the side chains.

The conversion of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-ester always stopped at the second product (Figure 28). It could not be driven further by the addition of NAD^+ and/or free coenzyme A which could serve as co-factors for a subsequent β-hydroxyacyl-CoA dehydrogenase or a β-oxoacyl-CoA thiolase, respectively. Also a reverse thiolase assay with *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-

esters in cell free extracts, which should elongate one of the side chains by an acetyl-unit, was not observed.

5.04.02. Mass-spectrometric analyses

The conversion products of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic CoA-ester were further analysed by mass-spectrometry. GC-MS analysis of the hydrolysed and BSTFA-derivatised substrate *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic CoA-ester (Figure 28, 0 min) revealed a highest mass fragment of 315.1 units (m/z), which corresponds to the cleavage of one methyl group from the trimethylsilyl-ester (Figure 30).

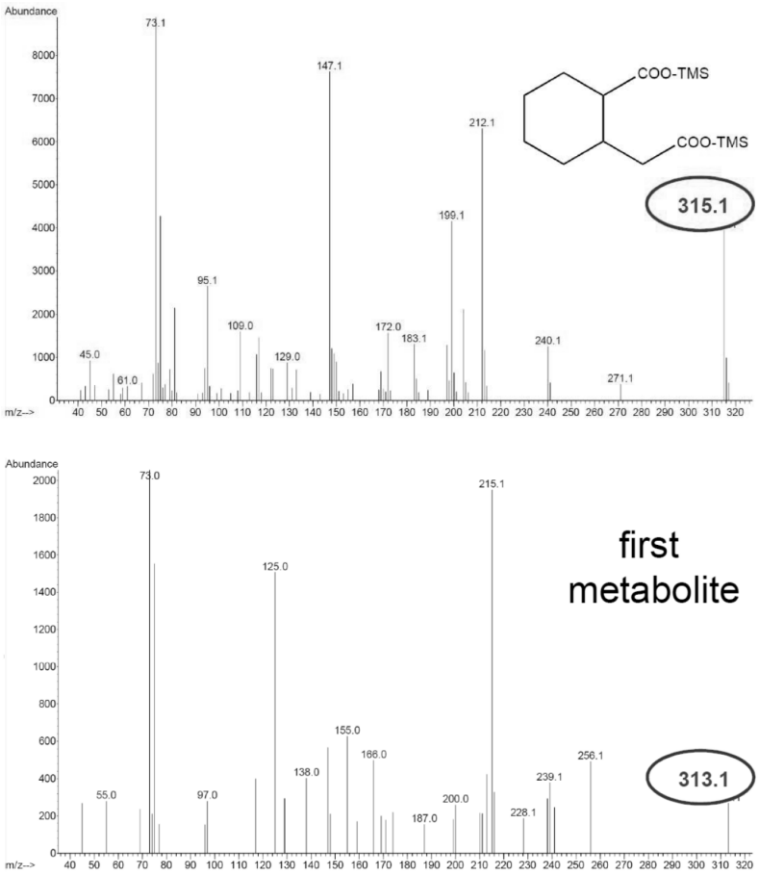


Figure 30 Mass-spectra of hydrolysed and BSTFA-derivatised *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic CoA-ester (top) and the first observed metabolite (bottom) obtained by GC-MS.

The respective fragment ion of the first metabolite appearing after 5 min in the enzyme assay had a mass of 313.1 units, accounting for a mass-difference of -2 units to the substrate. This clearly indicates the formation of a double-bond (Figure 28, 5 min). The second metabolite showed a mass-difference of +18 units to the first one, indicating the addition of water (Figure 28, 15 min).

Still the position of the introduced double bond and the subsequently added hydroxyl group remained unclear. The double-bond could have been introduced in three different positions as depicted in Figure 31.

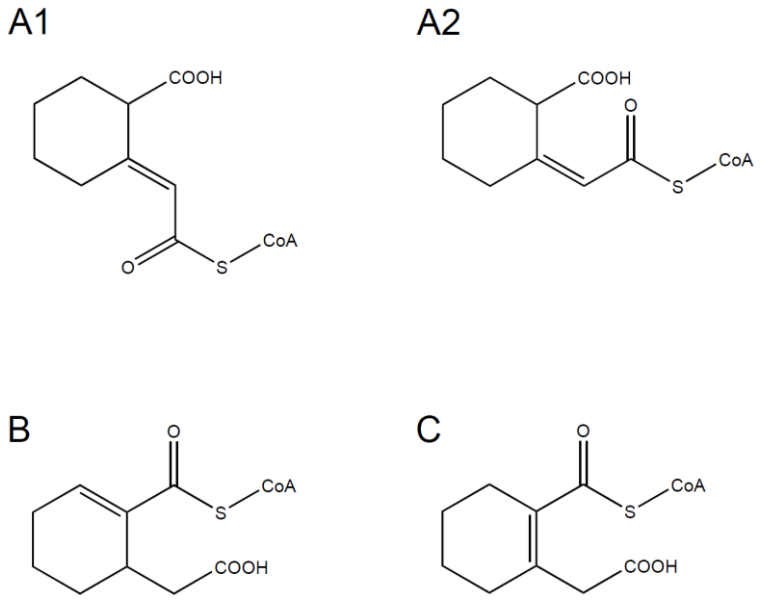


Figure 31 Possible conformations of the first conversion product of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic CoA-ester. (A1): (*E*)-2-(2-carboxycyclohexylidene)acetyl-CoA. (A2): (*Z*)-2-(2-carboxycyclohexylidene)acetyl-CoA. (B): 2-(carboxymethyl)cyclohex-6-ene-1-carboxyl-CoA. (C): 2-(carboxymethyl)-cyclohex-1-ene-1-carboxyl-CoA.

Compound C had already been tested for conversion in cell free extracts (Figure 29). Since no conversion was observed, this compound could be ruled out as potential metabolite. For a further discrimination between compounds A and B, the CoA-ester products were analysed by high-resolution LC-MS. This also confirmed the atomic

composition to be $C_{30}H_{46}N_7O_{19}P_3S$ (Figure 32) which is in accordance with the expected CoA-ester structures (Figure 31).

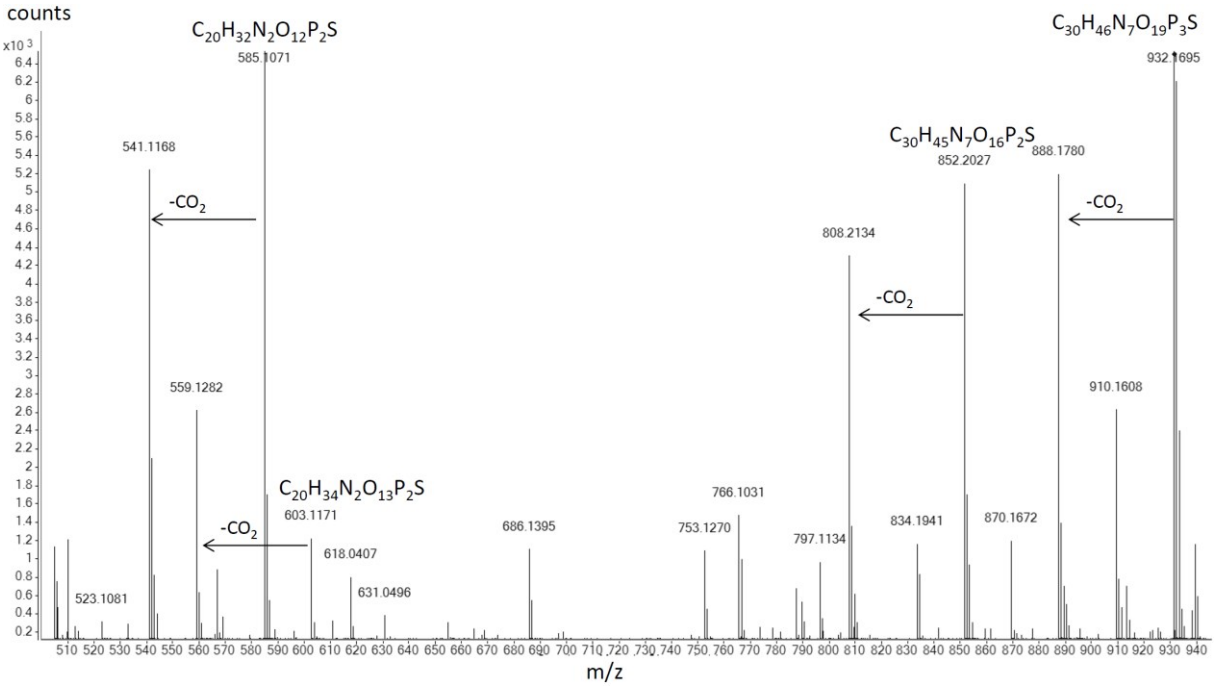


Figure 32 High-resolution mass-spectrum from MS/MS experiments with the first conversion product of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-ester.

The high-resolution mass-spectrum revealed several pairs of mass-fragments that resulted from the cleavage of a CO_2 group (Figure 32). In contrast, cleavage of a $C_2H_2O_2$-group was not observed. This points to compound A in Figure 31, 2-(2-carboxy-cyclohexylidene)acetyl-CoA, as metabolite which can release a CO_2-fragment from its 2-carboxyl group. For compound B in Figure 31, a release of both CO_2- and $C_2H_2O_2$-fragments would be expected. 2-(2-Carboxycyclohexylidene)acetyl-CoA might occur in *E*- (A1 in Figure 31) or *Z*-configuration (A2). MS-analysis did not allow to discriminate between the two stereoisomers.

The identification of 2-(2-carboxycyclohexylidene)acetyl-CoA as the conversion product of the dehydrogenase reaction implies the second reaction to be a water addition yielding a hydroxyl group in the only possible, position C1, resulting in 2-(1-hydroxy-2-

carboxy-cyclohexyl)acetyl-CoA (Figure 28, 15 min). The latter metabolite could be verified by high-resolution MS-analysis (data not shown).

5.04.03. Conversion of potential downstream metabolites

The degradation pathway downstream of 2-(1-hydroxy-2-carboxycyclohexyl)acetyl-CoA is assumed to involve a second ring-opening step and consecutive β-oxidation-like reactions which would generate pimeloyl-CoA and glutaryl-CoA. Both of these potential downstream metabolites were converted in cell free extracts of naphthalene-grown N47 cultures in a ferrocenium-dependant dehydrogenase reaction (Figure 33).

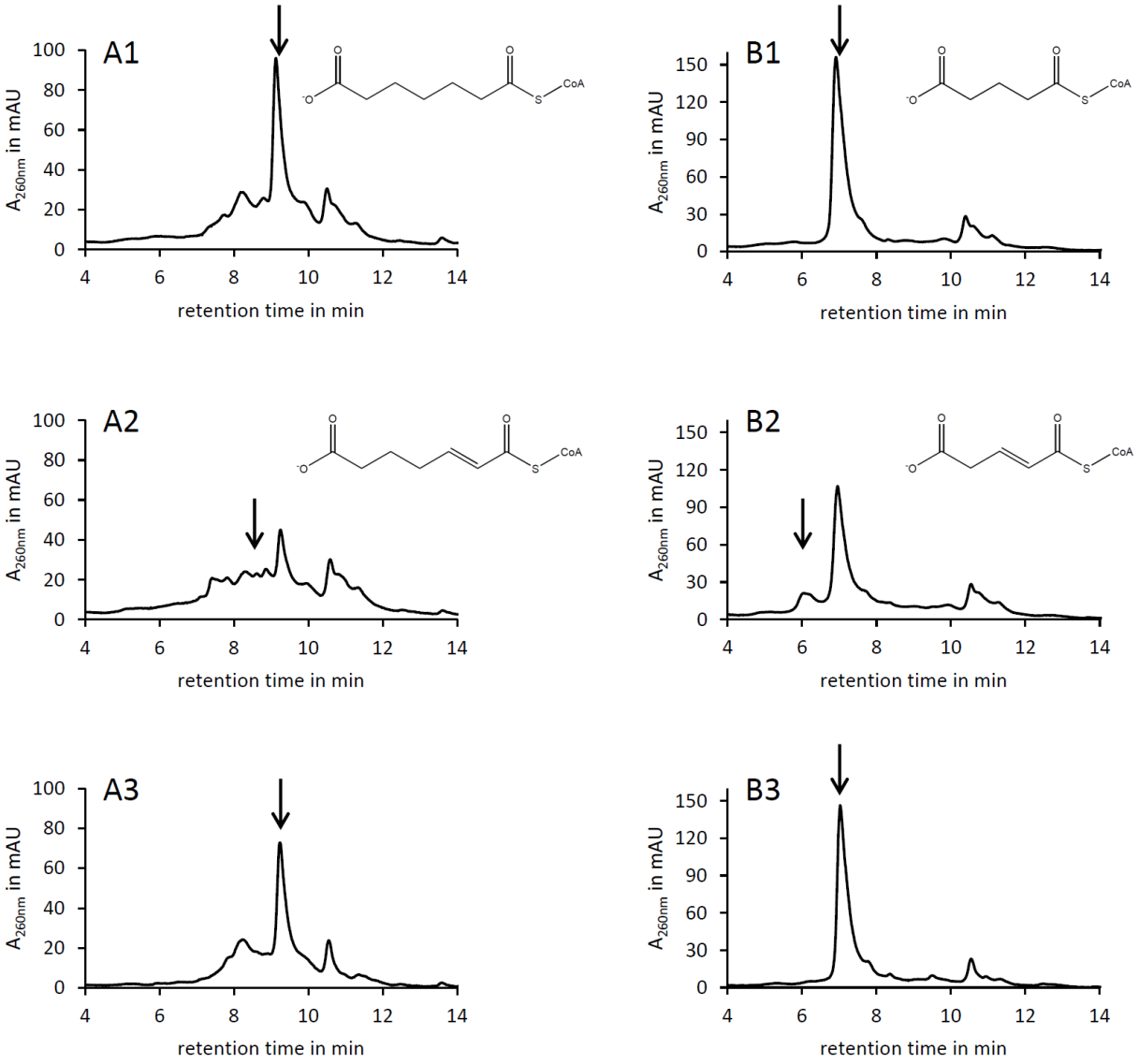


Figure 33 HPLC chromatograms depicting the conversion of pimeloyl-CoA and glutaryl-CoA in cell free extracts of N47. (A1): pimeloyl-CoA + ferrocenium, t = 0 min; (A2): t = 30 min; (A3): control without ferrocenium, t = 30 min; (B1): glutaryl-CoA + ferrocenium, t = 0 min; (B2): t = 30 min; (B3): control without ferrocenium, t = 30 min. Figure shows a representative example out of two replicates each with cell free extracts of N47 or NaphS2

The conversion of pimeloyl-CoA produced several new peaks, indicating that the initial unsaturated product was probably further transformed via water-addition and subsequent β-oxidation steps. Glutaryl-CoA was converted to one single earlier eluting product. According to its retention time, the product was assumed to be glutaconyl-CoA

rather than crotonyl-CoA, which could be formed by a decarboxylating glutaryl-CoA dehydrogenase reaction.

The same conversions were also observed for cell free extracts of 2-naphthoate-grown NaphS2 cultures. This indicates that both compounds occur as metabolites during anaerobic naphthalene degradation and can be metabolised via acyl-CoA dehydrogenases. In accordance with this finding, pimelic acid regularly occurred as metabolite in culture extracts of N47 or NaphS2 (data not shown).

5.04.04. Conversion with heterologously produced enzymes

cis-2-(carboxymethyl)cyclohexane-1-carboxylic acid CoA-ester was also tested for conversion by the two heterologously produced acyl-CoA dehydrogenases encoded within the *thn*-operon, ThnO and ThnT (3.03.05). None of the two enzymes showed activity towards this substrate like it was the case for cell free extracts of N47 and NaphS2.

5.05. Discussion

5.05.01. Proposed reaction scheme

Conversion studies of the CoA-esters of *cis*-2-(2-carboxycyclohexyl)acetic acid in cell free extracts of N47 and NaphS2 indicated the formation of a double-bond in α,β-position of *cis*-2-(2-carboxycyclohexyl)acetyl-CoA followed by water addition in β-position yielding a tertiary hydroxyl group (Figure 34).

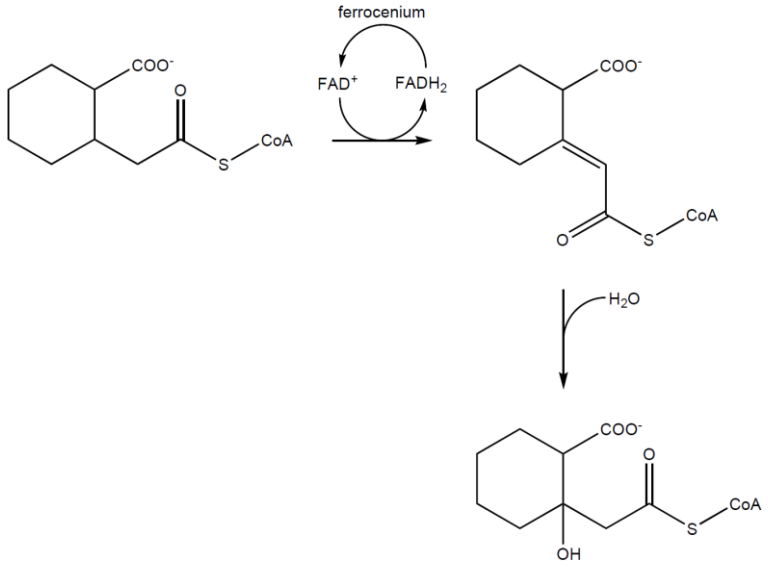


Figure 34 Proposed reaction scheme for the ferrocenium-dependent conversion of *cis*-2-carboxy-cyclohexylacetyl-CoA in cell free extracts of N47 and NaphS2. 2-(2-carboxycyclohexyl)acetyl-CoA is unsaturated in a ferrocenium-dependant reaction to 2-(2-carboxyclohexylidene)acetyl-CoA, which is further converted to 2-(1-hydroxy-2-carboxycyclohexyl)acetyl-CoA by water addition. The unsaturated intermediate is depicted in *E*-configuration but could be the *Z*-isomer.

The α,β-unsaturation of acyl-CoA thioesters is usually achieved by flavoenzymes from the ACAD superfamily of acyl-CoA dehydrogenases (Kim *et al.*, 1993). Typical members of this family have a homotetrameric structure (Kim and Miura, 2004). The conversion of *cis*-2-(2-carboxycyclohexyl)acetyl-CoA requires a special enzyme that can react on a tertiary carbon atom.

Acyl-CoA dehydrogenases acting on branched substrates are for example known from the acyclic terpene utilisation pathway. AtuD from the citronellol-degrading bacterium *Pseudomonas citronellolis* (Seubert, 1960) catalyses the α,β-unsaturation of citronellyl-CoA (Foerster-Fromme and Jendrossek, 2006; Foerster-Fromme *et al.*, 2008). Unlike common acyl-CoA dehydrogenases (Kim and Miura, 2004), AtuD produces *Z*-geranyl-CoA and not the *E*-isomer. A homologous enzyme is encoded in the genome of *Pseudo-monas aeruginosa* PAO1 (Aguilar *et al.*, 2006). The leucine/isovalerate utilisation pathway of *P. aeruginosa* PAO1 contains an additional enzyme with similar, but

E-specific function: LiuA catalyses the desaturation of isovaleryl-CoA to 3-methyl-glutaconyl-CoA (Foerster-Fromme and Jendrossek, 2008).

Other examples for acyl-CoA dehydrogenases with branched substrates are the (2*S*)-methylsuccinyl-CoA dehydrogenase Mcd from the ethylmalonyl-CoA pathway of *Rhodobacter sphaeroides* (Erb *et al.*, 2009) and its homologue from *Phaeobacter gallaeciensis* DSM 26640 (Frank *et al.*, 2014). A comparable reaction can also occur inside a ring structure like that known for the cyclohexanecarboxyl-CoA dehydrogenase AliB (formerly BadJ) from *Rhodopseudomonas palustris* (Egland *et al.*, 1997; Pelletier and Harwood, 2000) and its homologues from *Syntrophus aciditrophicus* and *Geobacter metallireducens* (Kung *et al.*, 2013; Kung *et al.*, 2014). However, further conversion of the products of Mcd or AliB (mesaconyl-C1-CoA or cyclohex-1-ene-1-carboxyl-CoA, respectively) by a hydratase does not produce a tertiary hydroxyl group as we proposed for the hydratase acting on 2-(2-carboxycyclohexylidene)acetyl-CoA (Figure 34).

Recently, a subfamily of $\alpha_2\beta_2$-heterotetrameric acyl-CoA dehydrogenases catalysing the dehydrogenation of steroid- or polycyclic-CoA substrates has been identified (Wipperman *et al.*, 2013). These enzymes have two active-sites and are encoded by two adjacent ACAD genes. One example is the acyl-CoA dehydrogenase ChsE1-ChsE2, formerly FadE28-FadE29, from the steroid metabolism of *Mycobacterium tuberculosis* (Thomas *et al.*, 2011; Thomas and Sampson, 2013). In similar enzymes like FadE34 from *M. tuberculosis* (Griffin *et al.*, 2011) or CasC from the cholate metabolism of *Rhodococcus jostii* RHA1 (Mohn *et al.*, 2012), the two different ACAD domains (α- and β-domain) are fused to one single gene product (Ruprecht *et al.*, 2015).

The *thn*-operon, which contains the genes involved in the downstream pathway of anaerobic naphthalene degradation (Table 7), encodes two ACAD-like enzymes, ThnO and ThnT. Regarding their amino acid sequence, ThnO and ThnT are affiliated with the common acyl-CoA dehydrogenases rather than with the heterotetrameric ACADs mentioned above (Figure 35).

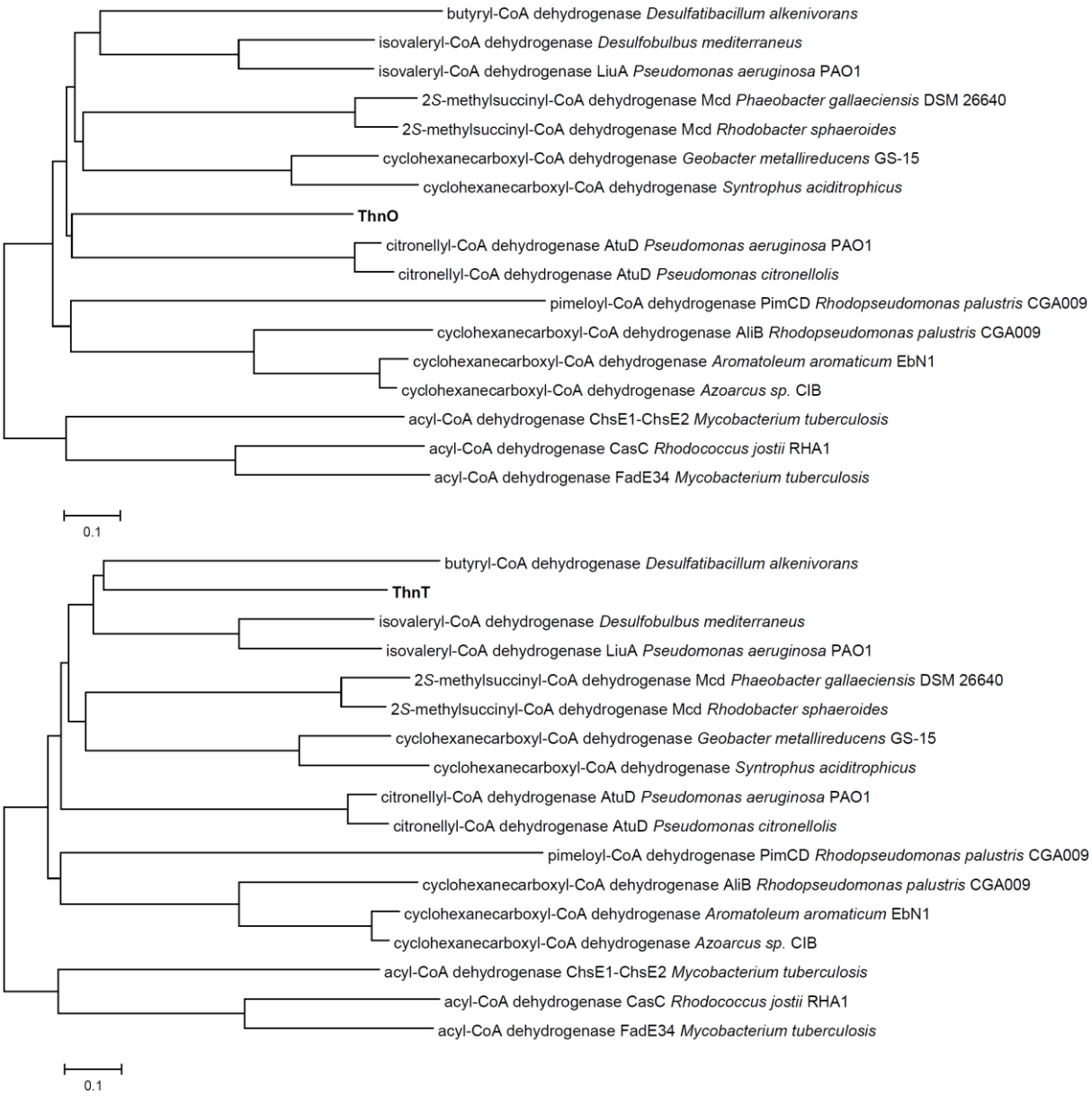


Figure 35 Phylogenetic tree (Neighbour-Joining) of known acyl-CoA dehydrogenases (ACADs) that act on branched substrates, aligned with the ACAD-like enzymes ThnO and ThnT encoded within the *thn*-operon. Alignment was performed with the MEGA6 software (Tamura *et al.*, 2013) using the MUSCLE algorithm (Edgar, 2004). Additional sequences of cyclohexanecarboxyl-CoA dehydrogenases from *Azoarcus evansii* sp. CIB (Martin-Moldes *et al.*, 2015) and *Aromatoleum aromaticum* EbN1 (Rabus *et al.*, 2005) that were not mentioned in the text were included for the alignment. Butyryl-CoA dehydrogenase from *Desulfatibacillum alkenivorans* AK-01 (Callaghan *et al.*, 2012) and the pimeloyl-CoA dehydrogenase PimCD of *Rhodopseudomonas palustris* (Harrison and Harwood, 2005) served as outgroup representing an ACAD-like enzyme with specificity for linear substrates and isovaleryl-CoA dehydrogenase from *Desulfobulbus mediterraneus* (Sass *et al.*, 2002) represented an enzyme from a closely related organism acting on the simplest branched substrate. The scale bar indicates 10% sequence divergence.

ThnO is related to the citronellyl-CoA dehydrogenases that convert branched substrates. In contrast, ThnT clusters with butyryl-CoA dehydrogenase, an enzyme specific for linear substrates. We therefore conclude that ThnO is most likely the acyl-CoA dehydrogenase from the *thn*-operon that reacts on *cis*-2-(2-carboxycyclohexyl)acetyl-CoA.

The subsequent water-addition on the ramified intermediate can for example be catalysed by a crotonase-like hydratase (Holden *et al.*, 2001) or by a MaoC-like *R*-specific hydratase (Engeland and Kindl, 1991; Fukui *et al.*, 1998). Two crotonase-like hydratases AtuE (3-isohexenylglutaconyl-CoA hydratase) and LiuC (3-methylglutaconyl-CoA hydratase) from *P. citronellolis* and *P. aeruginosa* PAO1 are proposed to be involved in the utilisation pathways of acyclic terpenes and leucine/isovalerate, respectively (Aguilar *et al.*, 2006; Foerster-Fromme *et al.*, 2006). The crotonase-like 3-methylglutaconyl-CoA hydratase has been studied in *Acinetobacter sp.* DSM 11652 (Mack *et al.*, 2006) and a homologue enzyme has been proposed to be involved in the anaerobic degradation pathway of 4-methylbenzoate in *Magnetospirillum sp.* pMbN1 (Lahme *et al.*, 2012). Recently, also the function of the 3-isohexenylglutaconyl-CoA hydratase AtuE from *P. aeruginosa* PAO1 has been proven (Poudel *et al.*, 2015).

Examples for MaoC-like hydratases reacting on branched substrates are the mesaconyl-CoA (2-methylfumaryl-CoA) hydratases Mch involved in the aforementioned ethylmalonyl-CoA pathway as well as in the autotrophic 3-hydroxypropionate CO_2 fixation cycle in *R. sphaeroides* and *Chloroflexus aurantiacus* (Alber *et al.*, 2006; Zarzycki *et al.*, 2008). The Mch reaction yields β-methylmalyl-CoA with a secondary hydroxyl group. In the 3-hydroxypropionate cycle of *C. aurantiacus*, an additional mesaconyl-C4-CoA (3-methylfumaryl-CoA) hydratase with only little relation to Mch was observed (Zarzycki *et al.*, 2009). The product of this enzyme, (*S*)-citramalyl-CoA, carries a tertiary hydroxyl group. A similar reaction is catalysed by the itaconyl-CoA hydratases Ich from *P. aeruginosa* PAO1 and *Yersinia pestis* KIM10 (Cooper and Kornberg, 1964; Sasikaran *et al.*, 2014) and from *Burkholderia xenovorans* LB400 (Kronen *et al.*, 2015). These are actually bifunctional enzymes acting as itaconyl-CoA isomerase and subsequently as mesaconyl-C4-CoA hydratase. Thus, they also yield (*S*)-citramalyl-CoA. Interestingly, the MaoC-like Ich enzymes from *P. aeruginosa* and *Y. pestis* are only distantly related although they catalyse the same reaction (Sasikaran *et al.*, 2014).

Also in cholesterol metabolism of *M. tuberculosis*, a MaoC-like hydratase is involved, namely the $\alpha_2\beta_2$-heterotetrameric *R*-specific enoyl-CoA hydratase ChsH1-ChsH2 (Thomas *et al.*, 2011; Yang *et al.*, 2014). Like the aforementioned subfamily of ACADs involved in steroid metabolism, this hydratase comprises of a unique heterotetrameric structure optimised for reactions on bulky steroid compounds.

Six potential hydratases are encoded within the *thn*-operon. A phylogenetic comparison with known hydratases acting on branched intermediates revealed that all crotonase-like enzymes (ThnA, ThnL, ThnM and ThnU) are closer related to ordinary crotonases than to 3-methyl- or 3-isohexenylglutaconyl-CoA hydratases and are thus rather not acting on branched substrates (Figure 36).

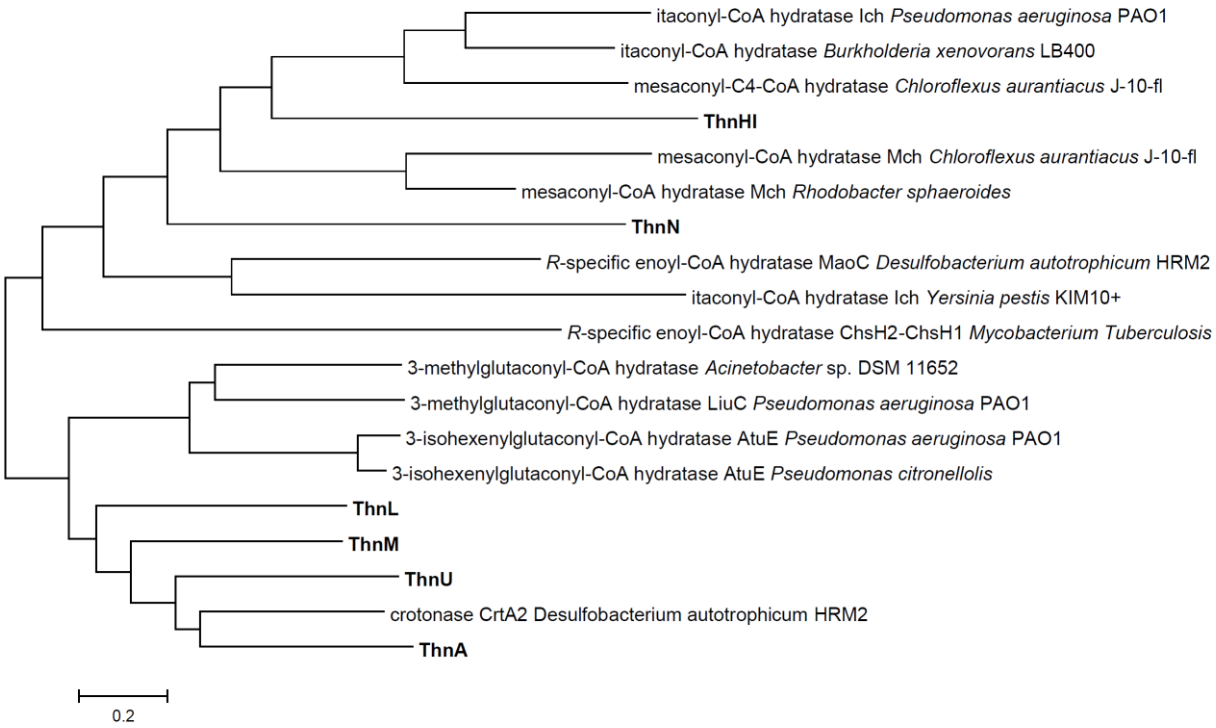


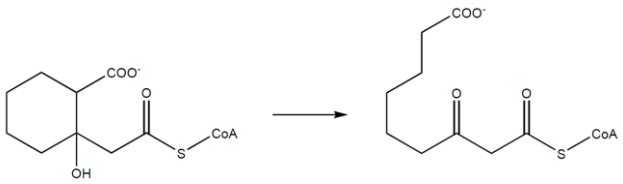
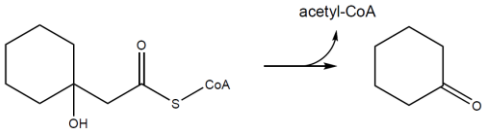
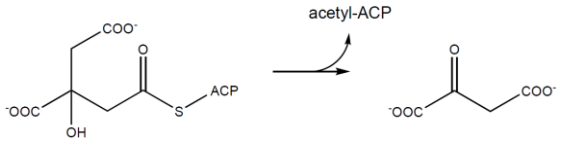
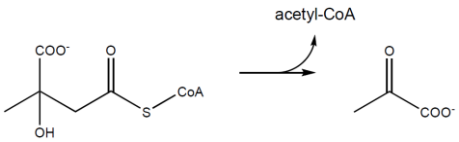
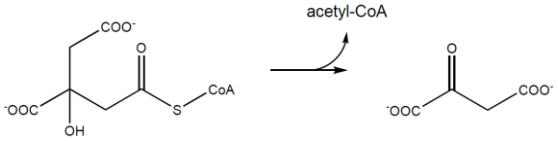
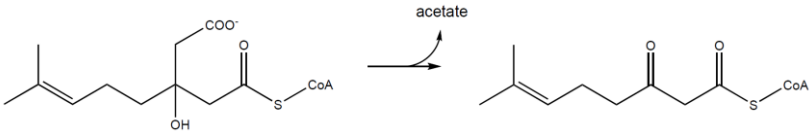
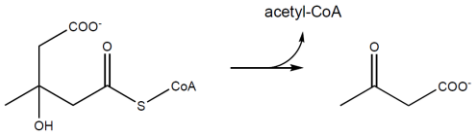
Figure 36 Phylogenetic tree (Neighbor-Joining) of known enoyl-CoA hydratases with specificity for branched substrates, aligned with proposed hydratases encoded within the *thn*-operon. Alignment was performed with the MEGA6 software (Tamura *et al.*, 2013) using the MUSCLE algorithm (Edgar, 2004). The 3-hydroxybutyryl-CoA dehydratase CrtA (Crotonase) and the *R*-specific enoyl-CoA hydratase MaoC from *Desulfobacterium autotrophicum* HRM2 (Strittmatter *et al.*, 2009) served as outgroup representing hydratases from a closely related organism with specificity for linear substrates. Scale bar represents 20% sequence divergence.

ThnN, which belongs to the structure-based superfamily of metallo-dependant hydratases (Holm and Sander, 1997), and the proposed MaoC-like hydratase ThnHI show no significant relation to hydratases with specificity for ramified substrates. However, enzymes within the MaoC-like superfamily may be rather distantly related but still catalyse the same reaction (Sasikaran *et al.*, 2014). Therefore, the function of these enzymes can only be reliably determined by testing them for conversion of the proposed substrate. ThnHI showed no activity towards ramified substrate analogues in previous studies (3.04.03), so ThnN, which could not yet be obtained as functional enzyme when heterologously produced in *E. coli* (3.04.02), remains as best enzyme candidate for the hydratisation of 2-(2-carboxycyclohexylidene)acetyl-CoA.

5.05.02. Cleavage of the cyclohexane ring

The observed conversion product of *cis*-2-(2-carboxycyclohexyl)acetyl-CoA, 2-(1-hydroxy-2-carboxycyclohexyl)acetyl-CoA, carries a tertiary hydroxyl group which implies a subsequent lyase reaction opening one of the three C-C bonds at the respective carbon-atom. A very similar reaction is catalysed by a 2-(1-hydroxycyclohexyl)acetyl-CoA:acetyl-CoA lyase in *Arthrobacter sp.* CA1 (Ougham and Trudgill, 1982), which cleaves its substrate to cyclohexanone and acetyl-CoA (Table 11).

Table 11 Reaction schemes of lyases acting on substrates with a tertiary hydroxyl group

Enzyme	Reaction scheme	Reference
ThnQS (proposed)		This study
unknown		Ougham & Trudgill, 1982
CitE		Bott & Dimroth, 1994
R- or *S*-specific Ccl		Friedmann *et al.*, 2007; Zarzycki *et al.*, 2009
citryl-CoA lyase		Aoshima *et al.*, 2004
AtuA (proposed)		Forster-Fromme & Jendrossek, 2006
LiuE		Chavez-Aviles *et al.*, 2009

Our newly identified metabolite of the anaerobic naphthalene degradation pathway differs from 1-hydroxycyclo-hexylacetyl-CoA only by an additional 2-carboxy group.

A prototype of lyases acting on substrates with a tertiary hydroxyl group is the citrate lyase complex which cleaves citrate to oxaloacetate and acetate (Dagley and Dawes, 1955; Bowen and Raman, 1960). The actual lyase enzyme in this complex is the β-subunit CitE releasing oxaloacetate from acyl-carrier-protein bound citrate (citryl-ACP, see Table 11) as studied in *Klebsiella pneumoniae* (Dimroth and Eggerer, 1975; Bott and Dimroth, 1994). A CitE-like lyase is also involved in the 3-hydroxypropionate cycle of *C. aurantiacus*, namely the trifunctional (*S*)-malyl-CoA/β-methylmalyl-CoA/(*S*)-citramalyl-CoA lyase Ccl (Herter *et al.*, 2002; Zarzycki *et al.*, 2009). This enzyme produces pyruvate and acetyl-CoA from (*S*)-citramalyl-CoA (Table 11). Further (*S*)-citramalyl-CoA lyases are present in the gene cluster for itaconate degradation in *P. aeruginosa* and *B. xenovorans* (Stover *et al.*, 2000; Chain *et al.*, 2006; Kronen *et al.*, 2015).

For the equivalent reaction on (*R*)-citramalyl-CoA, *C. aurantiacus* employs a completely different *R*-citramalyl-CoA lyase (Friedmann *et al.*, 2007). This enzyme is not related to CitE but belongs to the family of (*S*)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase like enzymes (Bachhawat *et al.*, 1955). Additionally, a citryl-CoA lyase different from CitE is known from *Hydrogenobacter thermophilus* TK-6 (Aoshima *et al.*, 2004). This lyase is similar to the citrate (*Si*)-synthase from the tricarboxylic acid cycle (Lenz *et al.*, 1971) and does not, unlike CitE, produce an enzyme-bound acetyl-residue but releases acetyl-CoA and oxaloacetate (Table 11).

Another lyase acting on a substrate similar to 2-(1-hydroxy-2-carboxycyclohexyl)acetyl-CoA, the newly identified intermediate of anaerobic naphthalene degradation, is the postulated (*S*)-3-hydroxy-3-isohexenylglutaryl-CoA:acetate-lyase (HIHG-CoA lyase) from the citronellol-degrading *P citronellolis* (Seubert and Fass, 1964). In contrast to all other lyases mentioned above, this lyase-reaction cleaves the C-C bond to acetate (Table 11). The enzyme associated with this reaction has not yet been identified, but there are clear indications that it is catalysed by AtuA which is encoded within the gene cluster for acyclic terpene utilisation (Foerster-Fromme *et al.*, 2006; Foerster-Fromme and Jendrossek, 2010). However, it was also speculated that the observed HIHG-CoA lyase activity might be a side-reaction of LiuE from the leucine/isovalerate utilisation pathway (Chavez-Aviles *et al.*, 2009; Chavez-Aviles *et al.*, 2010). The substrate of LiuE, 3-hydroxy-3-methylglutaryl-CoA, is quite similar to that of HIHG-CoA lyase, only carrying a methyl-

instead of an isohexenyl residue. In contrast to HIHG-CoA lyase, LiuE is an acetyl-CoA-lyase and therefore releases acetyl-CoA and not free acetate (Table 11). An HMG-CoA lyase reaction has also been proposed to occur during the anaerobic degradation of 4-methylbenzoate (Lahme *et al.*, 2012).

Two proteins encoded by genes of the *thn*-operon, ThnQ and ThnS, share significant sequence similarities with the HIHG-CoA lyases. On closer inspection, they seem to represent subunits of a heteromeric protein ThnQS, as the two adjacent genes *thnQ* and *thnS* both align with the *atuA*-gene (Figure 37).

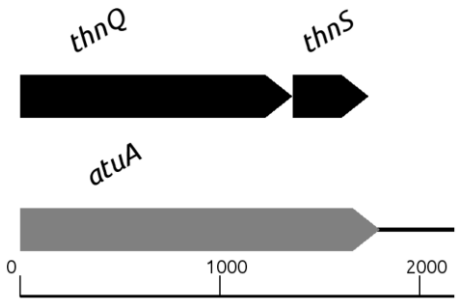


Figure 37 Alignment of the *thnQ*- and *thnS*-genes from *Deltaproteobacterium* N47 and the *atuA*-gene from *Pseudomonas aeruginosa PAO1*. Picture was created via the Genome2D tool (Baerends *et al.*, 2004). Scale bar indicates length in bp.

ThnQS shares 32% sequence identities and 49% positive substitutions with the proposed HIHG-CoA lyases AtuA from *P. aeruginosa* PAO1. Phylogenetically, ThnQS and the two AtuA homologues build their own branch and are not affiliated with CitE-like enzymes or with the HMG-CoA lyase and similar enzymes (Figure 38).

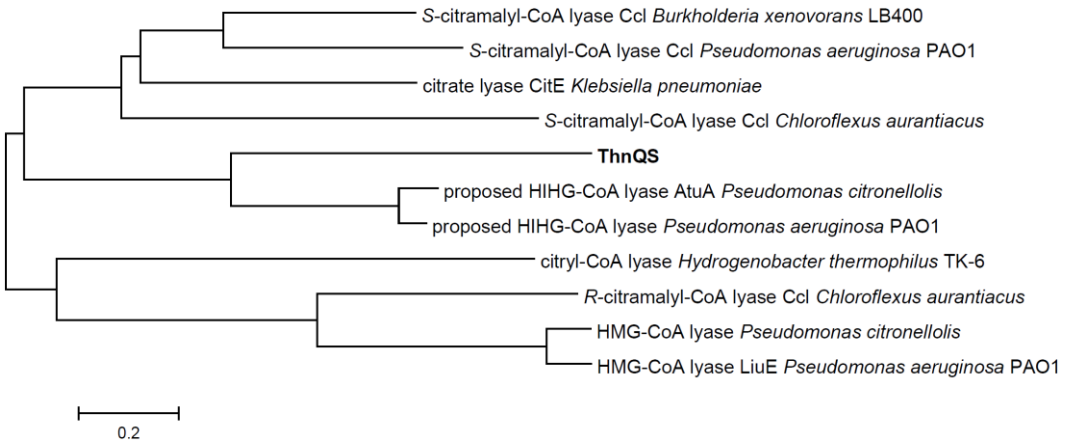


Figure 38 Phylogenetic tree (Neighbor-Joining) of known lyase enzymes acting on tertiary hydroxyl groups, aligned with ThnQS from *Deltaproteobacterium* N47. Alignment was performed with the MEGA6 software (Tamura *et al.*, 2013) using the MUSCLE algorithm (Edgar, 2004). Scale bar represents 20% sequence divergence.

Owing to its similarity to AtuA, the only acetate-lyase among the above-quoted enzymes, ThnQS is expected to perform a similar reaction. From a chemical point of view, the release of acetate is less likely than releasing an acetyl-CoA, since the latter can better stabilise a negative charge occurring during the cleavage process. Acetate-lyases might overcome this constraint by intramolecular CoA-transferase activity. The lyase-reaction would initially release an acetyl-CoA, but the CoA-thioester would be transferred to another carboxyl group of the substrate so that finally acetate is released. A similar transfer occurs during the reaction cycle of the citrate lyases, which is a two-step sequence of consecutive acyl-transfer and cleavage reactions (Buckel *et al.*, 1971; Buckel *et al.*, 1973). After the cleavage of citryl-ACP to oxaloaxetate and acetyl-ACP (Table 11), the enzyme complex binds a new molecule of citrate to ACP and releases acetate in exchange for it.

In the anaerobic naphthalene degradation pathway, an acetate-lyase might either act on the observed intermediate 2-(1-hydroxy-2-carboxycyclohexyl)acetyl-CoA or on 2-hydroxy-2-(carboxymethyl)cyclohexane-1-carboxyl-CoA. The latter could be formed from the observed intermediate by an intramolecular CoA-transfer from the acetyl- to the carboxyl group (Figure 39).

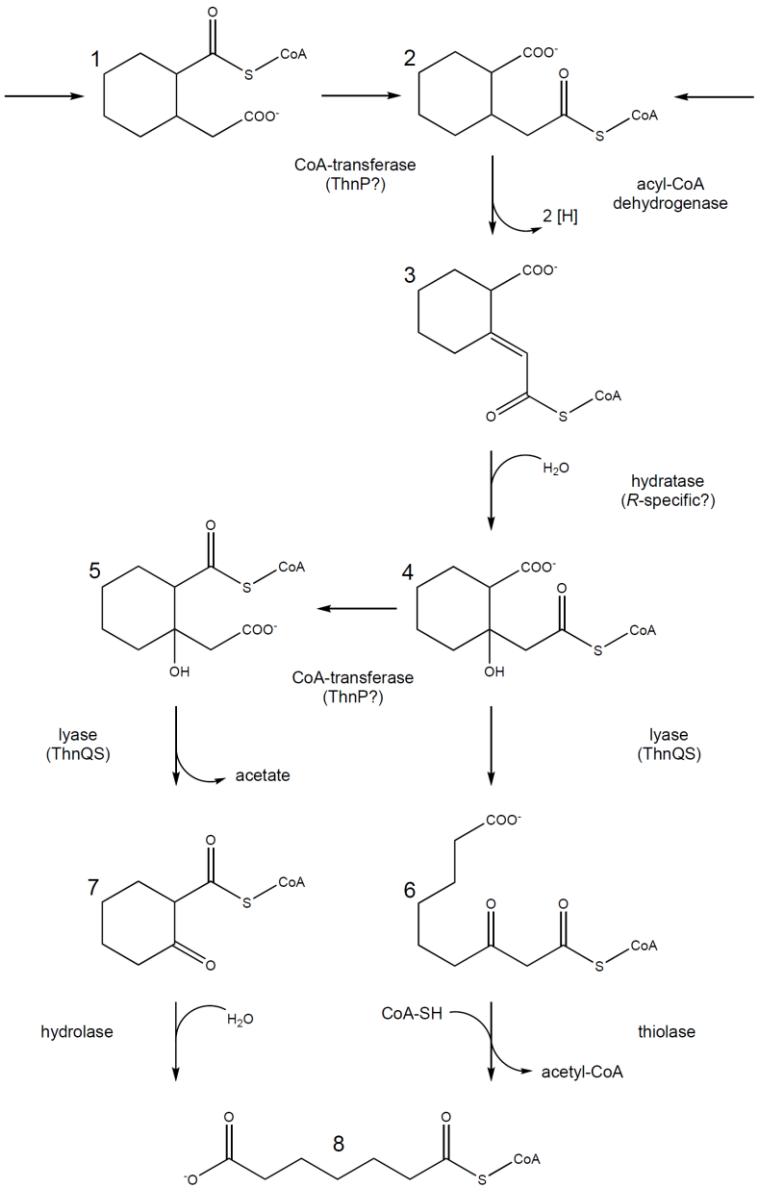


Figure 39 Proposed second ring cleavage pathway from 2-(carboxymethyl)cyclohexane-1-carboxyl-CoA or 2-(2-carboxycyclohexyl)acetyl-CoA to pimeloyl-CoA with hypothetical function of enzymes encoded by the *thn*-operon. (1): 2-(carboxymethyl)cyclohexane-1-carboxyl-CoA. (2): 2-(2-carboxycyclohexyl)acetyl-CoA. (3) 2-(2-carboxycyclohexylidene)acetyl-CoA, *E*-conformation. (4): 2-(1-hydroxy-2-carboxycyclohexyl)acetyl-CoA. (5): 2-hydroxy-2-(carboxymethyl)cyclohexane-1-carboxyl-CoA. (6): 3-oxoazeloyl-CoA. (7): 2-oxocyclohexane-1-carboxyl-CoA. (8): pimeloyl-CoA.

The corresponding CoA-transferase encoded by the *thn*-operon might be ThnP, which shares sequence homologies with enzymes of the CoA-transferase family (Scherf and Buckel, 1991; Jacob *et al.*, 1997). Albeit, ThnP might as well fulfil a role up-stream in this pathway and catalyse the CoA-transfer between the acyl-chains of *cis*-2-(carboxymethyl)cyclohexane-1-carboxylate, thereby channelling *cis*-2-(carboxymethyl)cyclohexane-1-carboxyl-CoA to *cis*-2-(2-carboxycyclohexyl)acetyl-CoA for further conversion (Figure 39). Indeed the analysis of metabolites downstream of the 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase product indicated that a β-oxidation-like sequence starting from 4,4a,5,6,7,8-hexahydro-2-naphthoyl-CoA leads to 2-(carboxymethyl)cyclohexane-1-carboxyl-CoA (Figure 26), which would make a CoA-transferase reaction necessary.

A ThnQS-catalysed lyase reaction on *cis*-2-hydroxy-2-(2-carboxycyclohexyl)acetyl-CoA would lead to a ring-opening, since in this compound an acetyl-like structure is integrated into the cyclohexane-ring (Figure 39). This reaction would yield 3-oxoazeloyl-CoA (a CoA-thioester of 3-oxoazelaic acid, i.e. 3-oxononanedioic acid), which could be thiolytically cleaved to pimeloyl-CoA and acetyl-CoA. Alternatively, a lyase-reaction on *cis*-2-hydroxy-2-(carboxymethyl)cyclohexane-1-carboxyl-CoA would resemble a real acetate-lyase resulting in 2-oxocyclohexane-1-carboxyl-CoA and acetate. 2-oxocyclohexane-1-carboxyl-CoA is known from the benzoyl-CoA pathway of *R. palustris* and can be converted to pimeloyl-CoA by a ring-cleaving hydrolase (Pelletier and Harwood, 1998). The first scenario is regarded as more likely to occur in anaerobic naphthalene degraders, as it enables the advantageous conservation of an energy rich bond by thiolytic cleavage of acetyl-CoA in a subsequent step. In contrast, the latter scenario would result in the release of non-activated acetate and therefore cause a net-loss of one acetyl-CoA (Figure 39).

5.05.03. From pimeloyl-CoA to central metabolism

All possible downstream pathways depicted above converge at the intermediate pimeloyl-CoA. In accordance with that, we could demonstrate the conversion of pimeloyl-CoA and the potential downstream metabolite glutaryl-CoA by cell free extracts of both anaerobic naphthalene degraders N47 and NaphS2. A catabolic pathway

for pimelate or pimeloyl-CoA, respectively, was originally proposed for *Pseudomonas* strain LP-1 (Gallus and Schink, 1994). The enzymes catalysing the conversion of pimeloyl-CoA to glutaryl-CoA are encoded by the *pim*-operon as previously described for *R. palustris* (Harrison and Harwood, 2005). The conversion proceeds in a β-oxidation-like manner (Figure 40).

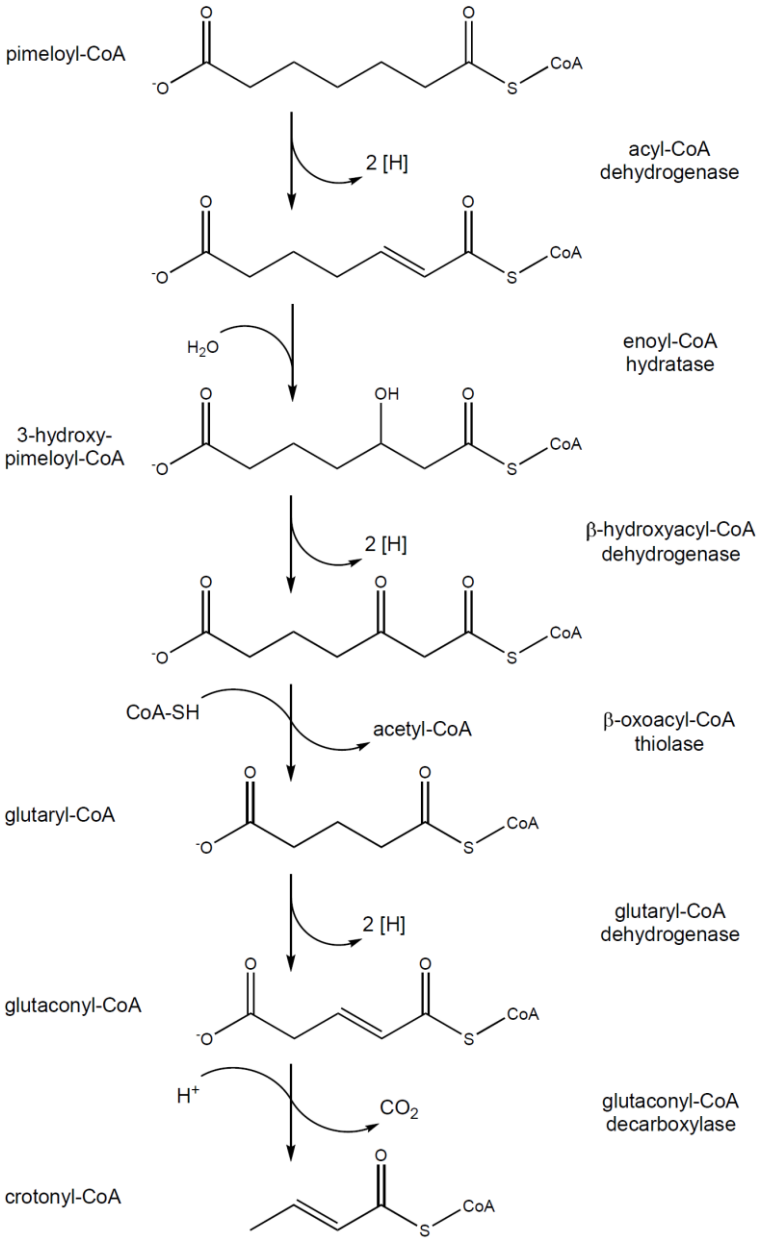


Figure 40 Proposed catabolic pathway from pimeloyl-CoA to crotonyl-CoA (Gallus and Schink, 1994).

The key-reaction that links this pathway to the central metabolism is the transformation of the dicarboxylic intermediate glutaryl-CoA to crotonyl-CoA. Facultative anaerobic bacteria perform this conversion by a decarboxylating glutaryl-CoA dehydrogenase that directly produces crotonyl-CoA from glutaryl-CoA (Haertel *et al.*, 1993; Gallus and Schink, 1994; Harrison and Harwood, 2005). In contrast, obligate anaerobes normally use a combination of a non-decarboxylating glutaryl-CoA dehydrogenase and a subsequent glutaconyl-CoA decarboxylase (Schoecke and Schink, 1999; Mueller and Schink, 2000; Elshahed *et al.*, 2001b; Wischgoll *et al.*, 2009) as depicted in Figure 40. The only known exception is *G. metallireducens* which is a strict anaerobe employing a decarboxylating glutaryl-CoA dehydrogenase (Wischgoll *et al.*, 2009). Non-decarboxylating glutaryl-CoA dehydrogenases enable an additional conservation of energy by coupling the subsequent decarboxylation of their product glutaconyl-CoA with a translocation of sodium ions across the cytoplasma membrane (Buckel and Semmler, 1982, 1983; Bendrat and Buckel, 1993; Braune *et al.*, 1999). Hence, the latter strategy is regarded as advantageous for energy-limited strictly anaerobic bacteria. The genome of *Desulfobacterium* N47 contains two genes coding for putative glutaconyl-CoA decarboxylases (*N47_I06890* and *N47_G40520*), whereas the genome of strain NaphS2 contains only one copy (*NPH_5426*). Adjacent to all of these genes, genes coding for acyl-CoA dehydrogenases are located (*N47_I06870* and *N47_G40550* in N47, *NPH_5425* in NaphS2). We therefore propose that sulphate-reducing naphthalene degraders utilise the intermediate glutaryl-CoA through a combination of a non-decarboxylating dehydrogenase and an energy-conserving glutaconyl-CoA decarboxylase to yield the more general intermediate crotonyl-CoA. From the latter, two additional molecules of acetyl-CoA can be generated via central metabolism.

5.05.04. Conclusion

Based on conversion studies with cell free extracts of sulphate-reducing naphthalene degraders, we propose that the downstream pathway of anaerobic naphthalene degradation proceeds via α,β-unsaturation of *cis*-2-(2-carboxycyclohexyl)acetyl-CoA and subsequent introduction of a tertiary hydroxyl group yielding 2-(1-hydroxy-2-carboxycyclohexyl)acetyl-CoA. Regarding phylogenetic analyses of the enzymes involved in the

downstream pathway which are encoded by the previously identified *thn*-operon (3.05.01), this metabolite supposedly serves as substrate for a novel kind of ring-opening lyase, which catalyses the conversion to 3-oxoazeloyl-CoA. We could demonstrate that further degradation proceeds via pimeloyl-CoA and glutaryl-CoA. The proposed pathway involves β-oxidation and the combination of a non-decarboxylating glutaryl-CoA dehydrogenase and a glutaconyl-CoA decarboxylase producing crotonyl-CoA. Central metabolism can form two molecules of acetyl-CoA from crotonyl-CoA and final oxidation may occur via the citric acid cycle.

For the first time, our studies allow to sketch a downstream pathway including all steps necessary to channel previously reported intermediates to central metabolism.

6. Summary

6.01. Initial activating and dearomatising reactions in the anaerobic degradation pathway of naphthalene

In the anaerobic degradation pathway of naphthalene, initial activation of this unsubstituted bicyclic aromatic hydrocarbon is achieved via a naphthalene carboxylase (Mouttaki *et al.*, 2012). Dearomatisation is then realised through aryl-CoA reductases (Eberlein *et al.*, 2013a) which require a prior activation of the substrate by the formation of a CoA-thioester. The 2-naphthoate:CoA ligase identified in this work (chapter 2) therefore serves as a link for the previously described metabolic steps. The substrate specificity of this enzyme was characterised in cell free extracts of the sulphate-reducing naphthalene degraders N47 and NaphS2. In addition, the enzyme from NaphS2 could also be heterologously produced in *E. coli*. Further studies will focus on the characterisation of the pure ligase enzymes.

6.02. The downstream pathway

An antecedent study showed that the intermediate 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) is further reduced to a hexahydro-2-naphthoyl-CoA by an ATP-dependent and oxygen-sensitive reductase with NADH as electron donor (Eberlein *et al.*, 2013a). However, only a partial conversion of the substrate could be achieved in the referred study and the conformation of the product remained unclear. This work provides evidence that 2-oxoglutarate is the natural electron donor of the THNCoA reducing system and that most likely 4,4a,5,6,7,8-hexahydro-2-naphthoyl-CoA is the product of this reduction (chapter 4). This implies a downstream pathway analogous to the benzoyl-CoA pathway described for *T aromatica* (Harwood *et al.*, 1999) with cleavage of the first ring occurring between C2 and C3. Through β-oxidation-like reactions, one side-chain of the cleavage-product is then shortened by one acetyl-CoA unit resulting in *cis*-2-(carboxymethy)cyclohexane-1-carboxyl-CoA. The free acid of this compound was also identified as a metabolite of the naphthalene-degrading enrichment culture N47 in prior metabolomic analyses (Annweiler *et al.*, 2002).

The enzymes responsible for these metabolic steps are encoded by the *thn*-operon which was identified in this work (chapter 3). This operon contains genes coding for the four subunits of the THNCoA reductase as well as for putative hydratases, hydrolases, dehydrogenases and thiolases. Most of these enzymes could be heterologously produced in *E. coli* and in some cases the postulated enzyme function could also be verified via enzyme tests with general substrate analogues.

As elucidated in chapter 5, the further downstream pathway proceeds through *cis*-2-(2-carboxycyclohexyl)acetyl-CoA. Like the aforementioned *cis*-2-(carboxymethy)-cyclohexane-1-carboxyl-CoA, this compound is also a CoA-ester derivative of *cis*-2-(carboxymethy)cyclohexane-1-carboxylic acid but with the CoA-thioester attached at the other carboxyl group. The putative CoA-transferase ThnP encoded by the *thn*-operon might catalyse the required intramolecular transfer of the CoA-thioester to form *cis*-2-(2-carboxycyclohexyl)acetyl-CoA. Subsequently, the latter is converted by an acyl-CoA dehydrogenase and an enoyl-CoA hydratase yielding *cis*-2-(1-hydroxy-2-carboxy-cyclohexyl)acetyl-CoA, which might be the substrate for a novel ring-opening lyase. This lyase reaction was deduced from sequence homologies of two enzymes encoded by the *thn*-operon (ThnQ and ThnS, presumably forming an enzyme complex ThnQS) to another proposed lyase. The lyase would yield 3-oxoazeloyl-CoA which implies a link to central metabolism via pimeloyl-CoA and glutaryl-CoA. The conversion of the latter two metabolites in cell free extracts of N47 and NaphS2 was demonstrated in this work. This is the first time that a complete metabolic route towards central metabolism was delineated for the anaerobic degradation of naphthalene.

6.03. Key enzymes of the pathway

Regarding the involved enzymes, some discrepancies between the benzoyl-CoA and the 2-naphthoyl-CoA degradation pathway were observed: Although it was postulated that strict anaerobic benzoate degraders generally employ an ATP-independent benzoyl-CoA reductase (Loeffler *et al.*, 2011), it could be shown that the reduction of the benzoyl-CoA analogue THNCoA in sulphate-reducing naphthalene degraders is catalysed by an ATP-dependant enzyme. Furthermore, no enzyme similar to the crotonase-like 6-oxo-

cyclohex-1-ene-1-carboxyl-CoA hydratase/hydrolase that usually occurs in the benzoyl-CoA pathway proceeding via the cyclic diene intermediate cyclohexadienecarboxyl-CoA (Kuntze *et al.*, 2008) is present in N47 or NaphS2. The cyclic diene metabolite β'-hydroxy-β-oxodecahydro-2-naphthoyl-CoA identified in the downstream pathway of anaerobic naphthalene degradation (see chapter 4) might be converted by the concerted action of two separate enzymes, a hydratase and hydrolase, leading to the cleavage of the first ring. In general, the phylogeny of the crotonase-like enzymes encoded by the *thn*-operon indicates reactions on intermediates derived from the cyclic monoene octahydro-2-naphthoyl-CoA, but the metabolites detected downstream of the THNCoA reductase product clearly prove that the β-oxidation-like sequence starts from hexahydro-2-naphthoyl-CoA. Therefore, this sequence seems to be catalysed by unusual enzymes that might be specific for anaerobic of PAH degraders.

Furthermore, the putative ring-opening lyase ThnQS, which is assumed to mediate cleavage of the second ring, would represent a completely novel enzyme once its function is proven. The respective genes (*thnQ* and *thnS*) as well as the ones coding for the aforementioned unusual crotonase-like enzymes might therefore be suitable as gene markers for the detection of anaerobic bacteria capable of degrading naphthalene or other PAHs. Comparable lyase reactions might be a common principle of degradation pathways of polycyclic compounds since they offer a convenient solution for cleaving ring systems with more than one side-chain or for the linearisation of branched intermediates. Either of these types of metabolites inevitably occurs during the degradation of polycyclic compounds. Thus it will be interesting to see if enzymes similar to ThnQS can also be identified in other PAH-degrading cultures in the future.

6.04. Outlook

In this work, most of the metabolic steps in the downstream pathway of anaerobic naphthalene degradation starting from THNCoA were measured in cell free extracts of sulphate-reducing naphthalene degraders and furthermore the *thn*-operon coding for the enzymes involved in the downstream pathway was identified. The objective of

further studies should be the linkage of the observed reactions in cell free extracts to the catalytic function of specific proteins.

Up to now, this attempt was hampered by the fact that the conformation of the hexahydro-2-naphthoyl-CoA isomer produced by the THNCoA reductase could not be identified and the conformations of downstream metabolites were likewise unknown. Therefore, the heterologously produced enzymes could not be assayed towards their native substrates yet. The results obtained in this work revealed that the reductase product is most likely 4,4a,5,6,7,8-hexahydro-2-naphthoyl-CoA, albeit this needs to be verified by NMR-analysis. This identification enables a targeted chemical synthesis of the naturally occurring isomer. However, the appropriate activity of the 2-naphthoate:CoA ligase from N47 towards its substrate analogue 5,6,7,8-tetrahydro-2-naphthoate (see chapter 2) might enable a more convenient enzymatic synthesis via a coupled system consisting of a CoA-ligase reaction on 5,6,7,8-tetrahydro-2-naphthoate and a subsequent THNCoA reductase reaction in cell free extracts. When running the reductase assay with 2-oxoglutarate as electron donor, a complete conversion of THNCoA can be achieved and downstream processes can by inhibited by amending the assay mixture with an excess of NADH (see chapter 4). This strategy has the advantage that it should yield only the naturally occurring isomer of hexahydro-2-naphthoyl-CoA.

Starting from this hexahydro-2-naphthoyl-CoA isomer, a stepwise conversion through candidate enzymes encoded by the *thn*-operon should be possible, whereas the product of one conversion would serve as substrate for the subsequent enzyme. Finally, these coupled enzymatic conversions should result in a reproduction of the metabolic steps observed in assays with cell free extracts and to an assignment of a metabolic function to all enzymes encoded by the *thn*-operon.

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8. Anhang

Abkürzungsverzeichnis

°C	degree Celsius
[H]	reducing equivalent
A	Ampere (unit)
AU	absorbance unit
$A_{x\ nm}$	absorbance at x nm
ACAD	acyl-CoA dehydrogenase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair
BSTFA	bis(trimethylsilyl)-trifluoroacetamide
cDNA	complementary DNA
c	concentration
cis-	(1*R*,2*R*)- or (1*S*,2*S*) configuration at a ring
CoA	coenzyme A bound as thioester
CoA-SH	free coenzyme A
Da	Dalton (unit)
DNA	deoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxynucleoside triphosphate
DTE	dithioerythritol
DTT	dithiothreitol
E	electric potential
E-	conformation with high-priority substituents at opposite sides of a double-bond (*entgegen*)
F	Faraday constant, 96485.3329 s·A/mol
FAD	flavin adenine dinucleotide, oxidised
$FADH_2$	flavin adenine dinucleotide, reduced
g	gram (unit)

g	earth's gravitational acceleration, 9.80665 m/s^2
GC	gas-chromatography
gDNA	genomic DNA
h	hours (unit)
HHNCoA	hexahydro-2-naphthoyl-CoA
HIHG-CoA	(*S*)-3-hydroxy-3-iso-hexenylglutaryl-CoA
HMG-CoA	(*S*)-3-hydroxy-3-methyl-glutaryl-CoA
HMN	2,2,4,4,6,8,8-hepta-methylnonane
HPLC	High-performance liquid chromatography
HR-MS	high-resolution mass-spectrometry
i.e.	that means (*id est*)
J	Joule (unit)
K	Kelvin (unit)
k (prefix)	kilo (10^3)
LC	liquid chromatography
M	molar, mol/L
M (prefix)	mega, 10^6
m (prefix)	milli, 10^{-3}
µ (prefix)	micro, 10^{-6}
m/z	mass to charge ratio
MCS	multiple cloning site
min	minute (unit)
mol	mole (unit)
MS	mass-spectrometry
n (prefix)	nano, 10^{-9}
n.d.	not detected
n.p.	not present
NAD^+	nicotinamide adenine dinucleotide, oxidised
NADH	nicotinamide adenine dinucleotide, reduced
$NADP^+$	nicotinamide adenine dinucleotide phosphate, oxidised
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
$OD_{x\ nm}$	optical density at x nm
OHNCoA	octahydro-2-naphthoyl-CoA
ox	oxidised

OYE	old yellow enzyme
Pa	Pascal (unit)
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon
PCR	polymerase chain reaction
P_i	orthophosphate, PO_4^{3-}
PP_i	pyrophosphate, $P_2O_7^{4-}$
psi	pound-force per square inch, 6.9 kPa
Q	quinone, oxidised
QH_2	quinone, reduced
R	gas constant, 8.314 J/(mol·K)
R-	configuration with a right-handed (*rectus*) twist at a chiral atom
red	reduced
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RT-PCR	reverse transcription PCR
S-	configuration with a right-handed (*sinister*) twist at a chiral atom
s	second (unit)
SDS	sodium dodecylsulphate
sp.	species
T	temperature
TEMED	*N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine
THNCoA	5,6,7,8-tetrahydro-2-naphthoyl-CoA
trans-	(1*R*,2*S*)- or (1*S*,2*R*) configuration at a ring
U	unit of enzyme activity – conversion of 1 µmol substrate per min
V	Volt (unit)
v/v	volume per volume
w/v	weight per volume
z	number of electrons transferred
Z-	configuration with high-priority substituents at the same side of a double-bond (*zusammen*)

Publikationsliste

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Anstehende Publikationen

Weyrauch, P., Henning, L.A., Koelschbach, J.S. and Meckenstock, R.U. (2016) Identification of the *thn*-operon involved in the downstream pathway of anaerobic naphthalene degradation. *In preparation*, Environmental Microbiology.

Weyrauch, P., Zaytsev, A.V., Stephan, S., Schmitz, O.J., Golding, B.T. and Meckenstock, R.U. (2016) Conversion of *cis*-2-(carboxymethyl)cyclohexyl-1-carboxylic acid CoA-ester in the downstream pathway of anaerobic naphthalene degradation. *In preparation*, Environmental Microbiology.

Poster und Vorträge

Weyrauch, P. and Meckenstock, R.U. (2016) Conversion of *cis*-2-carboxycyclohexyl-acetic acid in the down-stream pathway of anaerobic naphthalene degradation. *Oral presentation at the Annual Conference 2016 of the Association for General and Applied Microbiology (VAAM), Jena, Germany*.

Weyrauch, P. and Meckenstock, R.U. (2015) Conversion of *cis*-2-carboxycyclohexyl-acetic acid in the down-stream pathway of anaerobic naphthalene degradation. *Poster presentation at the Gordon Research Conference Applied & Environmental Microbiology 2015, South Hadley, USA*.

Weyrauch, P. and Meckenstock, R.U. (2015) Conversion of *cis*-2-carboxycyclohexyl-acetic acid in the down-stream pathway of anaerobic naphthalene degradation. *Poster presentation at the 6th Congress of European Microbiologists (FEMS 2015), Maastricht, the Netherlands*.

Weyrauch, P. and Meckenstock, R.U. (2015) Proposal for the down-stream pathway of anaerobic naphthalene degradation. *Poster presentation at the Annual Conference 2015 of the Association for General and Applied Microbiology (VAAM), Marburg, Germany*.

Weyrauch, P., Henning, L.A. and Meckenstock, R.U. (2014) Identification of a gene cluster involved in the down-stream pathway of anaerobic naphthalene degradation. *Poster presentation at Microbiology and Infection 2014 – 4th Joint Conference of the German Society for Hygiene and Microbiology (DGHM) and the Association for General and Applied Microbiology (VAAM), Dresden, Germany*.

Lebenslauf

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Mikrobieller Abbau von Naphthalin unter anaeroben Bedingungen"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im Monat September 2016

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