Metabolic reconstruction of the genome of candidate *Desulfatiglans TRIP_1* and identification of key candidate enzymes for anaerobic phenanthrene degradation

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Summary
Polycyclic aromatic hydrocarbons (PAHs) are widely distributed pollutants. As oxygen is rapidly depleted in water-saturated PAH-contaminated sites, anaerobic microorganisms are crucial for their consumption. Here, we report the metabolic pathway for anaerobic degradation of phenanthrene by a sulfate-reducing enrichment culture (TRIP) obtained from a natural asphalt lake. The dominant organism of this culture belongs to the *Desulfobacteraceae* family of Deltaproteobacteria and genome-resolved metagenomics led to the reconstruction of its genome along with a handful of genomes from lower abundance bacteria. Proteogenomic analyses confirmed metabolic capabilities for dissimilatory sulfate reduction and indicated the presence of the Embden-Meyerhof-Parnas pathway, a complete tricarboxylic acid cycle as well as a complete Wood-Ljungdahl pathway. Genes encoding enzymes putatively involved in the degradation of phenanthrene were identified. This includes two gene clusters encoding a multisubunit carboxylase complex likely involved in the activation of phenanthrene, as well as genes encoding reductases potentially involved in subsequent ring dearomatization and reduction steps. The predicted metabolic pathways were corroborated by transcriptome and proteome analyses, and provide the first insights into the metabolic pathway responsible for the anaerobic degradation of three-ringed PAHs.

Introduction
Polycyclic aromatic hydrocarbons (PAHs) are widely distributed contaminants with hazardous effects on human health. PAHs are composed of two or more fused aromatic rings and mainly derive from natural gas and petroleum. They are also produced during coal processing and are present in exhaust emissions from vehicles (Cai et al., 2007). Due to their high hydrophobicity and low bioavailability, PAHs are persistent in the environment and recalcitrant to degradation.

Environmental remediation of PAHs is mainly achieved via microbial degradation. Many aerobic bacteria capable of using PAHs as carbon and energy source have been isolated and the corresponding degradation pathways have been widely studied (Peng et al., 2008). However, PAH-contaminated aquifers or sediments rapidly become anoxic due to the consumption of oxygen at high carbon loads. Under such conditions, molecular oxygen can be replaced by other electron acceptors for anaerobic respiration, but is critically lacking as a reactive co-substrate for the biochemical activation of hydrocarbons. Anaerobic PAH degradation under iron-reducing, sulfate-reducing and methanogenic conditions has been documented (Meckenstock et al., 2016). Nevertheless, very little is known about the responsible organisms or the biochemical mechanisms for PAHs degradation in the absence of molecular oxygen.

Naphthalene (C10H8) is the simplest PAH and has been used as a model compound for studying PAH-degradation. Anaerobic naphthalene degradation proceeds via carboxylation to 2-naphthoate (Mouttaki et al., 2012) followed by formation of the corresponding CoA ester. The further transformation of 2-naphthoyl-CoA occurs via reductive dearomatization yielding 5,6,7,8-tetrahydro-2-naphthoyl-CoA, which is then reduced in a stepwise
manner to a hexahydro-2-naphthoyl-CoA (Eberlein et al., 2013a,b; Estelmann et al., 2015).

Knowledge about the anaerobic metabolism of larger PAHs such as the three-ring PAH phenanthrene \( (C_{14}H_{10}) \) is very scarce. Some information is available on the degradation of phenanthrene under sulfate-reducing conditions. However, neither the genomes nor the degradation pathways have been described. A direct carboxylation of phenanthrene was suggested as a key initial reaction in two of these studies (Zhang and Young 1997; Davidova et al., 2007). Phenanthrene is one of the most abundant compounds present in PAH-contaminated sites, and due to its high toxicity, it is listed as a priority pollutant by the World Health Organization.

A major limitation for studying anaerobic PAH-degradation is the poor bacterial growth. Here, we report on phenanthrene degradation by a sulfate-reducing enrichment culture (TRIP), obtained from the Pitch Lake in Trinidad-Tobago, the world’s largest natural asphalt lake. This culture exhibits surprisingly fast growth with phenanthrene as substrate, with generation times of 10 days (Himmelberg et al., 2018), offering the unique opportunity to study anaerobic phenanthrene degradation in detail. We describe the key player for phenanthrene degradation by the TRIP culture by genome-resolved metagenomics, as well as the genes and enzymes involved in the anaerobic degradation of this three-ringed PAH.

Results and discussion

The metagenome of the TRIP culture was sequenced, and genome-resolved analyses (Gkanogiannis et al., 2016) led to the reconstruction of five nearly-complete genomes that were annotated using the MicroScope microbial genome annotation and analysis platform (Médigue et al., 2017; Himmelberg et al., 2018). A general overview of the main genomic features of these organisms is presented in Table 1. The most abundant organism in the TRIP culture accounted for about 60% of the raw reads and was classified as a member of the genus Desulfatiglans based on its full length 16S rRNA gene sequence (Fig. 1). This organism showed a close phylogenetic relationship to the naphthalene-degrading Deltaproteobacterial strain NaphS2 (Galushko et al., 1999), but not to 16S rRNA gene clones from the previously described phenanthrene-degrading Phe4 enrichment culture (Davidova et al., 2007; Himmelberg et al., 2018). The other microorganisms of the TRIP culture include another member of the genus Desulfatiglans, a Paludibacter, as well as members of the family Spirochaetaceae and the phylum Zixibacteria (Table 1).

In order to evaluate the metabolic potentials of the organisms in the TRIP culture, metabolic pathways were predicted from the genome sequences using the KEGG and MetaCyc databases (Kanehisa and Goto, 2000; Caspi et al., 2016), followed by a manual curation to evaluate the completeness of each predicted pathway. Furthermore, expression of the predicted genes was evaluated through a combined metatranscriptomic and metaproteomic analysis of the TRIP culture during growth with phenanthrene. Differential transcriptomic and proteomic analyses were not possible because the TRIP culture is not pure and shifts of the bacterial community were observed upon growth with other substrates (Himmelberg et al., 2018).

Previous biochemical studies performed with the TRIP culture suggested that anaerobic phenanthrene degradation involves mechanisms similar to anaerobic naphthalene degradation with an initial carboxylation reaction followed by CoA activation at the carboxyl group and a stepwise reduction of the aromatic rings (Himmelberg et al., 2018). Then, the genes and enzymes involved in the anaerobic degradation of phenanthrene might be similar to those involved in the anaerobic degradation of naphthalene. In order to test this hypothesis, DNA sequences of naphthalene-degradation genes from the Deltaproteobacteria N47 (Bergmann et al., 2011a) and NaphS2 (DiDonato et al., 2010) were used to search for

| Genome Classification | TRIP_1 Desulfatiglans | TRIP_2 Desulfatiglans | TRIP_3 Paludibacter | TRIP_4 Spirochaetaceae | TRIP_5 Zixibacteria |
|------------------------|-----------------------|-----------------------|---------------------|------------------------|---------------------|
| Completeness           | 99.35                 | 99.35                 | 95.61               | 98.85                  | 99.94               |
| Contamination          | 1.29                  | 0.97                  | 0.27                | 0                      | 5.59                |
| Strain heterogeneity   | 0                     | 0                     | 0                   | 0                      | 0                   |
| Length (bases)         | 5 429 328             | 4 801 105             | 3 156 633           | 2 943 264              | 3 508 505           |
| GC (%)                 | 47.01                 | 58.62                 | 35.02               | 55.55                  | 49.72               |
| Number of CDS          | 4868                  | 4891                  | 2634                | 2930                   | 3045                |
| Average CDS length (bp)| 888                   | 865                   | 1058                | 944                    | 1039                |
| Average intergenic length (bp) | 300           | 170                   | 149                 | 117                    | 143                 |
| Protein coding density (%) | 86.92           | 87.30                 | 90.61               | 92.32                  | 89.7                |

Table 1. Overview of the genomes reconstructed from the TRIP metagenome. Phylogenetic classification is based on the full length 16S rRNA gene (SILVA database, Quast et al., 2013).
genes encoding homologous enzymes in the metagenome of the TRIP culture. The pathway for anaerobic naphthalene degradation involves an initial carboxylation step for substrate activation (Mouttaki et al., 2012). This step is catalysed by a carboxylase enzyme encoded by a gene cluster containing several *ubiD*-like carboxylase genes both in Deltaproteobacteria N47 and NaphS2 (Bergmann et al., 2011a). Two similar gene clusters, encoding a putative phenanthrene carboxylase were found in the genome of candidate *Desulfatiglans* TRIP_1 and the products of those genes were detected during the proteomic and/or transcriptomic analysis (Fig. 2A and Table 2). These results are in concordance with a previously suggested carboxylation as the initial step for the anaerobic degradation of phenanthrene in other bacterial cultures (Zhang and Young, 1997; Davidova et al., 2007) and with the carboxylated derivatives of phenanthrene detected during metabolite analyses of the TRIP culture (Himmelberg et al., 2018). Moreover, our results provide evidence of the participation of UbiD-like carboxylases in the degradation of three-ring PAHs. The involvement of UbiD-like carboxylases was previously demonstrated not only in the anaerobic degradation of non-substituted PAHs such as naphthalene but also of benzene (Meckenstock et al., 2016).

During anaerobic naphthalene degradation, the produced 2-naphthoic acid is subsequently converted to 2-naphthoyl-CoA by an ATP-dependent 2-naphthoate-CoA ligase (Meckenstock et al., 2016). The formation of CoA esters enables further reduction steps and fosters product accumulation within the cell as CoA derivatives cannot permeate through membranes (Fuchs, 2008). The products of five genes of candidate *Desulfatiglans* TRIP_1 displayed between 30% and 33% sequence identity with the proposed 2-naphthoate-CoA ligases of Deltaproteobacteria N47 and NaphS2. Only three of these genes were expressed during growth of the TRIP culture in phenanthrene, and putatively encode phenanthroate-CoA ligases (Table 3(a)). Noteworthy, one of the expressed genes is located in the vicinity of one of the putative carboxylase gene clusters. In Deltaproteobacterium NaphS2, the naphthoyl-CoA ligase gene is located in the vicinity of

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**Fig. 1.** A. Relative abundance of genomes reconstructed from the TRIP metagenome, calculated by re-mapping raw reads to the assembled scaffolds using the BWA software (Li and Durbin, 2009). B. Operational taxonomic units (OTU) obtained by clustering 16S rRNA gene amplicon sequences (V3-V4 region) using MetaAmp Version 2.0 (Dong et al., 2017).
the gene cluster coding for 2-naphthoyl-CoA reductase, the enzyme catalysing the first reduction step of 2-naphthoyl-CoA (DiDonato et al., 2010). The genomic neighbourhood of the corresponding gene cluster in candidate Desulfatiglans TRIP_1 (see below) contains two genes encoding CoA-transferases, which were expressed during growth on phenanthrene (Table 3(b)). Since some microorganisms are known to use CoA transferases instead of CoA ligases under energy limitations (Meckenstock et al., 2016), it is likely that such gene products are involved in this metabolic step. Nevertheless, CoA ligase activity was measured in the TRIP culture through cell-free enzymatic assays (Himmelberg et al., 2018). The expressed CoA transferases of candidate Desulfatiglans TRIP_1 could also be part of other metabolic pathways. For example, genes encoding these enzymes are part of a synton containing genes involved in butanoate metabolism and fatty acid metabolism (not shown). The third expressed CoA transferase enzyme in candidate Desulfatiglans TRIP_1 shares 68% sequence identity with a protein of Deltaproteobacterium NaphS2 involved in late steps of benzoyl-CoA degradation (DiDonato et al., 2010).

In Deltaproteobacterium N47, further degradation of 2-naphthoyl-CoA proceeds through consecutive two-electron reduction steps (Eberlein et al., 2013a,b; Estelmann et al., 2015). The first two steps are catalysed by the enzymes 2-naphthoyl-CoA reductase and 5,6-dihydro-2-naphthoyl-CoA reductase, respectively, both belonging to the ‘old yellow enzyme’ (OYE) family (Williams and Bruce, 2002). Five genes encoding similar enzymes were found in the genome of candidate Desulfatiglans TRIP_1. The combined transcriptome/proteome analysis allowed narrowing down the candidate genes encoding enzymes involved in the reduction of phenanthroyl-CoA. Namely, the products of only two of those genes were detected during the proteomic analysis.
Table 2. Gene clusters identified in the genome of candidate Desulfatiglans TRIP_1, related to the naphthalene carboxylase gene clusters from the Deltaproteobacteria N47 and NaphS2 (Di Donato et al., 2010; Bergmann et al., 2011a).

| Annotated function in N47 | Gene in N47 | Gene in NaphS2 | Gene in TRIP_1 | Annotated function in TRIP_1 | Expression (RNA) | Expression (proteins) | Sequence identity with N47/ NaphS2 enzyme (%) |
|--------------------------|-------------|----------------|----------------|-----------------------------|-----------------|----------------------|---------------------------------------------|
| Phenylphosphate carboxylase, α subunit | N47_K27540 | NPH_5855 | PITCH_v1_a1330019 | Phenylphosphate carboxylase, alpha subunit | 1160 | 7 | 50 (N47_K27540)/(NPH_5855) |
| Phenylphosphate carboxylase γ subunit | N47_K27530 | NPH_5856 | PITCH_v1_a1330006 | Conserved protein of unknown function | 104 | 5 | 41 (N47_K27530)/41 (NPH_5856) |
| MRP, Fer4_NifH superfamily | N47_K27520 | NPH_5857 | PITCH_v1_a1350002 | Conserved protein of unknown function | 3921 | 2 | 65 (N47_K27520)/64 (N47_K27520) |
| ParA/MinD ATPase like, MRP, Fer4_NifH superfamily | N47_K27510 | NPH_5858 | PITCH_v1_a1340003 | Protein of unknown function | 390 | - | 61 (N47_K27510)/60 (NPH_5858) |
| UbID family carboxylase | N47_K27500 | NPH_5859 | PITCH_v1_a1350004 | 3-octaprenyl-4-hydroxybenzoate carboxylase | 6752 | 7 | 50 (N47_K27500)/50 (NPH_5859) |
| Conserved hypothetical protein | N47_K27490 | NPH_5860 | - | - | - | - | - |
| UbID family carboxylase | N47_K27480 | NPH_5861 | PITCH_v1_a1350006 | 3-octaprenyl-4-hydroxybenzoate carboxylase | 10404 | 9 | 53 (N47_K27480) |
| Conserved hypothetical protein | N47_K27470 | NPH_5862 | PITCH_v1_a1350007 | Conserved protein of unknown function | 12173 | - | 49 (N47_K27470) |
| UbID family carboxylase | N47_K27460 | NPH_5863 | PITCH_v1_a1350010 | Conserved protein of unknown function | 2521 | 32 | 39 (N47_K27460) |
| HAD hydrolase | N47_K27450 | - | - | - | - | - | - |
| HAD hydrolase | N47_K27440 | - | - | - | - | - | - |
| Membrane protein (aromatic hydrocarbon degradation) | N47_K27430 | - | PITCH_v1_a1350009 | Protein of unknown function | 3425 | - | 28 (N47_K27430) |
| IS4 transposase | N47_K27420 | - | - | - | - | - | - |
| Succinate dehydrogenase/ fumarate reductase iron-sulfur protein | N47_K27410 | NPH_5879 | - | - | - | - | - |
| Succinate dehydrogenase flavoprotein subunit | N47_K27400 | NPH_5880 | - | - | - | - | - |
| Succinate dehydrogenase flavoprotein subunit | N47_K27390 | - | - | - | - | - | - |
| Pyridoxamine 5'-phosphate oxidase family protein | N47_K27380 | NPH_5878 | - | - | - | - | - |
| Phenylphosphate carboxylase α subunit | N47_K27540 | NPH_5855 | PITCH_v1_a1920006 | Phenylphosphate carboxylase, alpha subunit | 64 | 4 | 46 (NPH_5855) |
| Phenylphosphate carboxylase γ subunit | N47_K27530 | NPH_5856 | - | - | - | - | - |
| MRP, Fer4_NifH superfamily | N47_K27520 | NPH_5857 | PITCH_v1_a2050011 | Conserved protein of unknown function | 453 | - | 66 (N47_K27520)/64 (N47_K27520) |
| ParA/MinD ATPase like, MRP, Fer4_NifH superfamily | N47_K27510 | NPH_5858 | PITCH_v1_a2050010 | Conserved protein of unknown function | 330 | 2 | 62 (N47_K27510)/61 (NPH_5858) |
| UbID family carboxylase | N47_K27500 | NPH_5859 | PITCH_v1_a2050009 | 3-octaprenyl-4-hydroxybenzoate carboxylase | 390 | 5 | 48 (N47_K27500)/59 (NPH_5859) |
| Conserved hypothetical protein | N47_K27490 | NPH_5860 | - | - | - | - | - |

(Continues)
| Annotated function in N47 | Gene in N47 | Gene in NaphS2 | Gene in TRIP_1 | Annotated function in TRIP_1 | Expression (RNA) | Expression (proteins) | Sequence identity with N47/ NaphS2 enzyme (%) |
|--------------------------|-------------|---------------|---------------|-----------------------------|----------------|---------------------|------------------------------------------|
| UbiD family carboxylase  | N47_K27480  | NPH_5861      | PITCH_v1_a2050007 | 3-octaprenyl-4-hydroxybenzoate carboxylase | 254            | 5                   | 57 (N47_K27480)/54 (NPH_5861)            |
| Conserved hypothetical protein | N47_K27470  | NPH_5862      | PITCH_v1_a2050006 | Conserved protein of unknown function | 436            | -                   | 50 (N47_K27470)/47 (NPH_5862)            |
| UbiD family carboxylase  | N47_K27460  | NPH_5863      | PITCH_v1_a2230002 | UbD family decarboxylase | 720            | 10                  | 45 (N47_K27460)/47 (NPH_5863)            |
| HAD hydrolase            | N47_K27450  | -             | PITCH_v1_a2030199 | HAD hydrolase, family IA, variant 3 | 548            | -                   | 27 (N47_K27450)                           |
| Membrane protein (aromatic hydrocarbon degradation) | N47_K27440 | -             | -              | -                           | -              | -                   | -                                         |
| HAD hydrolase            | N47_K27430  | -             | -              | -                           | -              | -                   | -                                         |
| IS4 transposase          | N47_K27420  | -             | -              | -                           | -              | -                   | -                                         |
| Succinate dehydrogenase/ fumarate reductase iron–sulfur protein | N47_K27410 | NPH_5879      | PITCH_v1_a2230004 | Conserved protein of unknown function | 418            | 9                   | 60 (N47_K27410)/59 (NPH_5879)            |
| Succinate dehydrogenase flavoprotein subunit | N47_K27400 | NPH_5880      | PITCH_v1_a2230003 | Succinate dehydrogenase flavoprotein subunit | 426            | 26                  | 61 (N47_K27400)/63 (NPH_5880)            |
| Succinate dehydrogenase flavoprotein subunit | N47_K27390 | -             | -              | -                           | -              | -                   | -                                         |
| Pyridoxamine 5'-phosphate oxidase family protein | N47_K27380 | NPH_5878      | -              | -                           | -              | -                   | -                                         |

Gene expression detected by transcriptome and mass spectroscopy proteome analysis of the TRIP culture grown on phenanthrene are expressed as read counts (fragments per kilobase of gene per million mapped reads) and number of unique peptides identified over runs performed on four independent samples respectively.
Table 3. Aryl-CoA ligase and CoA transferase genes identified in the genome of candidate Desulfatiglans TRIP_1, based on homology to previously characterized naphthalene degradation genes in the Deltaproteobacteria N47 and NaphS2 (Di Donato et al., 2010; Bergmann et al., 2011a).

| Annotated function in N47 | Gene in N47 | Gene in NaphS2 | Gene in TRIP_1 | Annotated function in TRIP_1 | Expression (RNA) | Expression (proteins) | Sequence identity with N47/NaphS2 enzyme (%) |
|--------------------------|-------------|----------------|----------------|----------------------------|-----------------|----------------------|------------------------------------------|
| 2-naphtoate-coenzyme A ligase | N47_I06840  | NPH_5477   | PITCH_v1_a1100006 | Putative Phenylacetate-coenzyme A ligase | 1226             | 3                    | 31 (N47_I06840)/33 (NPH_5477)           |
|                           |             |             | PITCH_v1_a133026 | Putative Phenylacetate-CoA ligase | 6008             | 16                   | 31 (N47_I06840)/30 (NPH_5477)           |
|                           |             |             | PITCH_v1_a760031 | Putative Phenylacetate-CoA ligase | 314              | 12                   | 31 (N47_I06840)/28 (NPH_5477)           |
|                           |             |             | PITCH_v1_a640050 | Conserved protein of unknown function | -                | -                    | 22 (N47_I06840)/21 (NPH_5477)           |
|                           |             |             | PITCH_v1_a1710015 | Conserved protein of unknown function | -                | -                    | 20 (N47_I06840)/22 (NPH_5477)           |
| CoA-transferases           | PITCH_v1_a420087 | Putative Coenzyme A transferase | 18 613 | 20 | - |
|                           | PITCH_v1_a420086 | Putative CoA transferase | 17 540 | 20 | - |
|                           | PITCH_v1_a920038 | CoA-acyltransferase | 13 761 | 11 | - |

- **a.** In the vicinity of a carboxylase gene cluster.
- **b.** In the vicinity of a reductase gene.

Gene expression detected by transcriptome and mass spectroscopy proteome analysis of the TRIP culture grown on phenanthrene are expressed as read counts (fragments per kilobase of gene per million mapped reads) and number of unique peptides identified over runs performed on four independent samples respectively.
Table 4. Reductase and oxidoreductase genes identified in the genome of candidate *Desulfatiglans* TRIP_1, based on homology to previously characterized naphthalene degradation genes in the Deltaproteobacteria N47 and NaphS2 (Di Donato et al., 2010; Bergmann et al., 2011a).

| Annotated function in N47 | Gene in N47 | Gene in NaphS2 | Gene in TRIP_1 | Annotated function in TRIP_1 | Expression (RNA) | Expression (protein) | Sequence identity with N47/NaphS2 enzyme (%) |
|----------------------------|-------------|----------------|----------------|----------------------------|-----------------|---------------------|---------------------------------------------|
| 2-naphthoyl-CoA reductase  | N47_G38220  | NPH_5475      | PITCH_v1_     | NADH-flavin oxidoreductases, Old Yellow Enzyme family | 94              | 17                   | 34 (N47_G38220)/33 (N47_G38210)/36 (NPH_5475)/35 (NPH_1753)/36 (NPH_5476) |
|                            |             | NPH_1753      | a190075       |                             | 67              | 5                    | 35 (N47_G38220)/33 (N47_G38210)/36 (NPH_5475)/37 (NPH_1753)/36 (NPH_5476) |
|                            |             |               | PITCH_v1_     |                             |                 |                      |                                             |
|                            |             |               | a20108        |                             |                 |                      |                                             |
| 5,6-dihydro-2-naphthoyl-CoA reductase | N47_G38210 | NPH_5476      | PITCH_v1_     |                             | 94              | 17                   | 34 (N47_G38220)/33 (N47_G38210)/36 (NPH_5475)/35 (NPH_1753)/36 (NPH_5476) |
|                            |             |               | a10001        |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     |                             |                 |                      |                                             |
|                            |             |               | a1860005      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     |                             |                 |                      |                                             |
|                            |             |               | a1940002      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     |                             |                 |                      |                                             |
|                            |             |               | a1000001      |                             |                 |                      |                                             |
| NADH-quinone oxidoreductase| N47_G38200  | NPH_5472      | PITCH_v1_     | NADH dehydrogenase          | 806             | 21                   | 52 (N5473/52 (NPH_1751) |
|                            |             | NPH_5474      | a1090011      |                             |                 |                      |                                             |
|                            |             | NPH_1750      | PITCH_v1_     | NADH-quinone oxidoreductase | 83              | 4                    | 55 (NPH_5473/57 (NPH_1751) |
|                            |             | NPH_1751      | a1100013      |                             |                 |                      |                                             |
|                            |             | NPH_1752      | PITCH_v1_     | NADH-quinone oxidoreductase | 232             | 6                    | 33 (NPH_5474/34 (NPH_1752) |
|                            |             |               | a1120003      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     | NADH-ubiquinone oxidoreductase | 267             | 10                   | 56 (N47_G38200)/55 (NPH_5473)/56 (NPH_1751) |
|                            |             |               | a1120005      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     | NADH-ubiquinone oxidoreductase | 530             | 5                    | 37 (NPH_5472)/36 (NPH_1750)/31 (NPH_1751) |
|                            |             |               | a1150094      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     | NADH dehydrogenase          | 553             | 20                   | 54 (NPH_5473)/51 (NPH_1751) |
|                            |             |               | a1150095      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     | 4Fe-4S binding domain protein | 340             | 6                    | 53 (NPH_5473/55 (NPH_1751) |
|                            |             |               | a1430008      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     | NADH dehydrogenase          | 120             | 3                    | 46 (NPH_5472/46 (NPH_1750) |
|                            |             |               | a1430009      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     | NADH ubiquinone oxidoreductase | 812             | 22                   | 57 (N47_G38200)/56 (NPH_5473/57 (NPH_1751) |
|                            |             |               | a1630007      |                             |                 |                      |                                             |
|                            |             |               | PITCH_v1_     | Benzoyl-CoA reductase       | 1437            | 20                   | 42 (NPH_5472/41 (NPH_1750) |
|                            |             |               | a1630008      |                             |                 |                      |                                             |

Gene expression detected by transcriptome and mass spectroscopy proteome analysis of the TRIP culture grown on phenanthrene are expressed as read counts (fragments per kilobase of gene per million mapped reads) and number of unique peptides identified over runs performed on four independent samples respectively.
of the TRIP culture (Table 4(a)). The expression of several other genomically clustered oxidoreductase genes was also detected during growth on phenanthrene. These genes bear convincing sequence identities (37%–57%) to the oxidoreductases of Deltaproteobacteria N47 and NaphS2, but unlike the situation in these naphthalene-degrading bacteria, they were not located in the direct vicinity of the putative reductase genes (Table 4(b)).

In the anaerobic degradation of naphthalene, the above-described reactions are followed by a reductive deaeromatization step catalysed by the enzyme 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase, which shows high sequence identity (70%–78%) to class I deaeromatizing benzoyl-CoA reductases (Meckenstock et al., 2016). In Deltaproteobacteria N47 and NaphS2, the genes encoding this enzyme are surrounded by hydrotases, dehydrogenases, hydrolases and thiolas genes, constituting the thn-operon. The products of this operon participate in β-oxidation-like reactions following the ring-deaeromatization step (DiDonato et al., 2010). Two gene clusters with 45% to 68% product sequence identity to the 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase of Deltaproteobacterium N47 were identified in the genome of candidate Desulfatiglans TRIP_1. The gene clusters in candidate Desulfatiglans TRIP_1 also contain other genes with high sequence identity to the thn-operon in Deltaproteobacterium N47 (Fig. 2B and Table 5). Products of both gene clusters were detected during the proteome and/or transcriptome analysis of the TRIP culture, providing further evidence for anaerobic degradation of phenanthrene involving reduction and ring deaeromatization steps, catalysed by similar enzymes to those involved in the anaerobic degradation of naphthalene. The detection of these reductase genes agrees with the phenanthrene derivatives detected in the TRIP culture showing different degrees of hydrogenation (Himmelberg et al., 2018).

Figure 3 shows an overview of the proposed phenanthrene degradation pathway employed by candidate Desulfatiglans TRIP_1, including the evidence obtained so far for each reaction at the level of genes, mRNA, proteins, metabolites and enzymatic activity. Interestingly, compared with the genomes of the naphthalene degraders Deltaproteobacteria N47 and NaphS2, the genome of candidate Desulfatiglans TRIP_1 contains (and expresses) a higher number of PAH-degradation genes. Considering the higher complexity of phenanthrene, a higher number of enzymatic steps is expected, particularly at the level of aromatic ring reductions.

Genes sharing sequence identities with the aforementioned naphthalene-degradation genes were not found in the other genomes composing the TRIP culture. Considering that the candidate Desulfatiglans TRIP_1 is the dominant organism of the TRIP culture, and since potential phenanthrene-degradation genes were only identified in its genome, it is likely that this bacterium is the one responsible for the anaerobic phenanthrene degradation. The fact that the community composition changes upon growth of the TRIP culture with different substrates (Himmelberg et al., 2018) further evidences that candidate Desulfatiglans TRIP_1 has a major role in the anaerobic degradation of phenanthrene by the TRIP culture.

The genome of candidate Desulfatiglans TRIP_1 contains all enzymes needed for dissimilatory sulfate reduction, including ATP sulfurylase (sat genes), adenosine-5'-phosphosulfate reductase (aprAB genes) and dissimilatory sulfite reductase (dsrAB genes) (Fig. 4). This is in concordance with the phylogenetic classification of this organism as well as with the growth of the TRIP culture with sulfate as electron acceptor. It has been proposed for gram-negative sulfate-reducing bacteria that electrons are transferred from the quinone pool to DsrAB reductase and AprAB by two different electron transfer complexes: DsrMKJOP together with the protein DsrC, and QmoABC respectively (Pereira et al., 2011). Consistently, genes encoding a DsrMKJOP redox complex are present in the genome of candidate Desulfatiglans TRIP_1 and a gene encoding the protein DsrC is located adjacent to the dsrAB genes. Moreover, genes close to the apr genes are putatively coding for an adenylylsulfate reductase-associated electron transfer protein (qmoABC genes). The expression of all of the aforementioned genes was detected by proteomic and/or transcriptomic analyses of the TRIP culture grown with phenanthrene as carbon source (Fig. 4 and Supporting Information Table S1). Unlike some sulfate-reducing bacteria, including the naphthalene-degrading Deltaproteobacterium N47, candidate Desulfatiglans TRIP_1 does not carry genes for the reduction of nitrate to ammonium (Bergmann et al., 2011b), in agreement with the inability of the TRIP culture to use nitrate as terminal electron acceptor (Himmelberg et al., 2018).

The genome of candidate Desulfatiglans TRIP_1 contains all the genes required to run the Embden-Meyerhof-Parnas pathway. Genes encoding the enzymes catalysing the reactions necessary for gluconeogenesis: fructose-1,6-bisphosphatase, phosphoenolpyruvate synthase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase, were also identified in the genome. The expression of these genes was detected by proteomic and/or transcriptomic analyses of the TRIP culture grown with phenanthrene (Fig. 4 and Supporting Information Table S1). The genome of candidate Desulfatiglans TRIP_1 also encodes the enzymes for the pentose phosphate pathway. Only the products of genes involved in the non-oxidative branch of this pathway, including transketolase, ribose 5-phosphate isomerase, ribulose-5-phosphate 3-epimerase and a transaldolase were detected in both the transcriptome and proteome analyses (Fig. 4 and Supporting Information Table S1).
| Annotated function in N47 | Gene in N47 | Gene in NaphS2 | Gene in TRIP_1 | Expression (RNA) | Expression (proteins) | Sequence identity with N47/ NaphS2 enzyme (%) |
|--------------------------|------------|----------------|----------------|-----------------|----------------------|---------------------------------------------|
| TetR-family transcriptional regulator | N47_E41510 | NPH_5886 | PITCH_v1_a1910018 | conserved protein of unknown function | - | 41 (N47_E41510)/32 (NPH_5886) |
| Enoyl-CoA hydratase/hydrolase/isomerase | N47_E41500 | NPH_5887 | PITCH_v1_a1910016 | 3-hydroxybutyryl-CoA dehydratase | 8194 | - |
| 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase | N47_E41490 | NPH_5888 | PITCH_v1_a1910015 | putative benzoyl-CoA reductase, bzd-type, N subunit | 6785 | 30 |
| 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase | N47_E41480 | NPH_5889 | PITCH_v1_a1910014 | putative benzoyl-CoA reductase, bzd-type, O subunit | 5685 | 19 |
| 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase | N47_E41470 | NPH_5890 | PITCH_v1_a1910013 | putative (R)-2-hydroxyglutaryl-CoA dehydratase activator | 5459 | 8 |
| 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase | N47_E41460 | NPH_5891 | PITCH_v1_a1910012 | Benzoyl-CoA reductase, bzd-type, Q subunit | 8639 | 16 |
| Ferredoxin | N47_E41450 | - | - | - | - | - |
| Oxidoreductase | N47_E41430 | NPH_5892 | PITCH_v1_a1910011 | BzdV protein | 2710 | 33 |
| R-3-hydroxyacyl-CoA dehydratase | N47_E41420 | NPH_5893 | PITCH_v1_a1910007 | conserved protein of unknown function | 1259 | 14 |
| R-3-hydroxyacyl-CoA dehydratase | N47_E41410 | NPH_5894 | PITCH_v1_a1910006 | Maac-like protein | 2272 | 7 |
| β-oxoacyl-CoA thiolase | N47_E41400 | NPH_5895 | PITCH_v1_a1910005 | Acetyl-CoA acetyltransferase | 4886 | 25 |
| β-oxoacyl-CoA dehydrogenase | N47_E41390 | NPH_5896 | PITCH_v1_a1910008 | putative 3-hydroxybutyryl-CoA dehydrogenase | 13225 | 9 |
| Enoyl-CoA hydratase/hydrolase/isomerase | N47_E41380 | NPH_5897 | PITCH_v1_a1910016 | 3-hydroxybutyryl-CoA dehydratase | 8194 | - |
| Enoyl-CoA hydratase/hydrolase/isomerase | N47_E41370 | NPH_5898 | PITCH_v1_a1910017 | putative enoyl-CoA hydratase/isomerase YngF | 1711 | 12 |
| Metallo-dependant hydrolase (TIM barrel fold) | N47_E41360 | NPH_5899 | PITCH_v1_a1910004 | putative amidohydrolase 2 | 4116 | 11 |
| acyl-CoA dehydrogenase | N47_E41350 | NPH_5900 | PITCH_v1_a1910010 | 3-oxoacyl-[acyl-carrier-protein] reductase FabG | - | - |
| CoA-transferase / acetyl-CoA hydrolase | N47_E41340 | NPH_5902 | - | - | - | - |
| Acyl-CoA:acetate-lyase AtuA-like | N47_E41330 | NPH_5903 | - | - | - | - |
| Acyl-CoA:acetate-lyase AtuA-like | N47_E41320 | NPH_5904 | - | - | - | - |
| β-oxoacyl-ACP reductase | N47_E41310 | NPH_5905 | PITCH_v1_a1910010 | 3-oxoacyl-[acyl-carrier-protein] reductase FabG | - | 10 |
| β-hydroxyacyl-CoA dehydrogenase | N47_E41300 | NPH_5906 | PITCH_v1_a1910008 | putative 3-hydroxybutyryl-CoA dehydrogenase | 13225 | 9 |
| Acyl-CoA dehydrogenase | N47_E41290 | NPH_5907 | PITCH_v1_a1910016 | 3-hydroxybutyryl-CoA dehydratase | 8194 | 21 |
Table S1). Such enzymes generate precursors for the synthesis of nucleic acids and amino acids (Zubay, 1983).

The genome further contains genes for the subunits of a pyruvate dehydrogenase complex required for transforming pyruvate into acetyl-CoA. All sequence analysis including similarities, synteny and protein domain predictions (Médigue et al., 2017) pointed to the classification of the aforementioned genes as encoding a 2-oxo-acid dehydrogenase and not a 2-oxo-acid:ferredoxin oxidoreductase. This excludes the possibility of producing reduced ferredoxin at this step which is needed for reduction of the ring system in anaerobic naphthalene degradation by Deltaproteobacterium N47 (Weyrauch, unpublished results). Expression of the pyruvate dehydrogenase complex was detected both by transcriptomics and proteomics (Fig. 4 and Supporting Information Table S1).

For oxidation of acetyl-CoA, genes for a complete tricarboxylic acid (TCA) cycle (citrate synthase, isocitrate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarate hydratase and malate dehydrogenase) were detected, with the exception of 2-oxoglutarate dehydrogenase. Instead, the cycle is closed by a 2-oxoglutarate:ferredoxin oxidoreductase. Expression of the genes encoding the aforementioned enzymes was detected during growth of the TRIP culture with phenanthrene (Fig. 4 and Supporting Information Table S1). The substitution of 2-oxoglutarate dehydrogenase by 2-oxoglutarate:ferredoxin oxidoreductase is well documented in microaerophilic and strictly anaerobic organisms (Spormann and Thauer, 1989; Yun et al., 2001). The reaction generates reduced ferredoxin, which can provide low-potential electrons for aryl-CoA reductases, required for anaerobic degradation of aromatic compounds (Bergmann et al., 2011a,b). Moreover, the participation of 2-oxoglutarate:ferredoxin oxidoreductase has been previously demonstrated during the anaerobic degradation of naphthalene by Deltaproteobacteria N47 and NaphS2 (Weyrauch, unpublished results). 2-oxoglutarate:ferredoxin oxidoreductase is known as a key enzyme in the reductive TCA cycle, which is used by some bacteria for carbon fixation (Buchanan and Arnon, 1990). Two other key enzymes of the reductive TCA cycle are the fumarate reductase and ATP citrate lyase. However, only genes for the fumarate reductase could be unambiguously detected in the genome of candidate Desulfatiglans TRIP_1 (Fig. 4 and Supporting Information Table S1). It cannot be excluded that the absence of the ATP citrate lyase in the genome of TRIP1 is due to genome incompleteness, as an estimated 0.7% of the genome is missing (Table 1). Nonetheless, lack of the ATP citrate lyase would not exclude the possibility of candidate Desulfatiglans TRIP_1 to run a reductive TCA cycle, as some citrate synthases have been reported to
perform the reverse reaction similar to the ATP citrate lyase (Mall et al., 2018). Hence, candidate Desulfatiglans TRIP_1 and the naphthalene-degrading Deltaproteobacterium N47 could resemble other sulfate-reducing bacteria such as Desulfobacter hydrogenophilus, which have the potential to switch between a hetero-organotrophic metabolism of growth using the TCA cycle for complete acetate oxidation and a litho-autotrophic metabolism of growth, with H₂, CO₂ and sulfate, assimilating CO₂ to acetate using the reductive TCA cycle (Schauder et al., 1987; Bergmann et al., 2011b). In fact, the genome of candidate Desulfatiglans TRIP_1 contains a gene encoding a NiFe Group 3 hydrogenase which catalyses hydrogen oxidation according to the HydDB classification system (Sendergaard et al., 2016), as well as genes for sulfide oxidation. However, the products of these genes were not detected in the proteomic analysis of the TRIP culture grown with phenanthrene as substrate (Fig. 4 and Supporting Information Table S1).

In addition to a complete TCA cycle, candidate Desulfatiglans TRIP_1 presents and expresses the genes for a Wood-Ljungdahl pathway (Wood et al., 1986), including the carbon monoxide dehydrogenase/acetyl coenzyme A (CoA) synthetase enzyme complex (Fig. 4 and Supporting Information Table S1). Many sulfate reducers are known to use the reversed Wood–Ljungdahl pathway for the oxidation of acetyl-CoA to CO₂ (Spormann and Thauer, 1989; Strittmatter et al., 2009), which suggests that candidate Desulfatiglans TRIP_1 both harbours and simultaneously expresses two pathways for the complete oxidation of acetyl-CoA during growth on phenanthrene. The implications of a joint expression of the Wood-Ljungdahl pathway and TCA cycle are still elusive, even though this has been previously observed for other PAH-degrading bacteria such as the sulfate reducer Deltaproteobacterium N47 (Bergmann et al., 2011b).

Candidate Desulfatiglans TRIP_1 expressed the genes for a NADH dehydrogenase complex, as well as for a succinate dehydrogenase/fumarate reductase complex needed for a potential electron transport chain. The genome also contains several genes encoding cytochromes, including a cytochrome bd complex and two gene clusters encoding subunits of a F0-F1 ATP synthase (Fig. 4 and Supporting Information Table S1).

The genome of candidate Desulfatiglans TRIP_1 also contains genes encoding acetate kinase, phosphate acetyltransferase and lactate dehydrogenase which were detected by both proteomic and transcriptomic analyses (Supporting Information Table S1). However, even though a fermentative lifestyle with less reduced substrates cannot be excluded for candidate Desulfatiglans TRIP_1, the TRIP culture was neither able to grow with phenanthrene without a terminal electron acceptor, nor is...
Enzymes for the anaplerotic reactions required to replenish intermediates of the TCA cycle: acetyl-CoA synthetase, pyruvate synthase, phosphoenolpyruvate synthase and phosphoenolpyruvate carboxykinase are encoded in the genome of candidate *Desulfatiglans* TRIP_1 (Fig. 4 and Supporting Information Table S1). This genome also encodes all the genes required for nucleotide anabolism and for the synthesis of 18 of the 20 essential amino acids, suggesting auxotrophy for the amino acids methionine and tyrosine. Enzymes for the synthesis of the unsaturated fatty acids palmitoleate, cis-vaccenate and (5Z)-dodec-5-enoate, and for the synthesis of diacylglycerol and the glycerophospholipids phosphatidylglycerol and phosphatidylethanolamine are encoded in the genome of candidate *Desulfatiglans* TRIP_1. Moreover, genes were found for the synthesis of the lipopolysaccharides 3-deoxy-o-manno-oct-2ulosonic acid and GDP-ß-glycerol-o-a-manno-heptose. This organism

![Figure 4. Metabolic pathways, membrane transporters and mobility machinery of candidate *Desulfatiglans* TRIP_1 predicted by genomic analysis.](image-url)
appears able to Synthesize the following vitamins and cofactors: thiamine, riboflavin, pyridoxine, nicotinate, nicotinamide, pantothenic acid, biotin, folate, coenzyme A, guanylyl molybdenum cofactor and cobalamin (not shown).

Additionally, the genome harbours 67 genes for different transporter systems, including ATP-binding cassette (ABC) transporters, tripartite ATP-independent periplasmic transporters (TRAP), energy-coupling factor (ECF) transporters and Tol/Ton-dependent transporters, as well as 46 genes encoding secretion systems. The expression of a subset of these genes was detected by proteomics and/or transcriptomics (Fig. 4 and Supporting Information Table S1). Although no ABC transporter system for sulfate could be found, two genes encoding sulfate transporters were present, one belonging to the Major Facilitator Superfamily (Pao et al., 1998) and a probable proton/sulfate cotransporter (Fig. 4 and Supporting Information Table S1). TonB-dependent transporters have been reported for the uptake of aromatic compounds in some bacteria (Miller et al., 2010). Multiple gene clusters containing proteins associated to TonB-dependent transport systems were identified in the genome of candidate *Desulfatiglans* TRIP_1 (Fig. 4 and Supporting Information Table S1). Hence, it is possible that candidate *Desulfatiglans* TRIP_1 uses TonB-dependent transporters for phenanthrene uptake. On the other hand, PAHs can enter bacterial cells through passive diffusion. Furthermore, proton motive force-dependent transport mechanisms of Gram-negative bacteria has been reported (Hua et al., 2004). Two genes encoding such proteins were identified in the genome of candidate *Desulfatiglans* TRIP_1, one being part of the putative phenanthrene carboxylase gene cluster. Expression of these two genes was detected during the transcriptomic assay (Table 3 and Supporting Information Table S1).

Moreover, 43 genes encoding flagella structure and regulation proteins, as well as 28 genes encoding chemotaxis proteins were identified, suggesting that candidate *Desulfatiglans* TRIP_1 has chemotactic sensing abilities. Most of these genes were detected in the transcriptomic analysis of the TRIP culture, but were absent from the proteomic data (Fig. 4 and Supporting Information Table S1).

Finally, the genome of candidate *Desulfatiglans* TRIP_1 harbours genes annotated as benzylsuccinate synthase, which is the first enzyme in the anaerobic toluene degradation pathway. These genes share between 40% and 50% sequence identity to those encoding the subunits of naphthyl-2-methyl-succinate synthase of Deltaproteobacterium N47 (Bergmann et al., 2011a), the first enzyme in the anaerobic degradation of 2-methylnaphthalene, suggesting that this glycol radical enzyme could be involved in the anaerobic degradation of methylphenanthrene. Consistently, these genes were not expressed during growth with phenanthrene (Supporting Information Table S1).

The other four nearly complete genomes of the TRIP culture also carry the genes for the Emden-Meyerhof-Parnas pathway (Table 6), the TCA cycle and at least one branch of the pentose phosphate pathway. Moreover, all bacteria encode at least one pathway for enabling a fermentative metabolism. Genes necessary for dissimilatory sulfate reduction were only present in the two candidate *Desulfatiglans* genomes. These two bacteria, as well as the candidate *Zixibacterium* present at least one putative CO2 fixation pathway. The candidate *Zixibacterium* also possesses genes for denitrification, candidate *Desulfatiglans* TRIP_2 possesses genes for thiosulfate disproportionation and candidate *Paludibacter* TRIP_3 genes for nitrogen fixation (Table 6).

As aforementioned, we did not find potential genes encoding enzymes directly involved in phenanthrene degradation in the non-dominant genomes of the TRIP culture. A potential role in phenanthrene degradation of the four microorganisms other than candidate *Desulfatiglans* TRIP_1 could thus only be indirect. The genome of candidate *Spirochaetaceae* TRIP_4 suggests metabolic capacities similar to *Rectinema cohabitans*. The rod-shape *Spirochaeta* *R. cohabitans* was isolated from the naphthalene-degrading enrichment culture N47 where it is involved in necromass recycling providing hydrogen and possibly nutrients to the naphthalene-degrading Deltaproteobacterium N47 (Dong et al., 2018). Although the genetic elements for hydrogen transfer between the candidate *Spirochaetaceae* TRIP_4 and the candidate *Desulfatiglans* TRIP_1 are present, the corresponding gene products were not detected during growth of the TRIP culture with phenanthrene. This includes a [NiFe] hydrogenase in candidate *Desulfatiglans* TRIP_1 (Supporting Information Table S1) and four genes encoding [FeFe] hydrogenases, as well as genes required for fermentation of carbohydrates in candidate *Spirochaetaceae* TRIP_4 (Table 6). *Paludibacter* species related to candidate *Paludibacter* TRIP_3 have also been reported in PAHs-contaminated sites (Yang et al., 2016). Also these microorganisms are able to grow with dead biomass, which could explain their persistence in the TRIP culture (Yang et al., 2016).

When we compared the contribution of each of the members of the TRIP culture to the N, C, S and Fe cycles using the MEBS (Multigenomic Entropy-Based Score) software (De Anda et al., 2017), none of the members of the TRIP culture showed a predominant role in any of the biogeochemical cycles. However, candidate *Zixibacteria* TRIP_5 presented a comparatively higher score for the iron cycle (not shown), mostly based on cytochromes b and c as well putative iron-siderophore transporters.

The multiomics metabolic reconstruction suggests that candidate *Desulfatiglans* TRIP_1 is the primary producer of biomass in the culture and the only phenanthrene
degrader with sulfate as electron acceptor. The other abundant members are most likely necromass degraders recycling dead biomass with a fermentative lifestyle.

Conclusion

For the first time, this work describes genes involved in the anaerobic degradation of the three-ring PAH phenanthrene. Our results show that the anaerobic degradation of phenanthrene follows the same biochemical principles known for the anaerobic degradation of the model two-ring PAH naphthalene, i.e. an initial activation of phenanthrene by carboxylation and CoA ligation followed by a stepwise ring reduction by type III and type I aryl-CoA reductases. Subsequently, the pathway proceeds by β-oxidation-like reactions and hydrolytic ring cleavage. The predominant microorganism in the TRIP culture, candidate *Desulfatiglans TRIP_1* most likely performs the complete degradation of phenanthrene whereas the other members of the TRIP culture are involved in secondary degradation of necromass.

Experimental procedures

*Cultivation of the TRIP enrichment culture*

The TRIP culture was enriched from the Pitch Lake in Trinidad-Tobago (Himmelberg et al., 2018) and was grown anaerobically in carbonate-buffered freshwater mineral medium (Widdel and Bak, 1992) at 30 °C in the dark. Sulfate (10 mM) was added as terminal electron acceptor and 1.5% w/v phenanthrene in a liquid carrier phase (2,2,4,4,6,8,8-heptamethylnonane) was used as carbon source. Cultures were transferred (10% v/v) to fresh medium every 8–10 weeks.

*DNA extraction*

The TRIP culture was grown as described above until middle exponential phase. Cells were harvested by centrifugation (20 min, 6000 × g, 4 °C) and DNA was extracted using the DNeasy™ Blood and Tissue Kit (Qiagen).

*Metagenome analysis*

The metagenome of the TRIP culture was sequenced (Illumina PE 250 on MiSeq) and genome-resolved analyses of the raw shotgun data were performed (Himmelberg et al., 2018). The preassembly protocol followed the steps described in (Gkanogiannis et al., 2016) and the Spades engine (Bankevich et al., 2012) was used for targeted assemblies. The resulting scaffolds were annotated using the MicroScope microbial genome annotation and analysis platform (Médigue et al., 2017).
The genome sequence of candidate *Desulfatiglans* TRIP_1 has been submitted to the ENA databases and can be accessed under contig accession numbers: LT984854-LT984881.

MicroScope’s syntactic annotation pipeline was used to identify protein coding genes, transfer RNA (tRNA), ribosomal RNA (rRNA), noncoding RNA (ncRNA) and repeat elements. Functional annotations were derived using generalist protein sequence (UniProtKB/Swiss-Prot), protein domain (Interpro, FIGFAM) and metabolic databases (KEGG, MetaCyc).

Completeness of genomic sequence

The completeness and contamination levels of the reconstructed bacterial genomes were evaluated with the checkM software (Parks et al., 2015), based on the distribution pattern of a set of ubiquitous and single-copy marker genes within a reference genome tree.

Microorganisms relative abundance

The relative abundance of the bacteria composing the TRIP culture was estimated by re-mapping the raw reads to the assembled scaffolds using the BWA software (Li and Durbin, 2009), and the estimates were confirmed by 16S rRNA gene amplicon sequencing. For the latter, the V3-V4 region of the 16S rRNA gene was amplified from the DNA of the TRIP culture and used for library construction. PCR products were converted into blunt ends using T4 DNA polymerase Klenow Fragment and T4 Polynucleotide Kinase previous to adaptor addition. After quality control, libraries were sequenced (Illumina PE 250 on MiSeq 2500). The raw data were filtered to eliminate adapters and low-quality calls to obtain clean reads. Overlapping paired-end reads were merged and clustered into operational taxonomic units (OTUs) using MetaAmp Version 2.0 (Dong et al., 2017). Amplicon sequences have been submitted to the GenBank database and can be accessed upon accession number PRJNA479783.

Phylogenetic analysis

The full-length 16S rRNA gene sequences of the organisms from the TRIP culture were compared with the SILVA (Quast et al., 2013) and NCBI (Benson et al., 2012) databases.

Metabolic reconstruction

The metabolic capabilities of the different microorganisms were predicted from their genome using the KEGG (Kanehisa and Goto, 2000) and MetaCyc (Casi et al., 2016) databases; followed by a manual curation of the predicted pathways. Candidate genes relevant for PAH degradation were identified by homology to previously characterized enzymes involved in the anaerobic degradation of naphthalene using the blastp program (Altschul et al., 1990).

Metatranscriptome analyses

The TRIP culture was grown as described above and cells were harvested in the mid-exponential phase by centrifugation (20 min, 6000 × g, 4 °C). Total RNA was immediately extracted using the Trizol™ reagent (Invitrogen). Samples were treated with the DNA-free™ kit (Invitrogen) to remove DNA. After rRNA depletion, the mRNA was used for strand-specific library construction and sequencing (Illumina PE100 in HiSeq 4000).

The raw RNA-seq reads were filtered to remove adapter sequences, contamination and low-quality reads. After quality control, the metatranscriptomic reads were mapped against the reconstructed genomes using bowtie2 (Langmead and Salzberg, 2012). Gene expression levels were estimated from the abundance of mapped reads using the featureCounts software (Liao et al., 2014), with results expressed as fragments per kilobase of gene per million mapped reads. The transcriptome sequences of the TRIP culture grown in phenanthrene have been submitted to the GenBank database and can be accessed under accession number PRJNA479783.

Mass spectroscopy

Sample preparation

Cells were harvested by centrifugation in the mid-exponential growth phase (20 min, 6000 × g, 4 °C) and immediately frozen at −20 °C. The frozen cell pellets were warmed to room temperature and taken up in phosphate buffered saline (PBS buffer, pH 7.4). Cells were lysed by ultrasonication in a BioRuptor™ (Diagenode) for 5 min to release the proteome and shear the genomic DNA. Samples were centrifuged (5 min, 12 000 × g, 4 °C) and supernatants were transferred to fresh 1.5 ml Eppendorf tubes. The protein concentration was determined using a modified Bradford assay (Ernst and Zor, 2010). An aliquot corresponding to 30 μg total protein per sample was removed and taken up in 2% w/v SDS and subsequently reduced with 5 mM DTT for 5 min at 90 °C. Next, reduced proteins were alkylated with 20 mM iodoacetamide (IAM) for 30 min at room temperature in the dark. Reduced and alkylated proteins were then subjected to methanol/chloroform precipitation (Wessel and Flügge, 1984). The protein pellets were then taken up in 50 μl 8 M Urea and 100 mM ammonium bicarbonate (ABC) and cleared by centrifugation (30 s; 10 000 × g).
Clear solutions of 25 μl (corresponding to 15 μg total protein) were transferred to fresh Eppendorf tubes, supplemented with 500 ng LysC (ratio 1/30) and incubated at 37 °C for 3 h while vigorously shaking. Samples were diluted with 100 mM ABC until the urea concentration was below 1 M. Then 1 μg Trypsin was added (ration 1/15) and the samples incubated overnight at 37 °C while vigorously shaking. The digestion was stopped the next morning by adding formic acid (final concentration 5% v/v).

**Sample clean-up for LC–MS**

Acidified tryptic digests were desalted on home-made C18 StageTips as previously described (Rappsilber et al., 2007). On each 2 disc StageTip around 15 μg peptides were loaded (based on the initial protein concentration). After elution from the StageTips, samples were dried using a vacuum concentrator (Eppendorf) and the peptides were taken up in 10 μl 0.1% v/v formic acid solution.

**LC–MS/MS**

Experiments were performed on an Orbitrap Elite instrument (Thermo, Michalski et al., 2012) that was coupled to an EASY-nLC 1000 liquid chromatography (LC) system (Thermo). The LC was operated in the one-column mode. The analytical column was a fused silica capillary (75 μm × 35 cm) with an integrated PicoFrit emitter (New Objective) encased by a column oven (Sonation) and attached to a EASY-nLC 1000 liquid chromatography (LC) system. The LC was operated in the one-column mode. The mass spectrometer was set in the positive ion mode. Precursor ion scanning was performed in the Orbitrap analyser (Fourier Transform Mass Spectrometry, FTMS) in the scan range of m/z 300–1800 and at a resolution of 60 000 with the internal lock mass option turned on (lock mass was 445.120025 m/z, polysiloxane, Olsen et al., 2005). Product ion spectra were recorded in a data dependent fashion in the ion trap (Ion Trap Mass Spectrometry, ITMS) in a variable scan range and at a rapid scan rate. The ionization potential (spray voltage) was set to 1.8 kV. Peptides were analysed using a repeating cycle consisting of a full precursor ion scan (1.0 × 10⁶ ions or 30 ms) followed by 15 product ion scans (1.0 × 10⁴ ions or 50 ms) where peptides are isolated based on their intensity in the full survey scan (threshold of 500 counts) for tandem mass spectrum (MS2) generation that permits peptide sequencing and identification. Collision-induced dissociation (CID) collision energy was set to 35% for the generation of MS2 spectra. During MS2 data acquisition, dynamic ion exclusion was set to 60 s with a maximum list of excluded ions consisting of 500 members and a repeat count of one. Ion injection time prediction, preview mode for the FTMS, monoisotopic precursor selection and charge state screening were enabled. Only charge states higher than 1 were considered for fragmentation.

**Peptide and protein identification**

Raw spectra were submitted to an Andromeda (Cox et al., 2011) search in MaxQuant (version 1.5.3.30) using the default settings (Cox and Mann, 2008). Label-free quantification and match-between-runs was activated (Cox et al., 2014). MS/MS spectra data were searched against the in-house generated databases of predicted proteins from candidate *Desulfatiglans* TRIP_1 (4868 entries), candidate *Desulfatiglans* TRIP_2 (4891 entries), candidate *Zixibacteria* TRIP_5 (3045 entries) and candidate *Paludibacter* TRIP_3 (2636 entries) and candidate *Spirochaetaeae* TRIP_4 (2930 entries). All searches included also a contaminants database (as implemented in MaxQuant, 267 sequences). The contaminants database contains known MS contaminants and was included to estimate the level of contamination. Andromeda searches allowed oxidation of methionine residues (16 Da), acetylation of protein N-terminus (42 Da as dynamic modification) and the static modification of cysteine (57 Da, alkylation with IAM). Enzyme specificity was set to ‘Trypsin/P’ with two missed cleavages allowed. The instrument type in Andromeda searches was set to Orbitrap and the precursor mass tolerance was set to ±20 p.p.m. (first search) and ± 4.5 p.p.m. (main search). The MS/MS match tolerance was set to ±0.5 Da. The peptide spectrum match FDR and the protein FDR were set to 0.01 (based on target-decoy approach). Minimum peptide length was seven amino acids. For protein quantification, unique and razor peptides were allowed. Modified peptides were allowed for quantification. The minimum score for modified peptides was 40.
Data analysis

Initial data analysis was performed using the PERSEUS computational platform version 1.5.5.3 (Tyanova et al., 2016). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al., 2016) partner repository (https://www.ebi.ac.uk/pride/archive/) with the dataset identifier PXD010151.

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Supporting Information

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Table S1. List of genes identified in the genome of candidate *Desulfatiglans* TRIP_1, classified according to their metabolic function. Gene expression detected by transcriptome and mass spectroscopy proteome analyses of the TRIP culture grown on phenanthrene are expressed as read counts (fragments per kilobase of gene per million mapped reads) and number of unique peptides identified over runs performed on four independent samples, respectively.