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# Gene clusters involved in anaerobic benzoate degradation of *Geobacter metallireducens*

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## Summary

**The degradation of aromatic compounds follows different biochemical principles in aerobic and anaerobic microorganisms. While aerobes dearomatize and cleave the aromatic ring by oxygenases, facultative anaerobes utilize an ATP-dependent ring reductase for the dearomatization of the activated key intermediate benzoyl-coenzyme A (CoA). In this work, the aromatic metabolism was studied in the obligately anaerobic model organism *Geobacter metallireducens*. The gene coding for a putative carboxylic acid-CoA ligase was heterologously overexpressed and the gene product was characterized as a highly specific benzoate-CoA ligase catalysing the initial step of benzoate metabolism. However, no evidence for the presence of an ATP-dependent benzoyl-CoA reductase as observed in facultative anaerobes was obtained. In a proteomic approach benzoate-induced proteins were identified; the corresponding genes are organized in two clusters comprising 44 genes. Induction of representative genes during growth on benzoate was confirmed by reverse transcription polymerase chain reaction. The results obtained suggest that benzoate is activated to benzoyl-CoA, which is then reductively dearomatized to cyclohexa-1,5-diene-1-carbonyl-CoA, followed by β-oxidation reactions to acetyl-CoA units, as in facultatively anaerobic bacteria. However, in *G. metallireducens* the process of reductive benzene ring dearomatization appears to be catalysed by a set of completely different protein components comprising putative molybdenum and selenocysteine containing enzymes.**

Accepted 12 September, 2005. *For correspondence. E-mail boll@biologie.uni-freiburg.de; Tel. (+49) 761 203 2685; Fax (+49) 761 203 2626. †Both authors contributed equally to this manuscript. ‡Dedicated to Professor Georg Fuchs, Freiburg on the occasion of his 60th birthday.

## Introduction

Aromatic compounds represent the second most abundant class of natural carbon compounds, which mostly derive from plant metabolism (e.g. lignin, flavonoids, tannins) and from the three aromatic amino acids. In addition, many man-made aromatic xenobiotics are continuously released into the environment and may cause severe problems due to their toxicity and their resistance to biomineralization. Mainly microorganisms are responsible for the complete mineralization of aromatic compounds to $CO_2$.

Aerobic bacteria metabolizing aromatic compounds make use of the cosubstrate dioxygen for most key processes. For example, oxygenases catalyse exergonic hydroxylations of the benzene ring or the oxygenolytic cleavage of the ring. Hence, anaerobic bacteria have to make use of different strategies to attack the aromatic ring. So far, the aromatic metabolism has mainly been studied in facultatively anaerobic bacteria; most information about the key enzymes derives from studies with denitrifying species of the genera *Thauera* and *Azoarcus* and from studies with the phototrophic *Rhodopseudomonas palustris* (for reviews see Schink *et al*., 2000; Boll *et al*., 2002; Gibson and Harwood, 2002).

In anaerobic bacteria, benzoyl-CoA is a key intermediate into which most aromatic compounds are converted via different channelling reactions. In facultative anaerobes the anaerobic metabolism of the model compound benzoate can be divided into four different reaction steps (I–IV, Fig. 1). (i) Activation to benzoyl-CoA catalysed by benzoate-CoA ligase. This enzyme couples benzoyl-CoA formation to a stoichiometric hydrolysis of ATP to AMP and diphosphate (Egland *et al*., 1995; Schühle *et al*., 2003). (ii) Benzene ring reduction. The key enzyme benzoyl-CoA reductase catalyses the reductive dearomatization of benzoyl-CoA to a cyclic, conjugated diene (Boll and Fuchs, 1995; Boll *et al*., 2000). The enzyme contains three [4Fe-4S] clusters and couples the transfer of electrons to the benzene ring to a stoichiometric ATP hydrolysis (1 ATP/e-transferred; Unciuleac and Boll, 2001). (iii) Modified β-oxidation of the dearomatized diene resulting

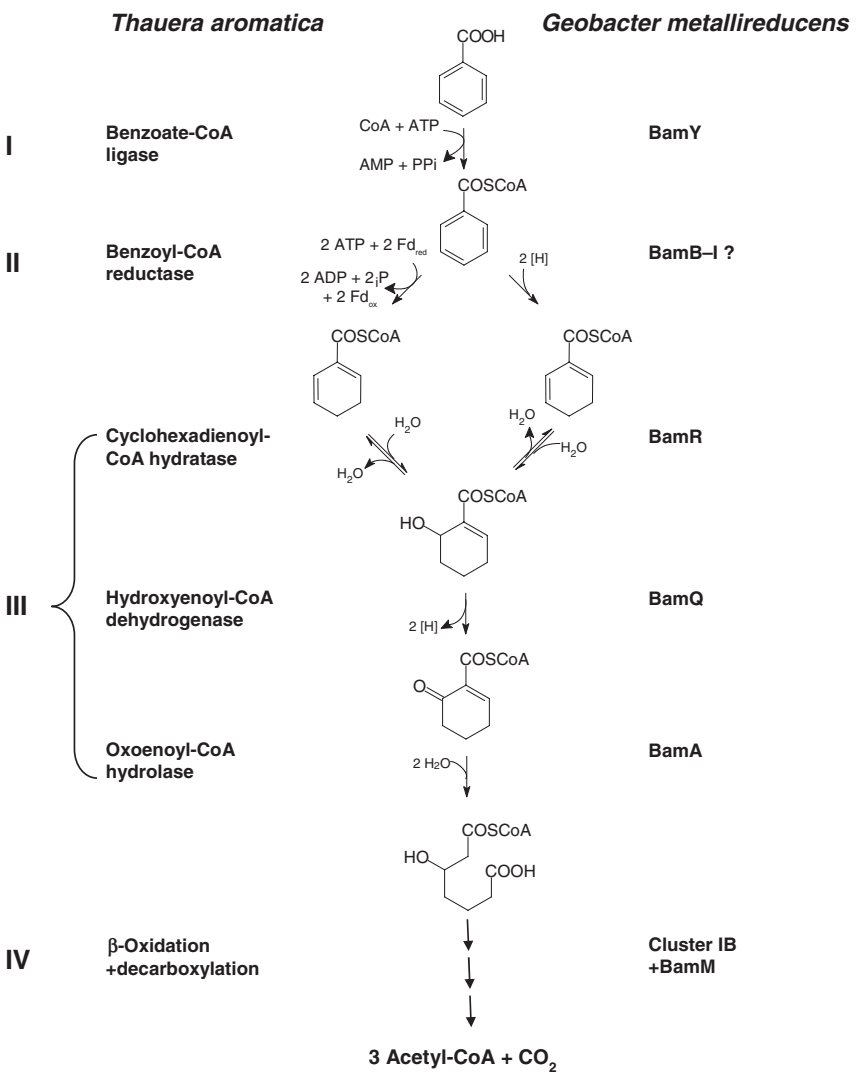


**Fig. 1.** Reactions involved in anaerobic benzoate metabolism of *Thauera aromatica* and those proposed in *Geobacter metallireducens*. The benzoate-induced genes/gene products of *G. metallireducens* were identified in this work; BamY was heterologously expressed and characterized. The product of benzoyl-CoA reduction is not known in *G. metallireducens*; however, the similarities of gene products involved in the following reactions (BamR, BamQ and BamA) to the corresponding enzymes in *T. aromatica* suggest the presented pathway. Heterologously expressed BamR catalysed the reaction as indicated (S. Wischgoll *et al.*, unpublished results). The following reaction steps can be summarized:
Step I. Activation of benzoate to benzoyl-CoA by benzoate CoA ligase.
Step II. Reductive dearomatization of benzoyl-CoA.
Step III. Modified β-oxidation resulting in ring cleavage.
Step IV. β-Oxidation of the 3-hydroxypimely CoA via glutaryl-CoA to three acetyl-CoA and one $CO_2$. BamM shows similarities to glutaryl-CoA dehydrogenase catalysing the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA.

in ring cleavage. The dearomatized product is converted to an aliphatic $C_7$-dicarboxyl-CoA by specific hydratases, dehydrogenases and ring cleaving hydrolases (Laempe *et al.*, 1998; 1999; Pelletier and Harwood, 2000). (iv) β-oxidation of the aliphatic $C_7$-dicarboxylic acid. The aliphatic thiol ester is converted to three acetyl-CoA and one $CO_2$ in a series of β-oxidation reactions including one decarboxylation step. In denitrifying bacteria, acetyl-CoA is fully oxidized to $CO_2$ in the TCA cycle; reducing equivalents are transferred to nitrate in an anaerobic respiratory chain.

In recent years, a number of strictly anaerobic bacteria have been isolated, which use aromatic compounds as sole source of cell carbon and electron donor for energy conserving reactions. They comprise organisms with anaerobic respiratory chains using Fe(III) or sulphate as terminal electron acceptors but also fermenting bacteria, often in syntrophic association with a methanogen (Lovley *et al.*, 1989; 2004; Schöcke and Schink, 1999; Elshahed and McInerney, 2001; Rabus *et al.*, 2001). So far, little is known about the aromatic metabolism in these organisms.

In studies of the sulphate reducing bacterium *Desulfococcus multivorans* (Deltaproteobacteria) initial evidence was obtained that key reactions of aromatic metabolism differ in strictly and facultatively anaerobic bacteria. Growth of *D. multivorans* on benzoate but not on non-aromatic substrates strictly depends on selenium and molybdenum (Widdel, 1980). Accordingly, $^{75}$Se-labelled benzoate-induced proteins were identified, including an unknown 30 kDa selenocysteine-containing protein (Peters *et al.*, 2004). Notably, no molybdenum and/or selenocysteine-containing enzyme is involved in benzoate metabolism of facultative anaerobes. From *D. multivorans*, a benzoate-CoA ligase was purified and characterized, which catalyses the initial step in benzoate metabolism (Peters *et al.*, 2004).

 

In this work we studied the aromatic metabolism of the obligately anaerobic, iron-reducing *Geobacter metallireducens*. This organism belongs to the family of *Geobacteraceae* (δ-group of proteobacteria) and is related to *D. multivorans*. *G. metallireducens* was isolated and described by Lovley and Phillips (1988) and Lovley *et al*. (1993); its genome has been sequenced (Accession number AAAS00000000). Besides their importance in the degradation of aromatic compounds, *G. metallireducens* and other members of the *Geobacteraceae* are renowned for their capacity to transfer electrons to insoluble metal oxides and even to electrodes; in the latter case electricity can be produced (for recent reviews see Lovley, 2003; Lovley *et al*., 2004). Notably, no homologues to genes coding for the four structural subunits of ATP-dependent benzoyl-CoA reductase are found in the genome of *G. metallireducens* suggesting the involvement of a different enzymology of benzene ring dearomatization.

The results obtained in this work suggest that benzoate activation and the modified β-oxidation reactions are highly similar in facultative anaerobes and the strictly anaerobic model organism *G. metallireducens* (Fig. 1). As an example, the initial enzyme of benzoate metabolism, benzoate-CoA ligase, was purified and briefly characterized. However, in *G. metallireducens* the reductive dearomatization process follows a yet unknown, most possibly ATP-independent, biochemical principle with selenocysteine and molybdenum containing proteins apparently playing a crucial role.

## Results and discussion

### *Heterologous overexpression, purification and characterization of BamY as benzoate-CoA ligase*

A BLAST search in the genome of *G. metallireducens* revealed a single open reading frame (gi 68004719) with deduced amino acid sequence identities higher than 50% to benzoate-CoA ligases from *Thauera aromatica* (Accession number AAN 32623), *Azoarcus evansii* (CAD 21640) and *Magnetospirillum magnetotacticum* (ZP 00055039). The putative gene of benzoate-CoA ligase was cloned and expressed in *Escherichia coli*. For easier purification a sixfold His-tag was fused at the C-terminus.

The deduced molecular mass of the of putative benzoate-CoA ligase is 58.5 kDa corresponding to 61.8 kDa including the His-tag. Heterologous expression of the corresponding gene yielded a highly expressed protein band in *E. coli* extracts (up to 50% of the soluble protein fraction) at approximately 60 kDa (Fig. 2). This protein was highly enriched from the soluble protein fraction of recombinant *E. coli* cell extracts by Ni-affinity chromatography. The gene product was eluted in a linear gradient between

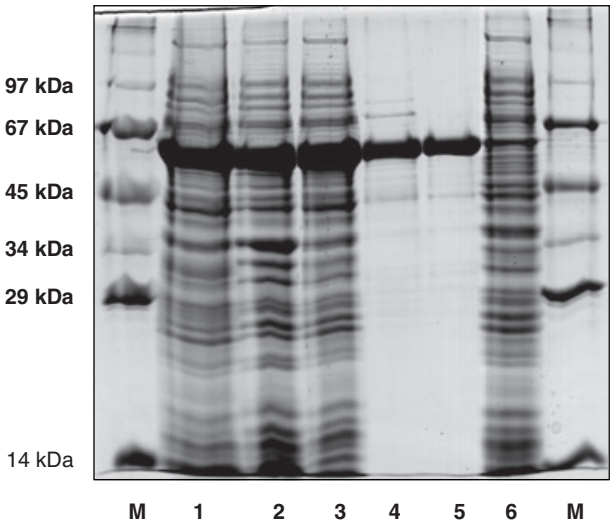


**Fig. 2.** SDS-PAGE analysis of protein fractions obtained during purification of heterologously expressed His-tagged benzoate-CoA ligase from *G. metallireducens*.
1. *E. coli* cell extract containing overexpressed benzoate-CoA ligase from *G. metallireducens* at approximately 60 kDa.
2. Supernatant after ultracentrifugation.
3. Resuspended pellet after ultracentrifugation.
4 and 5. Two different pools eluting between 80 and 200 mM imidazole from the Ni-affinity chromatography column.
6. Flowthrough from Ni-column.
M. Molecular-mass-standard proteins.

80 and 200 mM imidazole, whereas almost the total soluble protein of *E. coli* eluted with the equilibration buffer (Fig. 2). The native molecular mass of His-tagged was approximately 60 kDa as determined by gel filtration suggesting a monomeric architecture.

For activity determination of the overexpressed gene product, the typical coupled spectrophotometric assay for carboxylic acid coenzyme A ligases was used. The specific activity with benzoate was 16 μmol $min^{-1}$ $mg^{-1}$, which is higher than that reported for benzoate-CoA ligases from other obligate anaerobes (1.1–8.3 μmol $min^{-1}$ $mg^{-1}$, Auburger and Winter, 1992; Peters *et al*., 2004) and in the range of enzymes from facultative anaerobes (9–25 μmol $min^{-1}$ $mg^{-1}$, Geissler *et al*., 1988; Schühle *et al*., 2003; López Barragán *et al*., 2004).

Only some fluorinated benzoate analogues gave comparable rates; activities with other aromatic or alicyclic substrates were considerably lower. Notably glutarate, succinate and acetate were virtually not converted (Table 1). This narrow substrate preference has also been reported for other benzoate-CoA ligases, which next to benzoate only accept fluorinated and some alicyclic benzoate analogues (for exceptions see References in previous chapter). The apparent $K_m$-value for benzoate was 30 ± 3 μM, which is in the physiological range. The product formed from ATP was AMP + $PP_i$ as determined in the

 

**Table 1.** Substrate preference of heterologously expressed benzoate-CoA ligase of *G. metallireducens.*

| Substrate | Activity (%) |
|---|---|
| Benzoate | 100 |
| 2-Fluorobenzoate | 92 |
| 3-Fluorobenzoate | 23 |
| 4-Fluorobenzoate | 98 |
| 2-Aminobenzoate | 3 |
| 3-Aminobenzoate | < 1 |
| 4-Aminobenzoate | < 1 |
| 2-Hydroxybenzoate | < 1 |
| 3-Hydroxybenzoate | < 1 |
| 4-Hydroxybenzoate | < 1 |
| Cyclohexanecarboxylate | 7 |
| Cyclohex-1-enecarboxylate | 13 |
| Cyclohex-3-enecarboxylate | 40 |
| Cinnamate | < 1 |
| Phenylacetate | < 1 |
| Indolacetate | < 1 |
| Glutarate | < 1 |
| Succinate | < 1 |
| Acetate | < 1 |

All substrates were added at 2 mM concentrations. 100% refer to 16 μmol $min^{-1}$ $mg^{-1}$.

spectrophotometric assay (see *Experimental procedures*); this property is a common feature of all benzoate-CoA ligases tested so far (see References in previous chapter). The substrates listed in Table 1 were additionally investigated for inhibitory effects on the overexpressed benzoate-CoA ligase. With 2-hydroxybenzoate a strong inhibition of activity was observed. Dixon blot analysis revealed that the inhibition was competitive with $K_i = 16$ μM (not shown).

### *Benzoyl-CoA reductase activities in cell extracts*

In the genome of *G. metallireducens* no open reading frames with clear similarities to the four structural genes of benzoyl-CoA reductases from facultative anaerobes are present. To further test the presence of a benzoyl-CoA reductase activity in extracts from cells grown on benzoate, the established anaerobic radioactive assay for benzoyl-CoA reductase was used (Koch and Fuchs, 1992; Boll and Fuchs, 1995). Briefly, it follows the ATP- and electron donor-dependent reduction of [*ring*-$^{14}$C]-benzoyl-CoA to a cyclic dienoyl-CoA compound, which becomes further metabolized in following reactions (Fig. 1). Analysis of the products formed was by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). Various cell fractions before and after removal of low molecular compounds by dialysis were tested (see *Experimental procedures*). As electron donors dithionite, Ti(III)-citrate, NAD(P)H, reduced methyl viologen, formate, hydrogen gas (saturated gas phase in a 5 ml tube containing 350 μl of assay mixture) and 2-oxoglutarate (by means of an intrinsic 2-oxoglutarate ferredoxin oxidoreductase activity; S. Wischgoll *et al*., unpublished results) were used. However, under no circumstances a conversion of benzoyl-CoA was observed. In a control experiment, in which extract from *G. metallireducens* was replaced by extracts from the denitrifyers *T. aromatica* or *A. evansii* grown on benzoate, the typical pattern of product formation was observed (Boll and Fuchs, 1995; López Barragán *et al*., 2004; not shown). These results suggest that in comparison to facultative anaerobes benzoyl-CoA dearomatization is accomplished in a differing mode in the strictly anaerobic model organism *G. metallireducens*.

### *Effect of selenium and molybdenum on growth of* G. metallireducens *on benzoate*

In facultatively anaerobic bacteria, the metabolism of benzoate involves Fe-S proteins (e.g. benzoyl-CoA reductase or ferredoxin); no other metal cofactors are required. In contrast, benzoate metabolism in the obligate anaerobe *D. multivorans* strictly depends on the presence of selenium and molybdenum (Peters *et al*., 2004). We therefore studied the role of these trace elements also in benzoate metabolism of the phylogenetically related obligate anaerobe *G. metallireducens*.

*Geobacter metallireducens* was grown in a mineral salt medium containing molybdate (150 nM) and selenite (20 nM) with benzoate (3 mM) and Fe(III)-citrate (60 mM) as sole sources of cell carbon and energy. When selenium was omitted from the mineral medium, no growth on benzoate was observed (Fig. 3). In the presence of Se and absence of Mo, a slight but reproducible decrease in growth rate was observed. Growth rate in these cultures could be increased to the initial value upon addition of molybdate or tungstate (150 nM each; not shown). The results indicate that growth on benzoate depended on the presence of Se. Notably, the effect of Mo was probably obscured due to Mo-impurities in the electron acceptor Fe(III)citrate (60 mM). Due to the high amount of Fe vs. Mo (> $10^6$-fold), the Mo-content in the Fe(III)-citrate used could not be determined accurately by inductively coupled mass spectrometry analysis. In control experiments with acetate (30 mM) as growth substrate, omission of Se and Mo had no effect on growth (Fig. 3). The optimal concentration of selenite was determined. Half maximal density within 72 h was reached between 1 and 10 nM selenite, whereas concentrations higher than 1 μM inhibited growth (data not shown).

### *Identification of gene products involved in benzoate metabolism*

The data obtained so far suggest that key processes in the anaerobic aromatic metabolism involve different

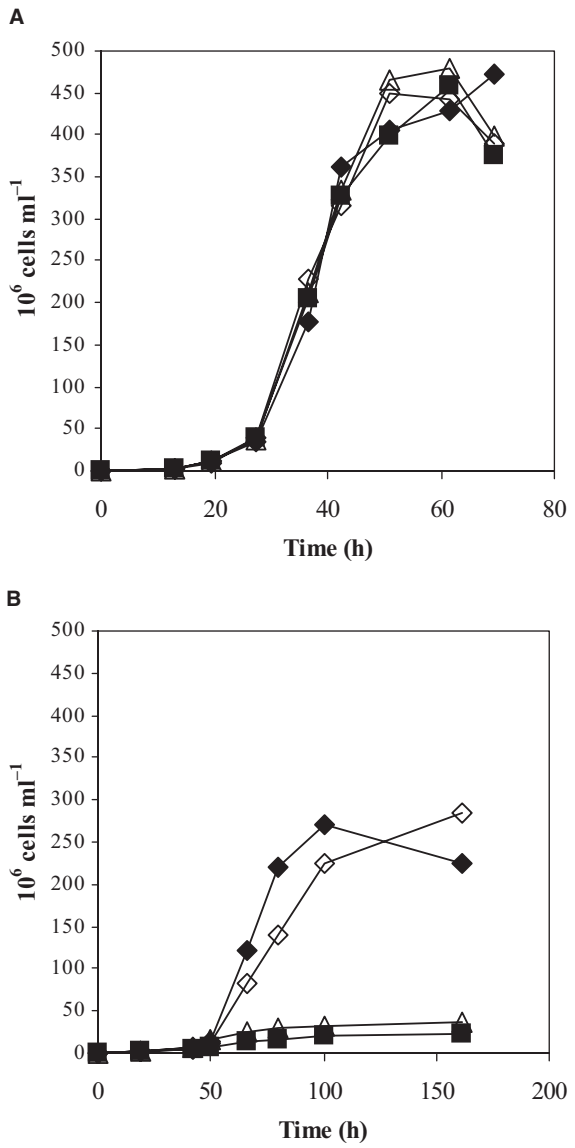


**Fig. 3.** Effects of Mo (150 nM) and Se (20 nM) on growth of *G. metallireducens* with different energy and carbon sources. Growth on acetate (30 mM) (A), and benzoate (B) (3 mM). The media contained Mo (150 nM) and Se (20 nM) (◆), Mo but no Se (△), Se but no Mo (◇), and neither Se nor Mo (■).

enzyme reactions in obligately and facultatively anaerobic bacteria. The sequenced genome of *G. metallireducens* enabled a proteomic approach to identify unknown proteins specifically involved in benzoate metabolism. For this purpose, extracts from cells grown on benzoate and butyrate, respectively, were separated by two-dimensional (2-D) gel electrophoresis analysis and the protein patterns were compared. For optimal separation different polyacrylamide gradients were used.

In Fig. 4, 14 protein spots are marked, which are at least fivefold more intense on 2-D gels of extracts from cells grown on benzoate compared with those grown on butyrate (visual estimation) and which could be identified by mass spectrometric analysis. The benzoate-induced spots were digested by trypsin and analysed by peptide mass fingerprinting (PMFP) using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. The results enabled the identification of benzoate-induced genes in the genome of *G. metallireducens* (Table 2, Fig. 5); in addition, they enabled an unambiguous assignment of many gene products to specific functions in benzoate metabolism. To obtain a complete picture, an additional liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis step was carried out, which enabled the identification of further benzoate-induced proteins. However, database algorithms in tandem mass spectrometry approach implicitly assume that the genome is accurately sequenced, and all protein coding genes are annotated. Therefore, an additional *de novo* peptide sequencing step was carried out, which consist of the reconstruction of a peptide sequence using a mass spectrum (Taylor and Johnson, 2001; Ma *et al.*, 2003).

The identified genes are organized in two clusters, termed cluster IA/B and II (for detailed description see below). Both clusters comprise 44 open reading frames; most of the deduced open reading frames of the individual clusters have the same orientation suggesting that they may form transcriptional units (Fig. 5). Cluster I is divided into IA and IB by a gene for a transposase (*tnpII*) in opposite orientation, and cluster IA is flanked by an additional transposase gene (*tnpI*).

To further test whether the genes of cluster IA/B and II were induced in benzoate-grown cells, reverse transcription polymerase chain reaction (RT-PCR) experiments were carried out. For this purpose total RNA was isolated from cells grown on 4-hydroxybenzoate, benzoate, butyrate and acetate respectively. After copying the total RNA of the individual cells into cDNA by reverse transcriptase, a set of 37 DNA oligonucleotide pairs were constructed as primers for PCR reactions for the analysis of gene expression (Fig. 5, for the detailed sequences of the primers used see Table S1). Two representative results of such experiments are shown in Fig. 6, where the induction of *bamB* (coding for an aldehyde : ferredoxin oxidoreductase-like protein, gi68004668) and *bamY*, coding for benzoate-CoA ligase (gi68004719), was investigated. With cDNAs from cells grown on aromatic growth substrates, DNA fragments of the expected sizes were amplified, whereas no DNA amplification was observed with cDNA from cells grown on acetate or butyrate. This result indicates that transcription of both genes is induced in cells grown on benzoate or 4-hydroxybenzoate but not in cells grown on butyrate or acetate. Similar results were obtained with all primer pairs amplifying internal DNA

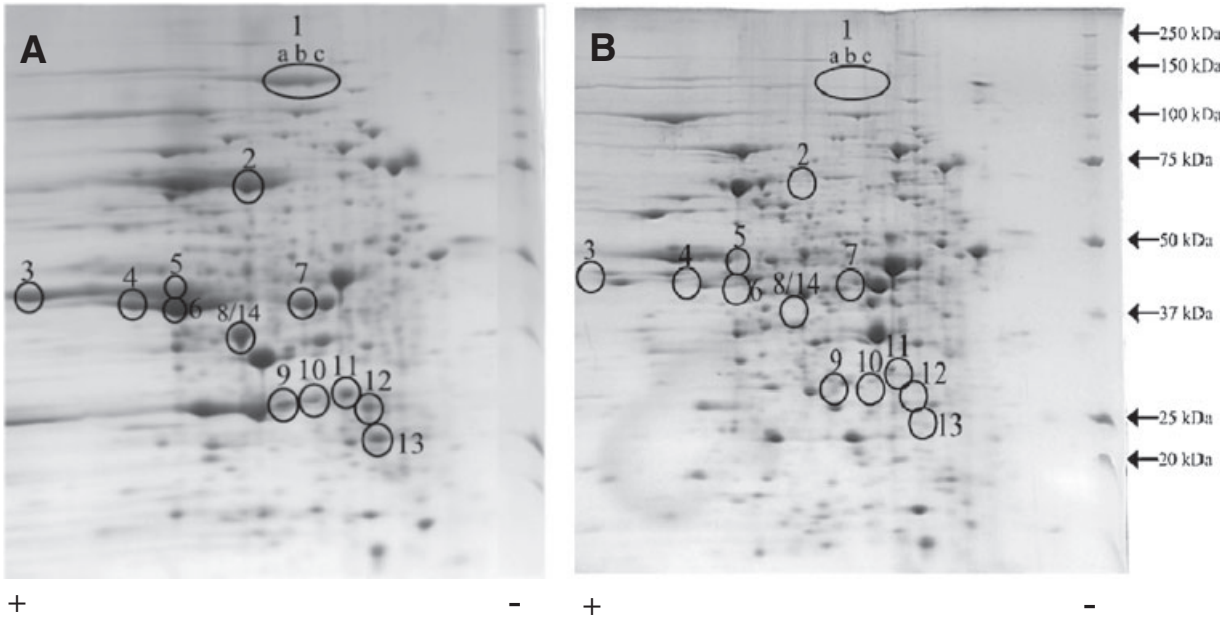


**Fig. 4.** Representative 2-D gel electrophoresis (IEF/SDS-PAGE) of extracts from *G. metallireducens*. Extracts from cells grown on benzoate (A) or butyrate (B). The pH gradient applied was from pH 3 (+, left side of gel) to pH 10; the polyacrylamide gradient was from 8% to 14%. The numbered protein spots were analysed by MALDI-TOF MS and LC-MS/MS and the corresponding genes were identified. The numbers refer to those in Table 2. Only benzoate-induced spots, which gave a clear result in MS analysis, are marked.

fragments of genes from clusters IA/B and II (primer pairs 3–29, 33–41). Notably, also primer pairs 12–14 gave positive results suggesting that *tnp*II, although orientated in opposite direction, is coexpressed with other genes of cluster I, which is typical for an accidentally inserted transposon. No amplificates were obtained with primer pairs 20 and 23 for DNA fragments between two open reading frames in opposite direction or with primer pairs outside clusters IA/B and II (primer pairs 1, 2, 30–32 and 42; Fig. 5). As a result cluster I is flanked by *tnpI* and *orf1/2* (coding for putative tRNA-isopentenyl transferase and a DNA-mismatch repair protein), and cluster II is flanked by *orf3/4* (coding for two hypothetical proteins, respectively) and by *orf5* (coding for a putative N-acetyltransferase). In control PCR experiments in which genomic DNA instead of cDNA were used, positive results were obtained in all cases. In further control experiments, a primer pair for the amplification of an internal DNA fragment of the gene coding for the β-subunit of RNA polymerase was tested. The results indicated that a gene of this house-keeping enzyme was transcribed in all cells, independent of the carbon source utilized (Fig. 6A).

### *Possible role of gene products in benzoate metabolism*

The assumed functions of the predicted gene products are described below (Table 2).

*Cluster IA and II.* The genes of cluster IA and II are suggested to code for enzymes involved in benzoate activation (reaction I in Fig. 1), benzene ring dearomatization (reaction II) and modified β-oxidation reactions, which convert the dearomatized cyclic product to an aliphatic thiol ester of a $C_7$-dicarboxylic acid (reaction III). For this reason the genes are termed *bamA–bamY* (benzoic acid metabolism). The former annotation in the genome is summarized in Table 2.

*BamY.* The gene product of *bamY* was heterologously overexpressed and its function as structural gene for benzoate-CoA ligase was demonstrated in this work (Fig. 1). Although the gene is located in the opposite DNA strand, transcription of *bamY* was clearly induced in cells grown on aromatic compounds (Fig. 6).

*BamA, BamQ and BamR.* The gene products of *bamA, bamQ* and *bamR* were all identified as benzoate-induced proteins on 2-D gels. They are annotated in the genome as two enoyl-CoA hydratases/isomerases and an alcohol dehydrogenase respectively. However, a BLAST search revealed exceptionally high similarities of these gene products with enzymes involved in β-oxidation reactions of benzoyl-CoA metabolism in *T. aromatica* and *M. magnetotacticum* (68–77% sequence identity). This finding allowed the prediction of the following functions: cyclohexa-1,5-diene-1-carbonyl-CoA hydratase (*bamR*), 6-hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase (*bamQ*), and the ring opening 6-oxocyclohex-

**Table 2.** Gene clusters involved in benzoate metabolism of *G. metallireducens.*

| gi | Gene | Annotation | m (kDa) | Spot-Nr. | Coverage (%) | Error p.p.m. |
|---|---|---|---|---|---|---|
| Cluster IA | | | | | | |
| **68004669** | ***bamA*** | **Enoyl-CoA hydratase/isomerase** | **41.9** | **6** | **51** | **22** |
| **68004668** | ***bamB*** | **Aldehyde : ferredoxin oxidoreductase** | **73.8** | **2** | **41** | **29** |
| **68004667** | ***bamC*** | **Fe-S-cluster-containing hydrogenase small subunit** | **20.6** | **13** | 36 | **12** |
| 68004666 | *bamD* | **Heterodisulfide reductase, fusion of subunits BC** | 43.2 | | | |
| **68004665** | ***bamE*** | **Heterodisulfide reductase, subunit A and** | **111.5** | **1a** | **38** | **15** |
| | | **related polyferredoxins** | | **1b** | **35** | **14** |
| | | | | **1c** | **36** | **17** |
| 68004664 | *bamF* | Coenzyme $F_{420}$ non-reducing hydrogenase, d subunit[a] | 28.4[a] | | | |
| 68004663 | *bamG* | NADH : ubiquinone oxidoreductase 24 kDa subunit | 16.5 | | | |
| 68004662 | *bamH* | NADH : ubiquinone oxidoreductase, NADH-binding 51 kDa subunit | 67.6 | | | |
| **68004661** | ***bamI*** | **Uncharacterized anaerobic dehydrogenase** | **24.7** | **12** | **16** | **183** |
| 68004660 | *bamJ* | Aspartate/tyrosine/aromatic aminotransferase | 47.3 | | | |
| 68004659 | *bamK* | N-Acetyltransferases | 16.6 | | | |
| 68004658 | *bamI* | Formate dehydrogenase, subunit FdhD | 28.4 | | | |
| **68004657** | *bamM* | **Glutaryl-CoA dehydrogenase** | **42.0** | **5** | **49** | **53** |
| 68004656 | *bamN* | Acetyl-CoA acetyltransferase | 41.1 | | | |
| Cluster IB | | | | | | |
| 68004654 | *acd* | 3-Hydroxyacyl-CoA dehydrogenase | *28.9* | | | |
| **68004653** | ***ech*** | **Enoyl-CoA hydratase/isomerase** | **27.4** | **9** | **55** | **9** |
| 68004652 | *oxr* | Fe-S oxidoreductase | 73.2 | | | |
| **68004651** | ***scsB*** | **Succinyl-CoA synthetase, β subunit** | **41.9** | **7** | **35** | **168** |
| **68004650** | ***scsA*** | **Succinyl-CoA synthetase, α subunit** | **30.7** | **8** | **30** | **144** |
| **68004649** | ***etfB*** | **Electron transfer flavoprotein, β subunit** | **29.3** | **14** | **56** | **204** |
| 68004648 | *etfA* | Electron transfer flavoprotein, α subunit | 48.6 | | | |
| 68004647 | *oxr* | Fe-S oxidoreductase | 73.1 | | | |
| 68004646 | *tre* | Transcriptional regulator, IclR | *27.4* | | | |
| 68004644 | *the* | Predicted thioesterase | *36.2* | | | |
| 68004645 | *rsbU* | Serine phosphatase RsbU, regulator of σ subunit | 39.8 | | | |
| 68004643 | *ssy* | Na+/proline symporter | 73.9 | | | |
| 68004642 | *pmp* | Predicted membrane protein | 68.2 | | | |
| 68004641 | *adh* | Short-chain alcohol dehydrogenases | 26.3 | | | |
| **68004640** | ***act*** | **Thiolase** | **42.5** | **3** | **41** | **208** |
| 68004639 | *ech* | Enoyl-CoA hydratase/isomerase | 28.5 | | | |
| 68004638 | *hyd* | Predicted metal-dependent hydrolase | 31.0 | | | |
| 68004637 | *tre* | Transcriptional regulator | 65.4 | | | |
| 68004636 | *act* | Acetyl-CoA hydrolase/transferase | 48.7 | | | |
| Cluster II | | | | | | |
| 68004729 | *bamO* | Electron transfer flavoprotein, β subunit | 28.1 | | | |
| **68004728** | ***bamP*** | **Electron transfer flavoprotein, α subunit** | **31.0** | **11** | **25** | **21** |
| **68004727** | ***bamQ*** | **Zn-dependent alcohol dehydrogenases** | **36.7** | **4** | **25** | **153** |
| **68004726** | ***bamR*** | **Enoyl-CoA hydratase/carnithine racemase** | **27.1** | **10** | **47** | **44** |
| 68004725 | *bamS* | Hypothetical protein | 30.0 | | | |
| 68004724 | *bamT* | Hypothetical protein | 47.3 | | | |
| 68004723 | *bamU* | Predicted metal-dependent hydrolase (TIM-barrel-fold) | 31.7 | | | |
| 68004722 | *bamV* | Signal transduction histidine kinase | 67.2 | | | |
| 68004721 | *bamW* | Response regulator containing CheY-like | 50.0 | | | |
| 68004720 | *bamX* | hypothetical protein | 45.4 | | | |
| 68004719 | *bamY* | Benzoate-CoA ligase | 58.5 | | | |

**a.** Selenocysteine containing, mass corrected for different translational start as previously assumed; see text.
The gene products identified by proteome analysis are shown in bold. The genes coding for transposase-like components are not included. The annotation is taken from the gene bank (NZ_AAAS00000000). The proteins were identified by the combination of peptide mass finger printing using MALDI-TOF MS and LC-MS/MS Searches were carried out with the MASCOT program (Matrix Science, London, UK) in the NCBI (National Center for Biotechnology Information) non-redundant protein database. m = molecular mass.

1-ene-1-carbonyl-CoA hydrolase (*bamA*) (Fig. 1). In *T. aromatica* the three enzymes catalyse the reaction sequence from the dearomatized dienoyl-CoA to the aliphatic thiol ester (reaction III, Fig. 1). The sequence identities to the corresponding enzymes involved in benzoate metabolism of *Azoarcus* strain CIB, *A. evansii* (López Barragán *et al*., 2004) and *A.* EbN1 were 34–52% (Rabus *et al*., 2005). Similarities to enzymes involved in β-oxidation reactions of aliphatic fatty acids are significantly lower (< 27% sequence identity). No homologues of *bamA*, *bamR* and *bamQ* with sequence identities higher than 25% are present in the genome of

 

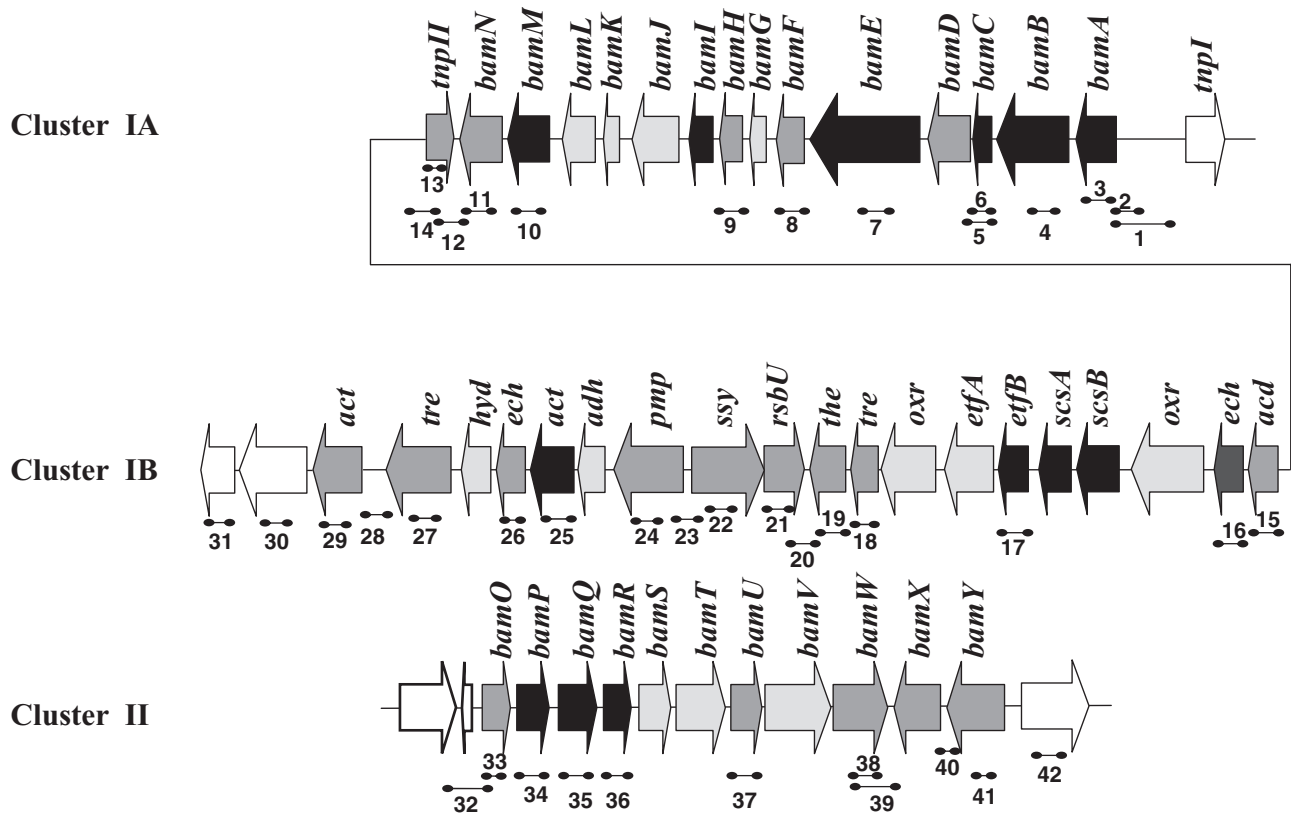


**Fig. 5.** Gene clusters of the genome of *G. metallireducens* containing benzoate-induced open reading frames. The abbreviations of genes and their annotations in the gene bank are listed in Table 2. Black arrows indicate genes coding for proteins which have been identified by mass spectroscopy on 2-D gels of extracts from cells grown on benzoate but not on 2-D gels of cells grown on butyrate; dark grey arrows indicate genes which are benzoate-induced as determined in RT-PCR experiments; light grey arrows are assigned to clusters I/II but expression has not been tested; white arrows indicate genes which were not expressed during growth on benzoate as determined in PCR experiments. The numbered short lines below the genes symbolize DNA fragments that were checked for amplification in RT-PCR experiments (for sequences of the primers used see supplementary information).

the closely related *Geobacter sulfurreducens* (Accession number NC 002939), which is unable to utilize aromatic compounds.

The *bamA*, *bamQ* and *bamR* gene products are less similar to the corresponding enzymes involved in benzoate metabolism in the phototrophic bacterium *R. palustris* (sequence identities < 28% for all three enzymes). This can be explained by the different mode of benzoyl-CoA metabolism in *R. palustris*. In this species benzoyl-CoA is reduced twice by two electrons to a cyclic monoenoyl-CoA (Gibson and Gibson, 1992). As a consequence, the following β-oxidation steps resulting in ring cleavage require enzymes with a different specificity compared with those acting on the two-electron reduced dienoyl-CoA product (Pelletier and Harwood, 2000). These features corroborate the conclusion that in *G. metallireducens* benzoyl-CoA appears to be reductively dearomatized, most possibly in a two-electron reduction to a dienoyl-CoA compound. Normally, the three genes coding for the three enzymes involved in reactions sequence 3 (Fig. 1) are usually found adjacent to each other in benzoate degradation operons in the facultative anaerobes *T. aromatica*, *M. magnetotacticum* and *Azoarcus* species. The separation of *bamA* from *bamR* and *bamQ* suggests that a transposition event may have separated them into two clusters. This assumption is supported by the presence of genes coding for transposases adjacent to *bamA* and *bamN* (Fig. 5).

*BamB*. The gene product of *bamB* was identified as a benzoate- and 4-hydroxybenzoate-induced 75 kDa protein by mass spectrometry; the induction of the gene was evident from reverse transcriptase PCR analysis (Fig. 6). BamB shows clear amino acid sequence similarities to tungsten- or molybdenum-containing aldehyde : ferredoxin oxidoreductases (AOR) of hyperthermophilic archaea or obligately anaerobic bacteria. The effect of molybdenum on growth of *G. metallireducens* on benzoate (see above) suggests that functional BamB contains molybdenum; however, it cannot be excluded that tungsten replaces molybdenum in native BamB.

 

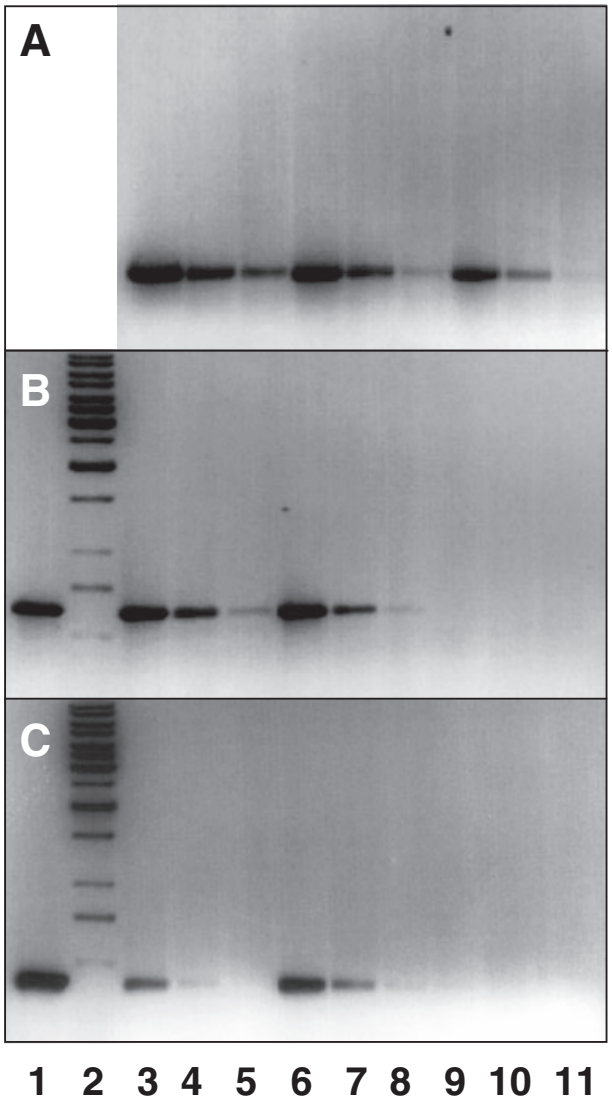


**Fig. 6.** Representative reverse transcriptase PCR studies of genes involved in benzoate metabolism. Ethidium bromide/agarose gel of PCR products formed with different primers using cDNA obtained from mRNA of *G. metallireducens* cells as template. Induction of (A) RNA-polymerase β-subunit (gi45593175), (B) *bamB* (gi68004668) coding for a putative aldehyde : ferredoxin oxidoreductase and (C) *bamY* (gi68004719) coding for benzoate-CoA ligase. The cDNA was produced from mRNA isolated from *G. metallireducens* cells grown on benzoate (lanes 3–5), 4-hydroxybenzoate (lanes 6–8), or acetate (lanes 9–11). In lane 1 genomic DNA of *G. metallireducens* was used as positive control. The cDNA templates used in these reactions were undiluted (lanes 3, 6, 9), diluted 10-fold (lanes 4, 7, 10) and 100-fold (lanes 5, 8, 11). Lane 2 shows a 1 kb ladder.

AOR-type enzymes usually contain a *bis*molybdo(tungsto)pterin mononucleotide cofactor and a [4Fe-4S] cluster (Chan *et al*., 1995; Roy *et al*., 2001). They consist of a single polypeptide with three domains and usually catalyse the oxidation of aldehydes to carboxylic acids by water using an oxidized ferredoxin as electron acceptor. BamB contains the conserved motifs involved in molybdenum/tungsten binding and [4Fe-4S] cluster coordination.

It has been proposed that during benzoyl-CoA reduction a one-electron reduced ketyl radical anion intermediate is transiently formed at the thiol ester functionality (Buckel and Keese, 1995). Such a species can be delocalized over the entire benzene ring, which facilitates its formation. It is interesting to note that thiol esters and aldehydes/ketones have similar chemical properties; thus an AOR-type enzyme is an attractive candidate for an alternative benzoyl-CoA reducing enzyme.

*BamC–I.* The putative gene products of *bamC–I* show sequence similarities to subunits of soluble heterodisulfide reductases, hydrogenases and soluble components of NADH : quinone oxidoreductases with at least 24 Fe/S-cluster-binding motives and two flavin binding sites. They are suggested to be involved in electron transfer to the aromatic ring and may replace the ATP-dependent electron transfer machinery of benzoyl-CoA reductases of facultative anaerobes.

*BamD and BamE.* The gene product of *bamE* (110 kDa) was identified as a benzoate-induced protein and showed high amino acid sequence similarity to the large subunit of FAD and [4Fe-4S] clusters containing soluble heterodisulfide reductases (encoded by *hdrA*, usually 85 kDa). The product of *bamD* is similar to the two other subunits of soluble heterodisulfide reductases (HdrBC), which appear to be joint together in a fusion protein (Hedderich *et al*., 1994).

*BamC* and *BamF.* The gene products of *bamC* and *bamF* are highly similar to individual subunits of hydrogenases. In particular, BamC is assigned to the small subunit of [NiFe]-hydrogenases carrying three Fe/S clusters. The N-terminal region of BamF shows high similarities to the δ-subunit of $F_{420}$ non-reducing hydrogenases (VhuD) and to the C-terminal part of the A-subunit of soluble heterodisulfide reductases found in methanogens and other anaerobes (HdrA, Table 2).

A careful analysis of the *bamF* gene revealed that no appropriate Shine-Dalgarno sequence is present at the expected position. Instead, an alternative start of translation with a much better Shine-Dalgarno sequence ($^{-13}$AAGGAG$^{-8}$) was found 129 bp upstream of the previously assigned one. The resulting reading frame would include a stop codon at position 54. However, the sequence conservation of the additional N-terminal 43 amino acids of BamF to the N-terminal region of VhuD-like proteins suggests that this TGA codon codes for a selenocysteine (Fig. 7). Notably, a cysteine or selenocysteine (see below) is found at this position in all VhuD-like proteins with sequence similarities to BamF (Fig. 7).

VhuD-like proteins have molecular masses of approximately 17 kDa (Sorgenfrei *et al*., 1997). The deduced mass of BamF is 28.4 kDa due to a long C-terminal exten-


**Fig. 7.** Alignment of the deduced N-terminal amino acid sequence of the *bamF* gene product with amino acid sequences from other proteins. Identical amino acids are marked in grey. Arrow 2 points to the start of translation as originally indicated in the genome; arrow 1 points to selenocysteine (U) encoded by the TGA stop codon. *Gmet*, *G. metallireducens*; VhuD, $F_{420}$ non-reducing hydrogenase d-subunit from *Methanococcus jannaschii* (*Mja,* Accession number NP_248185) and from *Methanococcus maripaludis* (*Mmar*, NP_988816). HydD, $F_{420}$ non-reducing hydrogenase d-subunits of *Methanococcus kandlerii*, (NP_613555); *Moorella thermoaceticum*, *Moth* (ZP_00329995). HdrA, C-terminal part of heterodisulfide reductase A subunit from *Methanosarcina mazei*, *Mmaz* (NP_632080) and *Archeoglobus fulgidus*, *Aful* (NP_070066).

sion showing some similarities to regulatory proteins. Very recently a benzoate-induced, selenocysteine-containing protein with a similar mass was identified in *D. multivorans* (Peters *et al*., 2004). This finding suggests that the expression of the 30 kDa selenocysteine containing proteins in *G. metallireducens* and in *D. multivorans* explains the selenium dependence during growth on aromatic compounds. Notably, no gene for such a selenocysteine-containing protein is present in the genome of *G. sulphurreducens*, which cannot utilize aromatic compounds.

So far, two VhuD-like proteins from methanogens are known to contain selenocysteine (Wilting *et al*., 1997; Rother *et al*., 2003; Fig. 7). VhuD is suggested to play a role in tight complex formation between soluble hydrogenase and heterodisulfide reductase (HdrABC) components (Stojanowic *et al*., 2003). Note, that in benzoate-induced cluster I of *G. metallireducens* no large [NiFe]-centre containing subunit of hydrogenases is present, excluding a role of *bamF* in $H_2$ reduction/evolution. Furthermore, no methyl viologen or $NAD^+$ reducing hydrogenase activity could be determined in extracts of *G. metallireducens* cells grown on benzoate (S. Wischgoll *et al*., unpublished results), and *G. metallireducens* cannot grow autotrophically with $H_2$ as electron donor (Lovley *et al*., 1993). Thus, the four hydrogenase- and heterodisulfide-like components (BamC-F) rather form a complex, thereby probably serving as electron transferring machinery involved in benzene ring reduction.

*BamG–I*. The gene products of *bamG–I* are similar to the soluble components of NADH:quinone oxidoreductases (Friedrich and Böttcher, 2004) and to the diaphorase module of a soluble hydrogenase of *Ralstonia eutropha* (Tran-Betcke *et al*., 1990). BamG–I might be involved in complex formation with the BamC-F components; their presence suggests that NAD(P)(H) may play a role as electron donor in enzymatic dearomatization and that the NADH:ubiquinone oxidoreductase-like products of *bamG–I* may interact with as yet unknown membrane-bound components.

*Cluster IB.* Most products of cluster IB are considered to be involved in β-oxidation reactions of the proposed aliphatic $C_7$-dicarboxylic acid intermediate (obtained after ring cleavage) to acetyl-CoA units (reaction sequence IV in Fig. 1). As they are not induced during growth on butyrate an alternative set of enzymes appears to be involved in β-oxidation of the $C_4$-carboxylic acid. As *G. metallireducens* is not growing on dicarboxylic acids such as pimelate ($C_7$), adipate ($C_6$) or glutarate ($C_5$, 3 mM each; Lovley *et al*., 1993; S. Wischgoll *et al*., unpublished results) it can be excluded that genes of cluster IB play a role in metabolism of medium chain dicarboxylic acids. The gene products include the following putative proteins: acyl-CoA/3-hydroxyacyl-CoA dehydrogenases (*acd*), enoyl-CoA hydratase (*ech*), thiolase (*act*), electron-transferring flavoprotein (Etf; α- and β-subunits, encoded by *etfAB*) and two membrane-bound Fe/S oxidoreductases (encoded by *oxr*, see below). Further benzoate-induced genes were annotated as α- and β-subunits of succinyl-CoA synthetases (*scsAB*), a thioesterase (*the*), a sodium-dependent symporter (*ssy*) and regulatory proteins (*tre* and *rsbU*).

*Oxr gene products.* Topology analysis and careful BLAST search analysis indicated high similarities of the putative

 

Fe/S oxidoreductases deduced from the *oxr* genes to subunits of membrane-bound heterodisulfide reductases from archaea consisting of two subunits (HdrDE; Deppenmeier, 2004). In *G. metallireducens*, both HdrDE-like subunits are fused to a single polypeptide of approximately 73 kDa.

The involvement of HdrDE-like membrane proteins in β-oxidation reactions has not been reported so far. During β-oxidation, Etf accept electrons from acyl-CoA dehydrogenases, the latter introducing a double bond into thiol esters of saturated fatty acids. Etf is usually reoxidized by membrane bound Fe/S/flavoprotein Etf : quinone oxidoreductases (Frerman, 1987). However, genes coding for the latter type of enzyme are absolutely absent in the genome of *G. metallireducens.* It appears that conventional Etf : quinone oxidoreductases are replaced by HdrDE fusion proteins, whose genes are always accompanied by genes coding for EtfA/B subunits.

### *Concluding remarks*

The results obtained in this work suggest that the major difference in benzoate metabolism of facultatively and strictly anaerobic bacteria is how the key process of benzoate metabolism, the reductive dearomatization of benzoyl-CoA, is achieved.

Due to energetic restraints some strictly anaerobic bacteria such as sulphate reducing or fermenting bacteria can hardly afford a stoichiometric ATP-dependent benzene ring reduction (Boll and Fuchs, 1995; Rabus *et al*., 2001; Peters *et al*., 2004). Therefore, an intriguing question remains whether electron transfer to the aromatic ring can be accomplished without a stoichiometric coupling to ATP hydrolysis in these bacteria. Notably, except benzoate-CoA ligase, no ATP-binding motif was found in any gene product of the benzoate-induced gene clusters in *G. metallireducens*.

Due to the high redox barrier for the first electron transfer to the aromatic ring (Boll *et al*., 2000), it is doubtful whether electron transfer to the aromatic ring can theoretically be accomplished without a coupling to an exergonic process. Alternatively to a stoichiometric ATP hydrolysis, electron transfer may be driven by a membrane potential; in such a process the energy input for driving benzoyl-CoA reduction could be reduced which might be essential for many strictly anaerobic bacteria. The benzoate-induced soluble modules of NADH : quinone oxidoreductase (BamG–I) suggest an association of ring reduction with membrane components.

## Experimental procedures

### *Growth of* G. metallireducens *and preparation of cell extracts*

*Geobacter metallireducens*, DSMZ-Nr. (7210) was cultured anaerobically in the so-called fresh water acetate medium (Lovley and Phillips, 1988). In our growth experiments the routinely added acetate (30 mM) was replaced by either benzoate, 4-hydroxybenzoate (3 mM each) or butyrate (10 mM); together with one of the carbon sources listed above, Fe(III)-citrate (60 mM) served as energy source. Larger cell amounts were obtained by culturing *G. metallireducens* in a 200 L fermenter. The growth of *G. metallireducens* was monitored by counting cells in a Neubauer counting chamber. Cells were harvested in the exponential growth phase by centrifugation (20 000 *g*) and were stored in liquid nitrogen. Trace elements were added from anaerobically prepared sodium selenite (20 μM) or sodium molybdate (150 μM) stock solutions. When the dependence on selenium and molybdenum was tested, both trace elements were omitted from the medium using bidistilled water (usually performed in 10 ml Hungate tubes). Before testing the dependence of growth on selenium and molybdenum, cells were transferred at least three times into fresh medium lacking the individual trace elements.

For the preparation of crude extracts, frozen cells were suspended in 20 mM triethanolamine hydrochloride/KOH buffer pH 7.8 (1 g cells in 1.5 ml buffer), 4 mM $MgCl_2$, 1 mM DTE, and 0.1 mg of DNAse I. Cell lysates were obtained by passage through a French Pressure Cell at 137 MPa. After centrifugation at 100 000 *g* (1 h, 4°C) the supernatant was used for further studies (see below).

### *Assay for benzoyl-CoA reductase*

Benzoyl-CoA reductase activity was tested in crude cell extracts using a radioactive assay as described (Koch and Fuchs, 1992; Boll and Fuchs, 1995); each step was performed under strictly anaerobic conditions (100% $N_2$). The test was carried out in gas-tight-sealed glass tubes at 30°C. The cell extracts used were prepared as described above under strictly anaerobic conditions. Tests were carried out with frozen and freshly harvested cells. Some extract preparations were centrifuged after French Press treatment at 6500 *g* instead of 100 000 *g*. The standard assay (0.35 ml) contained 100 mM morpholinopropanesulfonic acid/KOH buffer (pH 7.2); 10 mM $MgCl_2$, 0.5 mM CoA, 7 mM ATP, 200 μM [*ring*-$^{14}$C]-benzoate (4810 kBq μmol$^{-1}$; Amersham Biosciences) and 100 μl cell extract with a protein concentration of 25 mg ml$^{-1}$. The electron donors tested were dithionite (1 mM), Ti(III)-citrate (5 mM), dithionite reduced methyl viologen (1 mM), NADH (1 mM), NADPH (1 mM), Na-formate (10 mM) and $H_2$ (100% gas phase in a 5 ml tube). In a control experiment the assay was carried out with extracts from *T. aromatica* cells grown on benzoate (8 mg per assay); Ti(III)-citrate served as reductant. For the formation of benzoyl-CoA by benzoate-CoA ligase, the mixture was preincubated for 10 min at 30°C with 10 μl of a protein fraction containing benzoate-CoA ligase activity of *T. aromatica* (6 mg ml$^{-1}$, 0.3 μmol min$^{-1}$ mg$^{-1}$). The reaction was started by addition of crude cell extracts. Residual [$^{14}$C]benzoyl-CoA and derived $^{14}$C-labelled CoA ester products were hydrolysed under alkaline condition. Samples (50 μl) taken between 0.1 min and 2 h were added to 5 μl of 4 M KOH, and hydrolysed by heating at 80°C for 10 min. Analysis of the released radioactively labelled carboxylic acids (5 μl of each sample) was

carried out by TLC or HPLC as described (Koch and Fuchs, 1992; Boll and Fuchs, 1995).

### *Assay for benzoate-CoA ligase*

Benzoate CoA ligase activity was determined at 37°C using an indirect continuous spectrophotometric assay, as described previously (Ziegler *et al*., 1987). Briefly, the formation of AMP (i) was coupled enzymatically to myokinase (ii), pyruvate kinase (iii), and lactate dehydrogenase (iv) reactions, and the oxidation of 2 mol of NADH per mol of aromatic substrate was monitored spectrophotometrically at $\lambda$= 365 nm ($\varepsilon_{365} = 3.4 \times 10^3$ $M^{-1}$ $cm^{-1}$). The following reactions are part of the coupled assay: (i) carboxylic acid + MgATP + CoASH→acyl-CoA + MgAMP + PPi; (ii) MgAMP + MgATP→2 MgADP; (iii) 2 phospho*enol*pyruvate + 2 MgADP→2 pyruvate + 2 MgATP; (iv) 2 pyruvate + 2 NADH + 2 $H^+$→2 lactate + 2 $NAD^+$. The 500 μl assay mixture contained 100 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 2 mM ATP, 0.4 mM NADH, 2 mM phosphoenolpyruvate, 0.5 mM CoA, 8 nkat myokinase, 20 nkat lactate dehydrogenase and 8 nkat pyruvate kinase. The product of ATP hydrolysis was identified by adding limiting amounts of benzoate (10–50 μM). As myokinase converts AMP plus ATP to two ADP, two NADH are oxidized per benzoate added in case of AMP formation (and only one NADH in case of ADP formation). The apparent $K_m$-values for benzoate was determined by varying benzoate concentration (2 μM–2 mM), cosubstrates were kept at saturating concentration. $K_m$-values were obtained by fitting the data to Michaelis-Menten curves using the Prism GraphPad software package (Graphpad Software, San Diego). For testing the substrate preference carboxylic acids were added at 2 mM in the spectrophotometric assay. To determine any inhibiting effect of a non-converted carboxylic acid, the test was restarted by addition of 0.5 mM benzoate after incubation for 5 min with the tested compound. The type of inhibition was determined by incubating benzoate-CoA ligase with the inhibitor (2 μM–2 mM, 37°C) for 1 min before starting the reaction with 0.01–1 mM benzoate; a Dixon plot analysis was carried out.

### *Cloning and expression of benzoate-CoA ligase*

The gene putatively coding for a benzoate-CoA ligase (gi: 48844990 – *bamY*) was cloned into *E. coli.* Primers were designed to remove the native stop codon and to place the gene of interest in frame with the DNA encoding a C-terminal peptide containing six histidins. The *bamY* gene was amplified from *G. metallireducens* DNA using the primer pair ATGCCCTACAATCTCAACCTG (forward primer)/GGCC ACGTGCTGCAACTCT (reverse primer); primers were purchased from Biomers (Ulm, Germany). The following PCR program using a Taq- and Pfu-Polymerase (18:1) was applied for amplification of the gene for *bamY*: 30 cycles of 94°C, 30 s; 55°C, 45 s, 72°C, 1 min 45 s. The program started with 2 min of 94°C, after the 30 cycles samples were incubated for further 5 min at 72°C. The amplified gene was transferred into the pCR RT7/CT-TOPO R expression vector according to the manufacturer's instruction (InvitrogenTMLife Technologies, Karlsruhe). The construct (pBamY) was transformed into One Shot RTOP10F′ and One Shot R BL21(DE3)pLysS cells for propagation, analysis and maintenance, and expression of the fusion protein, respectively, according to the manufacturer's instructions. Expression of the His-tagged BamY protein was observed at 18°C for 21.5 h after induction with isopropyl-D-thiogalactopyranoside (IPTG) to a final concentration of 0.8 mM. Cells were harvested at an optical density of 1.4 by centrifugation at 13 700 *g* for 15 min at 4°C and stored at –80°C until further use. Cell extracts were prepared as described above for *G. metallireducens*.

### *Purification of His-tagged benzoate-CoA ligase*

The overexpressed His-tagged gene product was purified at 8°C using a Ni-Sepharose high performance affinity column (HisTrap HP, Amersham Biosciences Europe GmbH, Freiburg) in a single step. For purification 3 g of *E. coli* strain BL21(DE3)pLysS cells (wet mass) containing the individual recombinant fusion protein were resuspended in 6.5 ml 20 mM Tris-HCl buffer, pH 7.9 (buffer A). Cell extracts were prepared as described above for *G. metallireducens* after which 10% (by vol.) of glycerol were added giving 5 ml cell lysate. After equilibration of a Ni-Sepharose column (1 ml volume) with buffer A containing 250 mM KCl and 10% glycerol (equilibration buffer), the cell extract was applied to the column at a flow rate of 0.2 ml $min^{-1}$. The column was then washed with 6 column volumes of equilibration buffer at a flow rate of 0.5 ml $min^{-1}$, after which a linear gradient over 20 column volumes from 0 to 500 mM imidazole in equilibration buffer was applied (flow rate of 0.5 ml min-[1]). The putative benzoate-CoA ligase fusion protein eluted between 80 and 200 mM imidazole in 4 ml containing 35 mg protein. Purity control was analysed by SDS-PAGE.

### *Determination of native molecular mass of benzoate-CoA ligase*

The native molecular mass of overexpressed BamY was determined using an FPLC Superdex 200 HR 10/30 gel-filtration column (Amersham Bioscience; diameter: 10 mm; bed volume: 24 ml), which had been equilibrated with 10 mM Tris-HCl buffer pH 7.5 containing 100 mM potassium chloride. 2.5 mg protein in 100 μl were applied at a flow rate of 0.2 ml $min^{-1}$.

### *Two-dimensional gel electrophoresis*

Two-dimensional gel electrophoresis was performed by IEF in the first and SDS-PAGE in the second dimension (both with Protean II xi 2-D Electrophoresis Cell-System, Bio-Rad; O'Farrell *et al*., 1975). Before application to the first dimension, cell extracts were dialysed against 20 mM Tris-HCl pH 7.8 (4°C, 10 h) after which the proteins were precipitated by adding a fivefold volume of ice-cold acetone with 0.2% DTT overnight at –20°C. For the first dimension, carrier ampholyte (pH 4–7) gels were prepared in glass tubes (2.3 mm diameter, 16 cm length; Abromeit *et al*., 1992). Per separation, 500 μg protein was applied. If not otherwise stated, separation in the second dimension was by SDS-PAGE using a polyacrylamide gradient from 8% to 16% with

dimensions $20 \times 20 \times 0.1$ cm (Laemmli, 1970). The visual comparison after colloidal blue staining of the protein patterns of cells grown on benzoate or butyrate allowed the identification of benzoate-induced proteins.

### *In-gel digestion of proteins*

Each gel spot was dried under vacuum pressure. In-gel digestion was performed with an automated protein digestion system, MassPREP Station (Micromass, Manchester, UK). The gel slices were washed twice with 50 µl of 25 mM $NH_4HCO_3$ and 50 µl of acetonitrile. The cysteine residues were reduced by 50 µl of 10 mM dithiothreitol at 57°C and alkylated by 50 µl of 55 mM iodoacetamide. After dehydration with acetonitrile, the proteins were cleaved in gel with 40 µl of 12.5 ng $\mu l^{-1}$ of modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM $NH_4HCO_3$ at room temperature for 14 h. The resulting tryptic peptides were extracted with 60% acetonitrile in 5% formic acid. The peptide extracts were used for MALDI-TOF MS and LC-MS/MS.

### *MALDI-TOF mass spectrometry*

MALDI-TOF mass spectrometry measurements were carried out with an Ultraflex TOF/TOF (Bruker Daltonik GmbH, Bremen, Germany). This instrument was operated in the reflectron positive mode as described previously by Heintz *et al*. (2004). Proteins were identified by PMFP with the MASCOT program (Matrix Science, London, UK) in the SWISSPROT and TrEMBL databases. Searches were carried out for all species.

### *Liquid chromatography-tandem mass spectrometry*

Nanoscale capillary LC-MS/MS analysis of the digested proteins was performed using a CapLC capillary LC system (Waters) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer Q-TOF II (Waters) as described elsewhere (Heintz *et al*., 2004). Mass data collected during a LC-MS/MS analysis were submitted to the search software MASCOT (Matrix Science, London, UK) for protein identification. Searches were carried out with a tolerance on mass measurement of 0.25 Da in MS mode and 0.3 Da in MS/MS mode. MS/MS searches were conducted using the NCBI (National Center for Biotechnology) nonredundant protein database. If no significant hits were obtained, MS-MS ion spectra were interpreted manually and using *de novo* peptide sequencing software (PEAKS) to obtain amino acid tags.

### *Reverse transcriptase PCR*

Total RNA from *G. metallireducens* grown on benzoate, 4-hydroxybenzoate, butyrate and acetate was used for RT-PCR. RNA was isolated from cells harvested in the exponential growth phase and was cleaned after DNAse treatment with an RNeasy Mini Kit (Quiagen, Hilden, Germany) for total RNA. Contaminating DNA was eliminated by on-column DNAse digestion with an RNAse-free DNAse Set (Quiagen, Hilden, Germany) and treatment with DNAse I (RNAse-free) 1 U per µg of total RNA; Fermentas, St. Leon-Rot, Germany for 30 min at 37°C. Complete removal of DNA was verified by amplifying the long intergenic region between two genes coding in different directions with cDNA as template. One microgram of purified total RNA was used to prepare cDNA by using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) using the provided random hexamer primer. Gene expression was studied by amplifying internal sequences of genes or intergenic regions from undiluted, 10-fold and 100-fold diluted cDNA, using genomic DNA of *G. metallireducens* as positive control. PCR-products (approximately 600–700 bp) were visualized by agarose gel electrophoresis. Standard protocols (Ausubel *et al*., 1987) were used for DNA amplification.

### *Further determinations*

Protein concentrations were determined according to Bradford (1976). Proteins on gels were stained with colloidal Coomassie (Neuhoff *et al*., 1990). Scanning of the gel was performed using a Fluor-S multiimager (Bio-Rad). Sequence comparisons were carried out using the BLAST tool (http://www.ncbi.nlm.nih.gov/blast); topology analysis of membrane proteins was performed with the TmPred tool (http://www.ch.embnet.org/software/TMPRED_form.html).

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## References

Abromeit, M., Askman, P., Sarnighausen, E., and Dörfling, K. (1992) Accumulation of high-molecular-weight proteins to response in cold hardening and abscisic acid treatment in two winter wheat varieties with different frost tolerance. *J Plant Physiol* **140:** 617–622.

Auburger, G., and Winter, J. (1992) Purification and characterization of benzoyl-CoA ligase from a syntrophic, benzoate-degrading, anaerobic mixed culture. *Appl Microbiol Biotechnol* **37:** 789–795.

Ausubel, F.M., Brent, R., Kingston, E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987) *Current Protocols in Molecular Biology*. New York: John Wiley and Sons.

Boll, M., and Fuchs, G. (1995) Benzoyl-CoA reductase (dearomatizing), a key enzyme of anaerobic aromatic metabolism. ATP-dependence of the reaction, purification and some properties of the enzyme from *Thauera aromatica* strain K 172. *Eur J Biochem* **234:** 921–933.

Boll, M., Laempe, D., Eisenreich, W., Bacher, A., Mittelberger, T., Heinze. J., and Fuchs, G. (2000) Non-aromatic products from anoxic conversion of benzoyl-CoA with benzoyl-CoA reductase and cyclohexa-1,5-diene-1-carbonyl-CoA hydratase. *J Biol Chem* **275:** 21889–21895.

Boll, M., Fuchs, G., and Heider, H. (2002) Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr Opin Chem Biol* **6:** 604–611.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254.

Buckel, W., and Keese, R. (1995) One electron reactions of CoASH esters in anaerobic bacteria. *Angew Chem Int Ed Engl* **34:** 1502–1506.

Chan, M.K., Mukund, S., Kletzin, A., Adams, M.W., and Rees, D.C. (1995) Structure of a hyperthermophilic tungstopterin enzyme, aldehyde ferredoxin oxidoreductase. *Science* **267:** 1463–1469.

Deppenmeier, U. (2004) The membrane-bound electron transport system of *Methanosarcina* species. *J Bioenerg Biomembr* **36:** 55–64.

Egland, P.G., Gibson, J., and Harwood, C.S. (1995) Benzoate-coenzyme A ligase, encoded by *badA*, is one of three ligases able to catalyze benzoyl-coenzyme A formation during anaerobic growth of *Rhodopseudomonas palustris* on benzoate. *J Bacteriol* **177:** 6545–6551.

Elshahed, M.S., and McInerney, M.J. (2001) Benzoate fermentation by the anaerobic bacterium *Syntrophus aciditrophicus* in the absence of hydrogen-using microorganisms. *Appl Environ Microbiol* **67:** 5520–5525.

Frerman, F.E. (1987) Reaction of electron transfer flavoprotein ubiquinone oxidoreductase with the respiration chain. *Biochim Biophys Acta* **893:** 161–169.

Friedrich, T., and Böttcher, B. (2004) The gross structure of the respiratory complex I: a Lego System. *Biochim Biophys Acta* **1608:** 1–9.

Geissler, J.F., Harwood, C.S., and Gibson, J. (1988) Purification and properties of benzoate-coenzyme A ligase, a *Rhodopseudomonas palustris* enzyme involved in the anaerobic degradation of benzoate. *J Bacteriol* **170:** 1709–1714.

Gibson, K.J., and Gibson, J. (1992) Potential early intermediates in anaerobic benzoate degradation by *Rhodopseudomonas palustris*. *Appl Environ Microbiol* **58:** 696–698.

Gibson, J., and Harwood, C.S. (2002) Metabolic diversity in aromatic compound utilization by anaerobic microbes. *Annu Rev Microbiol* **56:** 345–369.

Hedderich, R., Koch, J., Linder, D., and Thauer, R.K. (1994) The heterodisulfide reductase from *Methanobacterium thermoautotrophicum* contains sequence motifs characteristic of pyridine nucleotide-dependent thioredoxin reductases. *Eur J Biochem* **225:** 253–261.

Heintz, D., Wurtz, V., High, A.A., Van Dorsselaer, A., Reski, R., and Sarnighausen, E. (2004) An efficient protocol for the identification of protein phosphorylation in a seedless plant, sensitive enough to detect members of signalling cascades. *Electrophoresis* **25:** 1149–1159.

Koch, J., and Fuchs, G. (1992) Enzymatic reduction of benzoyl-CoA to alicyclic compounds, a key reaction in anaerobic aromatic metabolism. *Eur J Biochem* **205:** 195–202.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

Laempe, D., Eisenreich, W., Bacher, A., and Fuchs, G. (1998) Cyclohexa-1,5-diene-1-carbonyl-CoA hydratase, an enzyme involved in anaerobic metabolism of benzoyl-CoA in the denitrifying bacterium *Thauera aromatica*. *Eur J Biochem* **255:** 618–627.

Laempe, D., Jahn, M., and Fuchs, G. (1999) 6-Hydroxycyclohex-1-ene-1-carbonyl-CoA dehydrogenase and 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase, enzymes of the benzoyl-CoA pathway of anaerobic aromatic metabolism in the denitrifying bacterium *Thauera aromatica*. *Eur J Biochem* **263:** 420–429.

López Barragán, M.J., Carmona, M., Zamarro, M.T., Thiele, B., Boll, M., Fuchs, G., *et al.* (2004) The bzd gene cluster, coding for anaerobic benzoate catabolism. *Azoarcus* sp. strain CIB. *J Bacteriol* **186:** 5762–5774.

Lovley, D.R. (2003) Cleaning up with genomics: applying molecular biology to bioremediation. *Nat Rev Microbiol* **1:** 35–44.

Lovley, D.R., and Phillips, E.J.P. (1988) Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron and manganese. *Appl Environ Microb* **54:** 1472–1480.

Lovley, D.R., Baedecker, M.J., Lonergan, D.J., Cozzarelli, I.M., Phillips, E.J.P., and Siegel, D.I. (1989) Oxidation of aromatic contaminants coupled to microbial iron reduction. *Nature* **339:** 297–299.

Lovley, D.R., Giovannoni, S.J., White, D.C., Champine, J.E., Phillips, E.J., Gorby, Y.A., and Goodwin, S. (1993) *Geobacter metallireducens* General nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. *Arch Microbiol* **159:** 336–344.

Lovley, D.R., Holmes, D.E., and Nevin, K.P. (2004) Dissimilatory Fe (III) and Mn (IV) reduction. *Adv Microb Physiol* **49:** 219–286.

Ma, B., Zhang, K., Lajoie, G., Doherty-Kirby, A., Hendrie, C., Liang, C., and Li, M. (2003) PEAKS: powerful software for peptide *de novo* sequencing by tandem mass spectrometry. *Rapid Commun Mass Spectrom* **17:** 2337–2342.

Neuhoff, V., Stamm, R., Pardowitz, I., Arold, N., Ehrhardt, W., and Taube, D. (1990) Essential problems in quantification of proteins following colloidal staining with coomassie brilliant blue dyes in polyacrylamide gels, and their solution. *Electrophoresis* **11:** 101–117.

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250:** 4007–4021.

Pelletier, D.A., and Harwood, C.S. (2000) 2-Hydroxycyclohexanecarboxyl coenzyme A dehydrogenase, an enzyme characteristic of the anaerobic benzoate degradation pathway used by *Rhodopseudomonas palustris*. *J Bacteriol* **182:** 2753–2760.

Peters, F., Rother, M., and Boll, M. (2004) Selenocysteine-containing proteins in anaerobic benzoate metabolism of *Desulfococcus multivorans*. *J Bacteriol* **186:** 2156–2163.

Rabus, R., Hansen, T., and Widdel, F. (2001) Dissimilatory sulfate- and sulfur reducing prokaryotes. In *The Prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds) www version. New York: Springer Press.

Rabus, R., Kube, M., Heider, J., Beck, A., Heitmann, K., Widdel, F., and Reinhardt, R. (2005) The genome

 

sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. *Arch Microbiol* **183:** 27–36.

Rother, M., Mathes. I., Lottspeich, F., and Böck, A. (2003) Inactivation of the *selB* gene in *Methanococcus maripaludis*: effect on synthesis of selenoproteins and their sulfur-containing homologues. *J Bacteriol* **185:** 107–114.

Roy, R., Menon, A.L., and Adams, M.W. (2001) Aldehyde oxidoreductases from *Pyrococcus furiosus*. *Methods Enzymol* **331:** 132–144.

Schink, B., Philipp, B., and Müller, J. (2000) Anaerobic degradation of phenolic compounds. *Naturwissenschaften* **87:** 12–23.

Schöcke, L., and Schink, B. (1999) Energetics of methanogenic benzoate degradation by *Syntrophus gentianae* in syntrophic coculture. *Microbiology* **143:** 2345–2351.

Schühle, K., Gescher, J., Feil, U., Paul, M., Jahn, M., Schägger, H., and Fuchs, G. (2003) Benzoate-coenzyme A ligase from *Thauera aromatica*: an enzyme acting in anaerobic and aerobic pathways. *J Bacteriol* **185:** 4920–4929.

Sorgenfrei, O., Müller, S., Pfeiffer, M., Sniezko, I., and Klein, A. (1997) The [NiFe] hydrogenases of *Methanococcus voltae*: genes, enzymes and regulation. *Arch Microbiol* **52:** 563–571.

Stojanowic, A., Mander, G.J., Duin, E.C., and Hedderich, R. (2003) Physiological role of the $F_{420}$ non-reducing hydrogenase Mvh from *Methanothermobacter margurgensis*. *Arch Microbiol* **180:** 194–203.

Taylor, J.A., and Johnson, R.S. (2001) Implementation and uses of automated *de novo* peptide sequencing by tandem mass spectrometry. *Anal Chem* **73:** 2594–2604.

Tran-Betcke, A., Warnecke, A., Böcker, C., Zaborosch, C., and Friedrich, B. (1990) Cloning and nucleotide sequences of the genes for the subunits of NAD-reducing hydrogenase of *Alcaligenes eutrophus* H16. *J Bacteriol* **172:** 2920–2929.

Unciuleac, M., and Boll, M. (2001) Mechanism of ATP-driven electron transfer catalyzed by the benzene ring-reducing enzyme benzoyl-CoA reductase. *Proc Natl Acad Sci USA* **98:** 13619–13624.

Widdel, F. (1980) Abbau von Fettsäuren und Benzoesäure durch neu isolierte Arten. PhD Thesis. Göttingen, Germany: University of Göttingen.

Wilting, R., Schorling, S., Persson, B.C., and Böck, A. (1997) Selenoprotein synthesis in archaea: identification of an mRNA element of *Methanococcus jannaschii* probably directing selenocysteine insertion. *J Mol Biol* **266:** 637–641.

Ziegler, K., Braun, K., Böckler, A., and Fuchs, G. (1987) Studies on the anaerobic degradation of benzoic acid and 2-aminobenzoic acid by a denitrifying *Pseudomonas* strain. *Arch Microbiol* **149:** 62–69.

## Supplementary material

The following supplementary material is available for this article online:

**Table S1.** Oligonucleotide primers used for reverse transcriptase experiments.

This material is available as part of the online article from http://www.blackwell-synergy.com