

Anaerobic degradation of aromatic amino acids by the hyperthermophilic archaeon *Ferroglobus placidus*

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Ferroglobus placidus was discovered to oxidize completely the aromatic amino acids tyrosine, phenylalanine and tryptophan when Fe(III) oxide was provided as an electron acceptor. This property had not been reported previously for a hyperthermophilic archaeon. It appeared that *F. placidus* follows a pathway for phenylalanine and tryptophan degradation similar to that of mesophilic nitrate-reducing bacteria, *Thauera aromatica* and *Aromatoleum aromaticum* EbN1. Phenylacetate, 4-hydroxyphenylacetate and indole-3-acetate were formed during anaerobic degradation of phenylalanine, tyrosine and tryptophan, respectively. Candidate genes for enzymes involved in the anaerobic oxidation of phenylalanine to phenylacetate (phenylalanine transaminase, phenylpyruvate decarboxylase and phenylacetaldehyde :ferredoxin oxidoreductase) were identified in the *F. placidus* genome. In addition, transcription of candidate genes for the anaerobic phenylacetate degradation, benzoyl-CoA degradation and glutaryl-CoA degradation pathways was significantly upregulated in microarray and quantitative real-time-PCR studies comparing phenylacetate-grown cells with acetate-grown cells. These results suggested that the general strategies for anaerobic degradation of aromatic amino acids are highly conserved amongst bacteria and archaea living in both mesophilic and hyperthermophilic environments. They also provided insights into the diverse metabolism of *Archaeoglobaceae* species living in hyperthermophilic environments.

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INTRODUCTION

Proteins account for ~10 % of the biomass of all living organisms (Yokoyama & Matsumura, 2008), and therefore their degradation products play a major role in carbon and nitrogen cycling on the planet. When organisms die, proteins are broken down to their monomers (amino acids) that can serve as carbon, nitrogen, sulfur and energy sources for numerous micro-organisms. Amino acids are also thought to be one of the first biologically relevant molecules ever formed on the planet. It has been proposed that the chemically reducing atmosphere, high temperatures and

abundance of precursor gases on the early Earth allowed the formation of certain basic compounds of life, such as amino acids and nucleic acids (Miller, 1953; Parker *et al.*, 2011). Hydrothermal vents are present-day environments that are reminiscent of the conditions that predominated at the onset of life. Therefore, biochemical studies of organisms isolated from hydrothermal vent sediments, such as *Ferroglobus placidus* and *Archaeoglobus fulgidus*, can provide considerable insight into amino acid metabolism on the early Earth.

To date, the majority of studies on amino acid catabolism have focused on the degradation of amino acids in the presence of oxygen, with anaerobic studies being mostly limited to partial oxidation (Baena *et al.*, 1998, 1999; Barker, 1981; Díaz *et al.*, 2007; Fonknechten *et al.*, 2010; Russell *et al.*, 2013). However, some anaerobic bacteria that can completely oxidize amino acids to carbon dioxide and respire such inorganic compounds as nitrate, sulfur, sulfate and iron oxides have also been documented (Bak & Widdel, 1986; Ebenau-Jehle *et al.*, 2012; Holmes *et al.*,

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Abbreviations: qRT, quantitative real-time.

A complete record of all oligonucleotide sequences used and raw and statistically treated data files are available in the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi>), accession number GSE59466.

One supplementary figure and 15 supplementary tables are available with the online Supplementary Material.

2004b; Kashefi *et al.*, 2002; Mechichi *et al.*, 2002; Schneider *et al.*, 1997).

In addition to providing an insight into the origins of life on the planet, studies of enzymes involved in amino acid degradation from hyperthermophilic archaea have broad industrial applications. For example, intermediates formed during the degradation of aromatic amino acids (phenylacetaldehyde, phenylacetic acid and indole-3-acetate) are used to scent perfume, to dye textiles and to synthesize antibiotics (Barden, 2011; Letizia *et al.*, 2003; Luengo *et al.*, 2001), and most of the aroma compounds associated with various cheeses are degradation products from aromatic amino acids (phenylalanine, tyrosine and tryptophan), branched-chain amino acids (leucine, isoleucine and valine) and methionine (van Kranenburg *et al.*, 2002). Thermostable enzymes such as those present in hyperthermophiles are particularly desirable for industrial and biotechnological applications, as they can be employed in harsh industrial environments where their specific catalytic activity is retained.

Although thermally stable enzymes involved in amino acid degradation have great potential, amino acid metabolism has been minimally explored in hot (>80 °C) environments. *Archaeoglobus fulgidus*, a phylogenetic relative of *F. placidus*, has only recently been shown to perform partial oxidation of aromatic amino acids (Parthasarathy *et al.*, 2013), and enzymic studies have been limited to *Thermococcales* and *Thermoprotei* species that cannot completely oxidize these compounds to carbon dioxide (Mai & Adams, 1994; Yokooji *et al.*, 2013).

This study focuses on amino acid degradation by the hyperthermophilic archaeon *F. placidus*. This organism is unique in that it can completely oxidize 15 of the 20 different proteinogenic amino acids to carbon dioxide and transfer those electrons to insoluble Fe(III) oxide. Here, metabolic pathways associated with the catabolism of non-aromatic and aromatic amino acids have been proposed, and enzymes involved in anaerobic phenylalanine degradation were identified through comparative genomics and transcriptomic analyses.

METHODS

Growth of *F. placidus*. *F. placidus* strain AEDII12DO (DSM 10642) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Strict anaerobic culturing and sampling techniques were used throughout (Balch *et al.*, 1979; Miller & Wolin, 1974). *F. placidus* cells were grown with various amino acids (0.5 mM) as the electron donor, and Fe(III) citrate (56 mM) or Fe(III) oxide (60 mM) as the electron acceptor.

F. placidus medium was prepared as described previously (Tor & Lovley, 2001). After autoclaving, FeCl₂ (1.3 mM), Na₂SeO₄ (30 µg l⁻¹), Na₂WO₄ (40 µg l⁻¹), APM salts (1 g MgCl₂ l⁻¹, 0.23 g CaCl₂ l⁻¹) (Coates *et al.*, 1995), DL vitamins (Lovley & Phillips, 1988) and all electron donors were added to the sterilized medium from anaerobic stock solutions. Cultures were incubated under N₂/CO₂ (80:20) at 85 °C in the dark.

Analytical techniques. Concentrations of phenylacetate, indole-3-acetate and 4-hydroxyphenylacetate were determined using HPLC (Agilent 1100 HPLC Series) with an Altima HP C18 HL column. The eluent consisted of 0.1% H₃PO₄ in MeOH/H₂O (60:40) and the compounds were quantified by A₂₈₀. Phenylalanine, tryptophan and tyrosine concentrations were determined with a ZORBAX Eclipse Amino Acid Analysis column. The eluent consisted of 40 mM NaH₂PO₄, pH 7.8, in acetonitrile/methanol/water (45:45:10, by vol.) and the compounds were quantified by A₃₃₈.

Fe(III) reduction was monitored by measuring the formation of Fe(II) over time with a ferrozine assay, quantified by A₅₆₂ in a split-beam dual-detector spectrophotometer (Spectronic Genosys2; Thermo Electron) after a 1 h extraction with 0.5 N HCl as described previously (Lovley & Phillips, 1987, 1988).

Cell numbers were determined by counting acridine orange-stained cells by fluorescence microscopy on a Nikon Eclipse E600 microscope as described previously (Lovley & Phillips, 1987).

Operon organization and gene annotation. Operon organization of the *F. placidus* genome was predicted using the commercial version of FGENESB software (V. Solovyev and A. Salamov, unpublished; Softberry), as previously described for the genomes of various *Geobacteraceae* (Mahadevan *et al.*, 2008; Tran *et al.*, 2008; Yan *et al.*, 2007). Sequence parameters used in Markov chain-based modelling of protein-coding genes were estimated by FGENESB via an iterative procedure using the sequence of each genome and a minimum ORF length of 100 bp.

Extraction of RNA from samples. RNA for microarray and quantitative real-time (qRT)-PCR analyses was extracted during exponential growth of *F. placidus* on phenylalanine, phenylacetate and acetate. In all cases, triplicate RNA samples were extracted.

For extraction of RNA, cultures (100 ml in 156 ml serum bottles) were divided into 50 ml conical tubes (Falcon) and cells were pelleted by centrifugation at 3000 g for 15 min. Pellets were then immediately frozen in liquid nitrogen and stored at -80 °C.

The pellets were resuspended in 10 ml HG extraction buffer, preheated to 65 °C. The HG extraction buffer consisted of 100 mM Tris/HCl, pH 8, 100 mM NaCl, 10 mM EDTA, 2.5% β-mercaptoethanol, 1% SDS, 2% Plant RNA Isolation Aid (Ambion), 5 mM ascorbic acid, Proteinase K (0.6 mg ml⁻¹) and lysozyme (5 mg ml⁻¹). The suspended cells were then dispensed into ten 2 ml screw-cap tubes and incubated at 65 °C for 10 min. After incubation, samples were placed on ice, and 2 µl Superscript-In (Ambion) and 0.025 mM CaCl₂ were added. Samples were then centrifuged at 16 100 g for 10 min and the supernatant was transferred to new 2 ml screw-cap tubes. Then, 50 µl Plant RNA Isolation Aid (Ambion), 4 µl linear acrylamide (5 mg ml⁻¹; Ambion), 600 µl hot acidic phenol (65 °C; pH 4.5; Ambion) and 400 µl chloroform/isoamyl alcohol (24:1; Sigma) were added to the supernatant. The tubes were then mixed on a Labquake rotator (Barnstead/ThermoLyne) for 10 min and centrifuged at 16 100 g for 5 min. The aqueous layer was removed and transferred to new 2 ml screw-cap tubes, and 600 µl hot acidic phenol (65 °C; pH 4.5; Ambion) and 400 µl chloroform/isoamyl alcohol (24:1; Sigma) were added. Tubes were mixed on a rotator for 5 min and centrifuged at 16 100 g for 10 min. The aqueous layer was removed again and transferred to new tubes, and 100 µl 5 M ammonium acetate (Ambion), 20 µl 5 mg ml⁻¹ glycogen (Ambion) and 1 ml cold 2-propanol (-20 °C; Sigma) were added.

Nucleic acids were precipitated at -30 °C for 1 h and pelleted by centrifugation at 16 100 g for 30 min. The pellet was then cleaned with cold (-20 °C) 70% ethanol, dried and resuspended in sterile diethylpyrocarbonate-treated water (Ambion). The resuspended pellets were combined and cleaned with an RNeasy RNA cleanup

kit (Qiagen) according to the manufacturer's instructions. The RNA cleanup product was then treated with DNA-free DNase (Ambion) according to the manufacturer's instructions.

High-quality RNA was extracted from these culture samples. All samples had A_{260}/A_{280} ratios of 1.8–2.0, indicating that they were of high purity (Ausubel *et al.*, 2001). In order to ensure that RNA samples were not contaminated with DNA, PCR amplification with primers targeting the 16S rRNA gene was attempted on RNA samples that had not undergone reverse transcription.

Microarray analysis. Whole-genome microarray hybridizations were carried out by Roche NimbleGen. A TransPlex Whole Transcriptome Amplification kit (Sigma) was used to amplify RNA prior to transcriptomic analyses. RNA was obtained from three biological replicates and triplicate technical replicates were conducted for microarray analyses. All cDNA samples were chemically labelled with Cy3 and hybridized by Roche NimbleGen. The oligonucleotide microarrays used in this study were designed based on the preliminary genome sequence data of *F. placidus* (GenBank accession number NC_013849) obtained from the Joint Genome Institute website (www.jgi.doe.gov).

A complete record of all oligonucleotide sequences used and raw and statistically treated data files is available in the NCBI Gene Expression Omnibus database (GEO data series number GSE59466).

Results from microarray hybridizations were analysed with ArrayStar 12 software (DNASTAR). *P* values were determined using Student's *t*-test. Multiple oligonucleotide probes (three or four) were analysed for each gene. For each of the six technical replicates in each experiment, a probe was considered valid if its signal intensity was above the mean signal from three probes for the *rgy* gene (Ferp_0787, DNA topoisomerase reverse gyrase) for either the control or the experimental condition. A probe was considered valid for a biological replicate if it was valid for one or both technical replicates thereof. A gene was considered expressed only if at least two probes were valid for at least two biological replicates each. A gene was only considered differentially expressed if at least two probes had $P \leq 0.01$.

qRT-PCR. Primer pairs used for qRT-PCR analysis of gene transcript abundance are listed in Table S1 (available in the online Supplementary Material). All primers were purchased from Eurofins MWG Operon and designed according to the manufacturer's specifications (amplicon size 100–200 bp). Representative products from each of these primer sets were verified by sequencing clone libraries.

The housekeeping gene *rgy* was used as an external control for qRT-PCR. Studies have shown that expression of this gene by *F. placidus* is stable under a variety of growth rates and conditions, and it has not been differentially expressed in any of the microarray studies conducted with *F. placidus* (Holmes *et al.*, 2011, 2012). A DuraScript Enhanced Avian First Strand Synthesis kit (Sigma) was used to generate cDNA as described previously (Holmes *et al.*, 2004a).

For clone library construction, PCR products were purified with a Gel Extraction kit (Qiagen), and clone libraries were constructed with a TOPO TA cloning kit, version M (Invitrogen) according to the manufacturer's instructions. One hundred plasmid inserts from each clone library were sequenced with the M13F primer at the University of Massachusetts Sequencing Facility.

Once the appropriate cDNA fragments were generated by reverse transcription, qRT-PCR amplification and detection were performed with a 7500 Real-Time PCR System (Applied Biosystems). Optimal qRT-PCR conditions were determined using the manufacturer's guidelines. Each PCR mixture consisted of a total volume of 25 μ l and contained 1.5 μ l of the appropriate primers (stock concentrations 15 μ M) and 12.5 μ l Power SYBR Green PCR Master Mix (Applied

Biosystems). Standard curves covering eight orders of magnitude were constructed with serial dilutions of known amounts of purified cDNA quantified with a NanoDrop ND-1000 spectrophotometer at A_{260} .

All qRT-PCR experiments followed the MIQE guidelines (Bustin *et al.*, 2009). The qRT-PCR efficiency (90–99%) was calculated based on the slope of the standard curve. All qRT-PCR assays had triplicate biological and technical replicates. Thermal cycling parameters consisted of an activation step at 50 °C for 2 min, a denaturation step at 95 °C for 10 min, and 50 cycles at 95 °C for 15 s and 60 °C for 1 min. This was followed by the construction of a dissociation curve by increasing the temperature from 60 to 95 °C at a ramp rate of 2 %. A single predominant peak was observed in the dissociation curve of each gene, supporting the specificity of the PCR product.

RESULTS

Anaerobic amino acid degradation by *F. placidus*

Growth of *F. placidus* was tested in media containing each of the 20 standard amino acids as an electron donor and insoluble Fe(III) oxide as the electron acceptor. *F. placidus* could couple the oxidation of 15 out of the 20 amino acids with Fe(III) respiration. It could utilize the majority of non-polar, polar, negatively charged, positively charged and aromatic amino acids (the exceptions were valine, methionine, asparagine, aspartate and histidine) as a sole carbon and energy source. To the best of our knowledge, *F. placidus* is the first organism found to grow via anaerobic respiration with such a wide range of amino acids as the sole electron donor. It is also the only known hyperthermophilic archaeon that can completely oxidize three of the four aromatic amino acids (tyrosine, phenylalanine and tryptophan, but not histidine) into carbon dioxide with Fe(III) oxide as an electron acceptor.

The genome of *F. placidus* was searched for genes that could potentially code for enzymes involved in the complete mineralization of these amino acids. Genes coding for biochemically characterized enzymes involved in α -amino acid catabolism from *Thermococcales* species were used as queries (Fig. 1) (Yokooji *et al.*, 2013). In *Thermococcales* species, the first enzyme of the pathway is an amino acid : 2-oxoacid aminotransferase that uses pyridoxal-phosphate as a cofactor to transfer the amino group from the amino acid (e.g. alanine) to a different 2-oxocarboxylate (e.g. 2-oxoglutarate) to form a 2-oxocarboxylate (e.g. pyruvate) and an L-amino acid (e.g. glutamate). *F. placidus* has eight genes coding for enzymes that are homologous to *Thermococcales* aminotransferase proteins, and a branched-chain amino acid aminotransferase without a homologue (Ferp_0064) that is likely to be the first enzyme in degradation of leucine and isoleucine (Table S2). Once the L-amino acid is formed, it is oxidized by a dehydrogenase protein (i.e. glutamate dehydrogenase) and its electrons are transferred to NAD^+ , regenerating 2-oxoglutarate. *F. placidus* has a protein (Ferp_0766) that is 72 % similar to glutamate dehydrogenase of *Pyrococcus furiosus* (Table S2). The 2-oxoacid (pyruvate) generated by the deamination

reaction is then converted to an acyl-CoA (acetyl-CoA in the case of pyruvate) by a 2-oxoacid:ferredoxin oxidoreductase and electrons are transferred to a ferredoxin protein. This enzyme consists of four subunits and the *F. placidus* genome contains two operons (Ferp_0892–0895; Ferp_1823–1826) with homologous genes for all four of these subunits (Table S2). Finally, an ADP-forming acyl-CoA synthetase, operating in reverse, catalyses the hydrolysis of the acyl-CoA coupled to ATP synthesis by substrate-level phosphorylation. This enzyme is a tetramer composed of two α and two β subunits, and the *F. placidus* genome contains two copies of both of these genes (Ferp_1489–1490 and Ferp_0287–0288) (Table S2).

Phenylalanine metabolism: initial steps to phenylacetate

F. placidus could grow with phenylalanine as the sole electron donor and insoluble Fe(III) oxide as the electron acceptor with a generation time of 8.9 days (Figs 2a and S1). When 0.51 mM phenylalanine was added, 19.06 mM Fe(III) were reduced. Therefore, 93.4% of the electrons available from the complete oxidation of phenylalanine were transferred to insoluble Fe(III) oxide according to the stoichiometry:

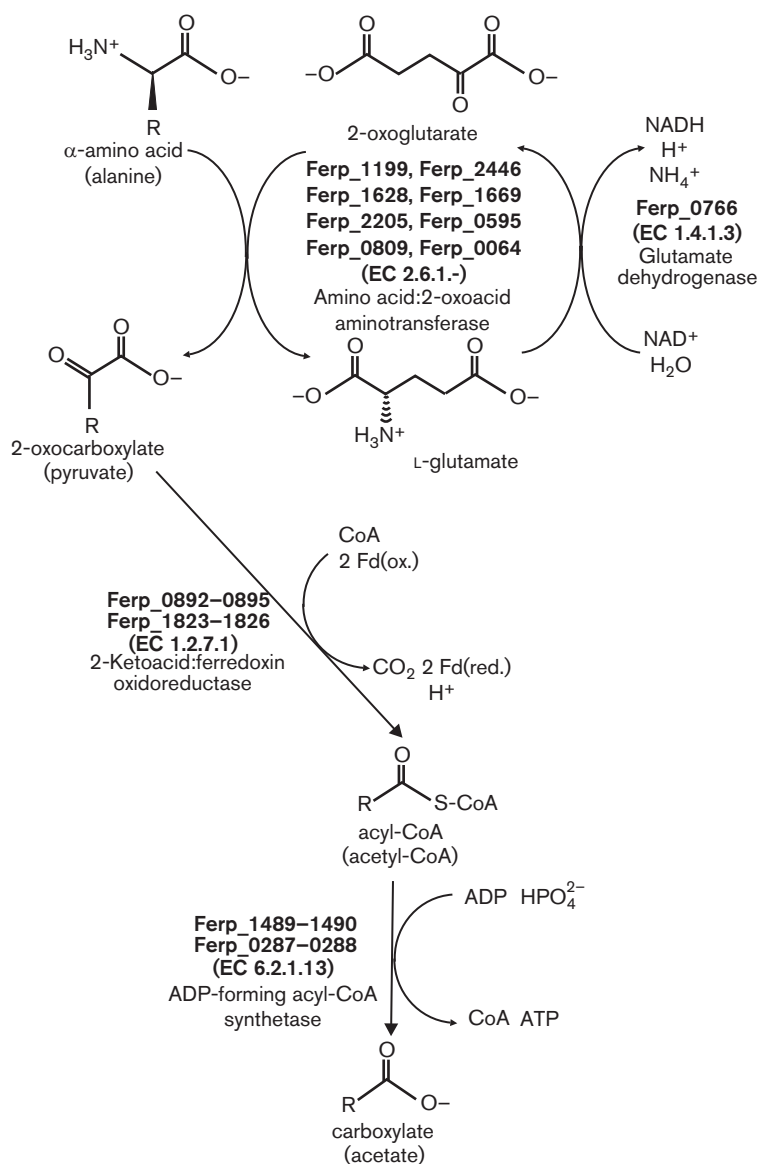
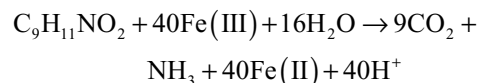


Fig. 1. Proposed pathway for degradation of α -amino acids by *F. placidus*.

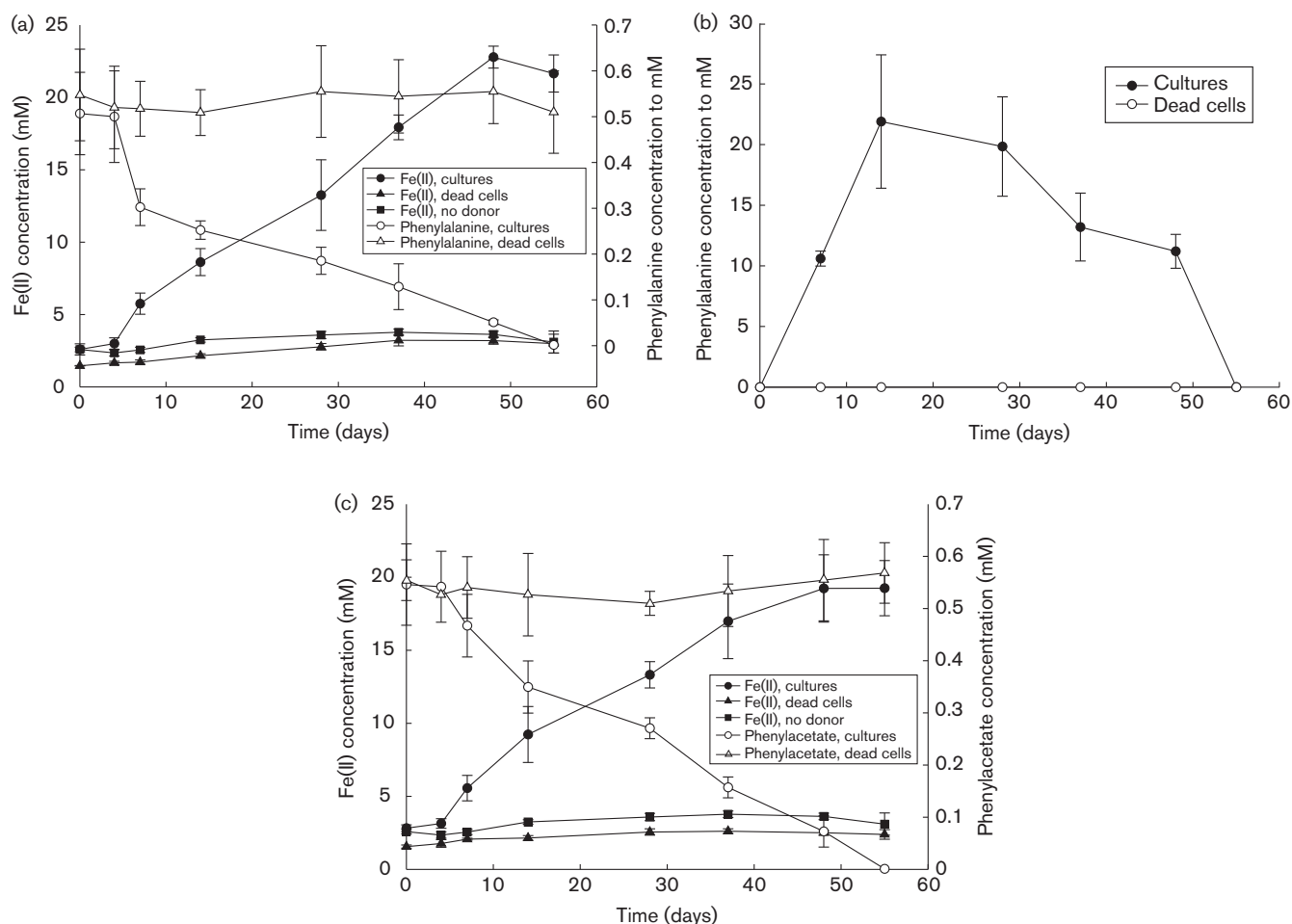


Fig. 2. (a) Fe(III) reduction and phenylalanine consumption when *F. placidus* was grown with phenylalanine (0.51 mM) as the electron donor and Fe(III) oxide (100 mM) as the electron acceptor. (b) Formation of phenylacetate as an intermediate in cultures grown with phenylalanine as electron donor. (c) Fe(III) reduction and phenylacetate consumption when *F. placidus* was grown with phenylacetate (0.5 mM) as the electron donor and Fe(III) oxide (100 mM) as the electron acceptor.

Anaerobic degradation of phenylalanine is well documented in the denitrifying bacterium *Thauera aromatica* (Schneider *et al.*, 1997). This organism oxidizes phenylalanine to phenylacetate and then to benzoyl-CoA with nitrate as an electron acceptor. Phenylacetate was detected during growth of *F. placidus* (Fig. 2b), suggesting that it utilizes a pathway for phenylalanine degradation that is similar to *Thauera aromatica*. Furthermore, *F. placidus* can grow with phenylacetate as the sole electron donor and insoluble Fe(III) oxide as electron acceptor with a generation time of 7.8 days (Figs 2c and S1).

In *Thauera aromatica*, the first step involves formation of a 2-oxoacid, phenylpyruvate (Fig. 3), by a pyridoxal-phosphate-dependent aminotransferase. There are eight genes coding for putative aminotransferases in the *F. placidus* genome that could catalyse this reaction (Table S3). Ferp_0595 encodes a protein with 45 % sequence identity to a characterized glutamate-dependent aminotransferase of

Thermococcus litoralis that is specific for phenylalanine, tyrosine and tryptophan (Andreotti *et al.*, 1994). However, Ferp_1628, which encodes one of two homologues of histidinol-phosphate aminotransferase in *F. placidus*, was the only amino acid aminotransferase gene that had significantly more mRNA transcripts during growth on phenylalanine compared with phenylacetate (5.68-fold) (Table S3).

The next step involves decarboxylation of phenylpyruvate and formation of phenylacetaldehyde by phenylpyruvate carboxylase. The gene responsible for this step is unclear. A BLAST search suggests Ferp_2163, which encodes the large subunit of acetolactate synthase involved in valine, leucine and isoleucine biosynthesis. Other genes encode decarboxylase proteins that could catalyse this reaction (Table S4). Three of these genes had higher transcript levels in cells grown with phenylalanine compared with phenylacetate. Of these, Ferp_0092 shares 55 % protein sequence identity

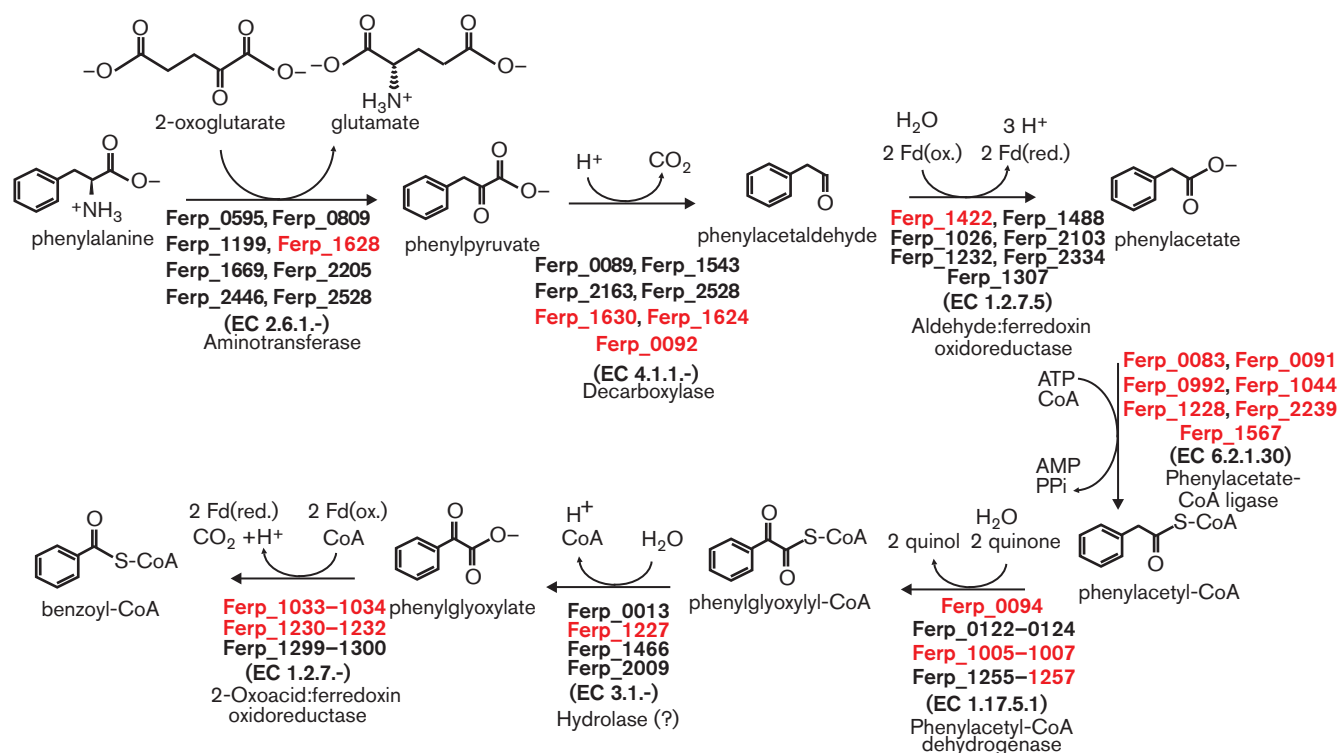


Fig. 3. Proposed pathway utilized by *F. placidus* to oxidize phenylalanine to phenylacetate and benzoyl-CoA. Genes highlighted in red were upregulated in phenylalanine- or phenylacetate-grown cells.

with a putative aromatic acid decarboxylase of *Thauera aromatica* that is encoded amongst phenol degradation genes (Breinig *et al.*, 2000). Although Ferp_0092 was also more highly transcribed in cells grown with phenylacetate compared with acetate, this may be due to positive feedback or an indication that this decarboxylase also acts in phenylacetate degradation. Both Ferp_1630 and Ferp_1624 were ~3.3 times more highly transcribed in phenylalanine-grown cells and were not differentially expressed in microarray or qRT-PCR comparisons of phenylacetate-grown cells to acetate-grown cells.

In *Thauera aromatica*, phenylacetaldehyde is oxidized to phenylacetate by an NAD-dependent aldehyde dehydrogenase. In contrast, the *F. placidus* genome encodes only three aldehyde dehydrogenases (Ferp_0927, Ferp_1128 and Ferp_1302) with specific functions in gluconeogenesis and amino acid biosynthesis (Table S5). Seven aldehyde:ferredoxin oxidoreductase proteins with *bis*-molybdopterin-oxotungsten cofactor-binding motifs and high sequence identity to the tungsten-containing aldehyde:ferredoxin oxidoreductase from *Aromatoleum aromaticum* (Debnar-Daumler *et al.*, 2014) were identified in *F. placidus* (Table S5), of which Ferp_1422 was significantly upregulated in phenylalanine-grown cells (2.86-fold) and may encode an enzyme responsible for ferredoxin-dependent oxidation of phenylacetaldehyde.

Phenylacetate degradation via benzoyl-CoA

To elucidate the pathway of phenylacetate degradation in *F. placidus*, transcriptomic studies followed by qRT-PCR were conducted to compare cells grown with either phenylacetate or acetate (control) as the electron donor and Fe(III) as the electron acceptor. Microarray studies showed significant ($P < 0.01$), at least twofold differences in expression for 257 genes (171 upregulated and 86 downregulated) when these two conditions were compared (Tables S6 and S7). Genes that were upregulated in phenylacetate-grown cells encode enzymes for degradation of phenol and benzoate, enzymes for tryptophan biosynthesis, numerous enzymes for acyl-CoA metabolism, including two (*R*)-2-hydroxyacyl-CoA dehydratases, four uptake transporters of the branched-chain amino acid group, four sodium/solute symporters, a periplasmic [NiFe]-hydrogenase, carbon monoxide dehydrogenase, various oxidoreductases, including four with molybdopterin cofactors, and enzymes for biosynthesis of NAD. Genes that were downregulated encode α -type cytochromes, quinone oxidoreductases, and transporters and enzymes for ammonium uptake and amino acid biosynthesis, phosphate uptake, sulfur metabolism, and acyl-CoA metabolism, including enzymes for oxidation of propanoyl-CoA through succinyl-CoA. The gene for 4-hydroxyphenylacetate 3-hydroxylase was downregulated 2.6-fold.

A hypothetical pathway was reconstructed by combining these transcriptomic results with genome analysis. It was

apparent from the data that metabolism of phenylacetate to benzoyl-CoA proceeded in a manner similar to *Thauera aromatica* (Heider *et al.*, 1998; Schneider *et al.*, 1997) (Fig. 3). Phenylacetate was first ligated to CoA, forming phenylacetyl-CoA and AMP. Thirteen genes could potentially code for the acyl-CoA synthetase (Table S8); however, microarray experiments showed that only six of these 13 genes had at least twice as many mRNA transcripts during growth on phenylacetate compared with acetate. Within these six genes, only one was highly similar to previously described phenylacetate-CoA ligase proteins (Ferp_1228) and was expressed at significantly higher levels by phenylacetate-grown cells compared with acetate-grown cells (3.70-fold higher according to microarray studies and 6.34-fold higher according to qRT-PCR studies).

In *Thauera aromatica*, phenylacetyl-CoA is oxidized to phenylglyoxyl-CoA by phenylacetyl-CoA dehydrogenase, which is a membrane-bound molybdenum-iron-sulfur enzyme that uses a quinone as an electron acceptor (Rhee & Fuchs, 1999). BLAST alignment with previously characterized phenylacetyl-CoA dehydrogenase catalytic subunits found that Ferp_1257 was most similar to these characterized proteins. Ferp_1257 forms a predicted operon with genes coding for a *c*-type cytochrome (Ferp_1255) and a 4Fe-4S ferredoxin-like iron-sulfur cluster-binding domain protein (Ferp_1256). Ferp_1257 was not differentially expressed in microarray experiments comparing phenylacetate-grown cells to acetate-grown cells (Table S9). However, in qRT-PCR studies, 2.3 times more Ferp_1257 mRNA transcripts were detected in phenylacetate-grown cells. Three other molybdopterin-binding oxidoreductase genes were examined (Ferp_0124, Ferp_0094 and Ferp_1005). Ferp_1005 and Ferp_0124 are associated with operons that encode a membrane protein (Ferp_1007 and Ferp_0122) and a 4Fe-4S ferredoxin-like iron-sulfur cluster-binding domain protein (Ferp_1006 and Ferp_0123). Two of the molybdopterin-binding oxidoreductase genes (Ferp_0094 and Ferp_1005) showed over sixfold higher expression during growth with phenylacetate as an electron donor (Table S9). In addition, the entire operon associated with Ferp_1005 was upregulated significantly, making this gene the most likely candidate for catalysis of this step (Table S6).

In the next step, phenylglyoxyl-CoA is somehow converted to phenylglyoxylate. It is possible that a thioester hydrolase protein (EC 3.1.2.-) catalyses this step and there are three genes coding for this protein in the *F. placidus* genome (Ferp_0013, Ferp_1466 and Ferp_2009), none of which were differentially expressed during growth on phenylacetate compared with acetate according to microarray or qRT-PCR analyses. There is also a gene (Ferp_1227) found in a cluster with other genes potentially involved in phenylacetate catabolism that codes for a cysteine hydrolase family protein. Evidence that this gene is involved in this pathway comes from the fact that this gene had 26.6–33.5 times more transcripts when *F. placidus* was grown with phenylacetate compared with acetate. It is also possible that

an acyl-CoA-carboxylate CoA transferase couples the conversion of phenylglyoxyl-CoA to phenylglyoxylate with the conversion of phenylacetate to phenylacetyl-CoA, which is more energy efficient than ligation followed by hydrolysis. There are 13 genes coding for acyl-CoA-carboxylate CoA transferase proteins in the *F. placidus* genome, six of which (Ferp_0747–0748, Ferp_1163–1164 and Ferp_2468–2469) had more than twice as many transcripts in *F. placidus* cells grown with phenylacetate as the electron donor compared with acetate (Table S10).

The next step is the oxidative decarboxylation of phenylglyoxylate to benzoyl-CoA. In *Azoarcus evansii*, NAD-dependent phenylglyoxylate dehydrogenase is composed of five subunits (Hirsch *et al.*, 1998) that correspond to a four-subunit 2-oxoacid:ferredoxin oxidoreductase plus an FAD-binding pyridine nucleotide-disulfide oxidoreductase subunit that transfers electrons from the ferredoxin subunit to NAD. Of the two four-subunit 2-oxoacid:ferredoxin oxidoreductases predicted from the *F. placidus* genome, the one most similar to the NAD-dependent phenylglyoxylate dehydrogenase subunits is encoded by Ferp_0892–0895, which have high sequence identity to subunits of an enzyme of *Methanothermobacter marburgensis* that is specific for pyruvate (Tersteegen *et al.*, 1997). The other, encoded by Ferp_1823–1826, is of uncertain substrate specificity and might oxidatively decarboxylate phenylglyoxylate to benzoyl-CoA with ferredoxin as the electron acceptor. However, neither of these gene sets was upregulated in phenylacetate-grown cells, suggesting that this step might be catalysed by a nonhomologous enzyme.

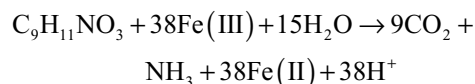
Two other gene sets (Ferp_1033–1034 and Ferp_1299–1300) encode ferredoxin oxidoreductases that could potentially catalyse oxidative decarboxylation of phenylglyoxylate to benzoyl-CoA with ferredoxin as the electron acceptor (Table S11). The number of Ferp_1033 mRNA transcripts was ~3.5 times greater in phenylacetate-grown cells than acetate-grown cells, suggesting that Ferp_1033–1034 may be involved in this step.

It is also possible that oxidoreductase proteins from another gene cluster (Ferp_1230–1232) are involved in formation of benzoyl-CoA. Not only were all three of these genes significantly upregulated in phenylacetate-grown cells, but also they are found in the *F. placidus* genome near other genes involved in phenylacetate metabolism, such as phenylacetate-CoA ligase (Erb *et al.*, 2008) (Ferp_1228). The Ferp_1230 protein may reduce NAD after receiving electrons from an iron-sulfur cluster-binding protein (Ferp_1231) that substitutes for ferredoxin with an aldehyde:ferredoxin oxidoreductase (Ferp_1232). Although the aldehyde:ferredoxin oxidoreductase might oxidize benzaldehyde to benzoate, the gene cluster does not encode an enzyme that could decarboxylate phenylglyoxylate to benzaldehyde. Therefore, proteins encoded by Ferp_1033–1034 may be more likely to carry out the direct conversion of phenylglyoxylate to benzoyl-CoA; however, further investigation into this possibility is required. Once benzoyl-CoA has

been synthesized, it enters the benzoyl-CoA reduction pathway for further degradation (Holmes *et al.*, 2012).

Tyrosine metabolism: initial steps via 4-hydroxyphenylacetate

Tyrosine as the sole electron donor supported growth of *F. placidus* with insoluble Fe(III) oxide as an electron acceptor with a generation time of 10.75 days (Figs 4a and S1). Reduction of 14.74 mM Fe(III) by oxidation of tyrosine (0.51 mM) accounted for 74 % of the electrons available according to the stoichiometry:



Although the anaerobic catabolism of tyrosine has not been as carefully studied as that of phenylalanine, the structural similarity of both amino acids suggests that their pathways are likely to be mechanistically similar. It has been reported that 4-hydroxyphenylpyruvate, 4-hydroxyphenylacetate and phenylacetate were formed as intermediates by a methanogenic consortium grown with tyrosine as an electron donor (Balba & Evans, 1980; Evans & Fuchs, 1988). Similar to the methanogenic consortium, 4-hydroxyphenylacetate was detected in cultures of *F. placidus* growing with tyrosine (Fig. 4b) and growth was observed when 4-hydroxyphenylacetate was provided as the sole electron donor with insoluble Fe(III) as electron acceptor (generation time 4.04 days) (Figs 4c and S1). Neither phenylacetate nor phenol was detected as an intermediate and genes coding for a tyrosine phenol-lyase (Kumagai *et al.*, 1970) are not present in the *F. placidus* genome. A hypothetical pathway is proposed for anaerobic tyrosine degradation by *F. placidus* based on these results (Fig. 5).

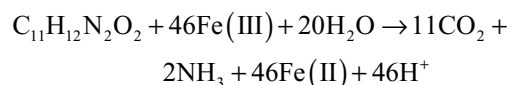
It is likely that the first step involves transfer of the amino group from tyrosine to 2-oxoglutarate to form 4-hydroxyphenylpyruvate and glutamate. As discussed above, the Ferp_0595 gene product is most likely to catalyse this step; however, there are seven other aminotransferases that could carry out this reaction (Table S3). For the next step, decarboxylation of 4-hydroxyphenylpyruvate, the candidate genes are Ferp_0092, Ferp_1624 and Ferp_1630. 4-Hydroxyphenylacetaldehyde is then oxidized to 4-hydroxyphenylacetate by one of several candidate aldehyde:ferredoxin oxidoreductases (Table S5).

The next steps are not clear; however, it is possible that CoA is ligated to 4-hydroxyphenylacetate to form 4-hydroxyphenylacetyl-CoA in a manner similar to denitrifying *Pseudomonas* species (Seyfried *et al.*, 1993). There are 13 different genes that could code for this protein (Table S8), four of which encode phenylacetate-CoA ligase homologues (Ferp_1228, Ferp_0992, Ferp_1946 and Ferp_2312). In the next steps, it is possible that a molybdopterin-binding

oxidoreductase catalyses the formation of 4-hydroxyphenylglyoxyl-CoA, followed by hydrolysis to 4-hydroxyphenylglyoxylate and oxidation to 4-hydroxybenzoyl-CoA by enzymes that are similar to those proposed in the phenylacetate degradation pathway (Tables S9–S11). Whilst it is known that *F. placidus* is capable of carrying out the dehydroxylation of 4-hydroxybenzoyl-CoA, as previously described for the degradation of phenol (Holmes *et al.*, 2012), the enzyme responsible for this activity is not clear. Homologues of previously characterized 4-hydroxybenzoyl-CoA reductases (Breese & Fuchs, 1998; Gibson *et al.*, 1997) are not present in the *F. placidus* genome; however, a gene that codes for an oxidoreductase protein (Ferp_0094), located near phenol catabolism genes, is a candidate. Alternatively, benzoyl-CoA reductase itself (Ferp_1184–1187) may dehydroxylate its substrate.

Tryptophan metabolism via indole-3-acetate

F. placidus grew with tryptophan as the sole electron donor and insoluble Fe(III) as the electron acceptor with a doubling time of 6.04 days (Figs 6a and S1). It was able to reduce 17.41 mM Fe(III) in the presence of 0.5 mM tryptophan. Therefore, 75.7 % of the electrons available from the complete oxidation of tryptophan were transferred to insoluble Fe(III) oxide according to this stoichiometry:



Pathways involved in the complete oxidation of tryptophan under strictly anaerobic conditions have not been well documented to date. Incomplete oxidation of tryptophan to indole-3-acetate via indole-3-pyruvate and indole-3-acetaldehyde has been observed in various plant-associated bacterial species and the hyperthermophilic archaeon *Sulfolobus* (Kaneshiro *et al.*, 1983; Spaepen *et al.*, 2007; Wakagi *et al.*, 2002). It has also been reported that a methanogenic consortium completely mineralized tryptophan to carbon dioxide and methane, and formed the intermediates indole-3-acetate, 2-aminobenzoate and benzoate (Balba & Evans, 1980; Evans & Fuchs, 1988). Tryptophan degradation by *F. placidus* most likely proceeds through indole-3-acetate (or its CoA thioester), as this key intermediate was detected in tryptophan-metabolizing cultures (Fig. 6b) and pure cultures of *F. placidus* could grow when indole-3-acetate was provided as the sole electron donor with insoluble Fe(III) as acceptor, with a doubling time of 4.96 days (Figs 6c and S1). In addition, indole was not detected as an intermediate and the genome of *F. placidus* did not contain a gene that could potentially code for a tryptophan indole-lyase (EC 4.1.99.1) found in a diversity of bacteria that can catalyse the conversion of tryptophan to indole, pyruvate and ammonia (DeMoss & Moser, 1969).

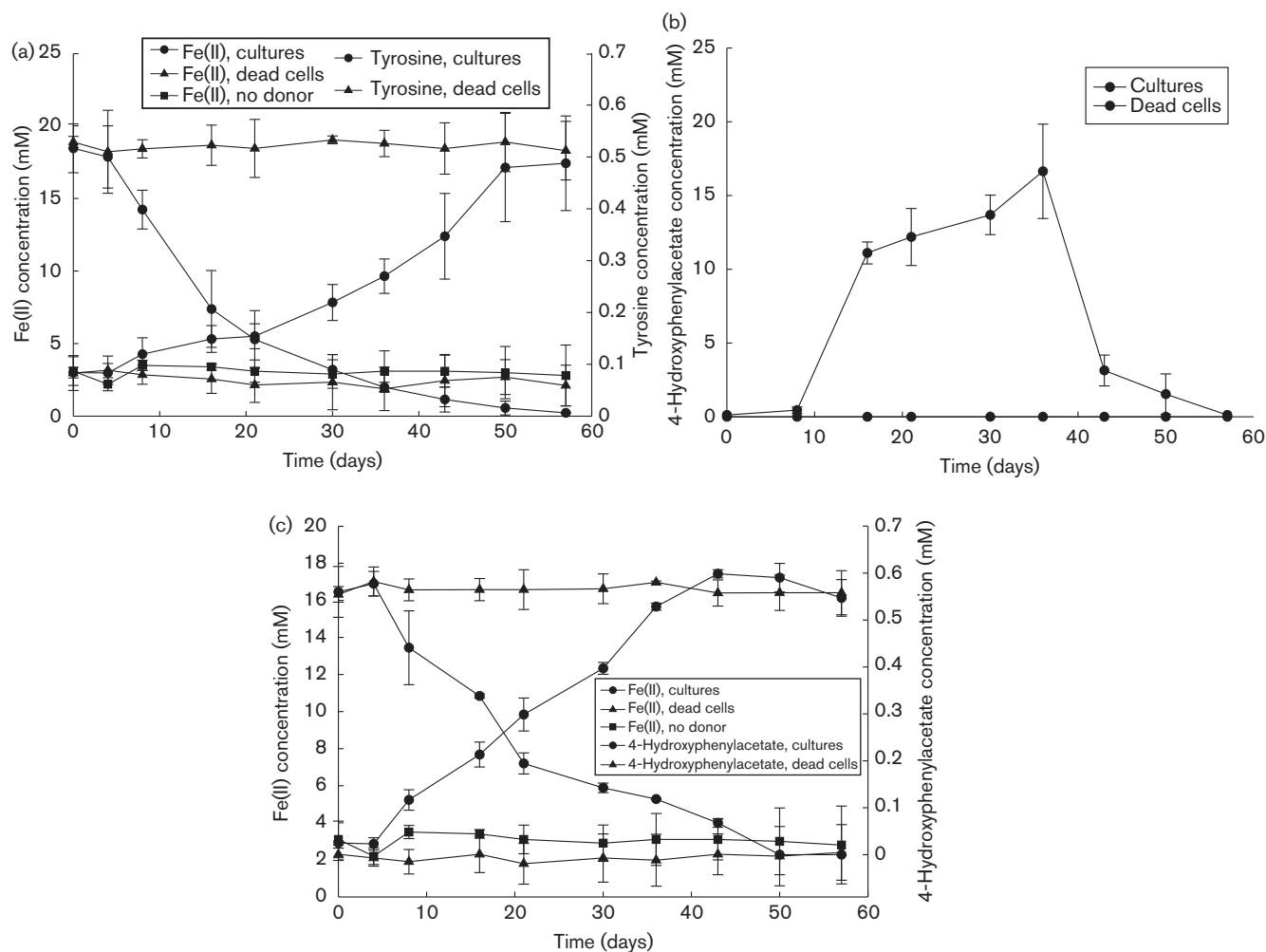


Fig. 4. (a) Fe(III) reduction and tyrosine consumption when *F. placidus* was grown with tyrosine (0.51 mM) as the electron donor and Fe(III) oxide (100 mM) as the electron acceptor. (b) Formation of 4-hydroxyphenylacetate as an intermediate in cultures grown with tyrosine as electron donor. (c) Fe(III) reduction and 4-hydroxyphenylacetate consumption when *F. placidus* was grown with 4-hydroxyphenylacetate (0.5 mM) as the electron donor and Fe(III) oxide (100 mM) as the electron acceptor.

It is likely that in the first step of the pathway, the amino group gets transferred from tryptophan to 2-oxoglutarate by an aminotransferase, forming indole-3-pyruvate and glutamate (Fig. 7). As discussed above, the Ferp_0595 gene product is most likely to catalyse this reaction. It is likely that indole-3-pyruvate is next converted to indole-3-acetyl-CoA. The *F. placidus* genome contains homologues for both subunits of an archaeal indole-3-pyruvate ferredoxin: oxidoreductase (Ferp_1033–1034) that can also oxidatively decarboxylate phenylpyruvate and form phenylacetyl-CoA (Schut *et al.*, 2001; Siddiqui *et al.*, 1997).

Once the CoA thioester of indole-3-acetate is formed, the subsequent steps could be similar to those proposed for indole-3-acetate degradation by *Aromatoleum aromaticum* EbN1 (Ebenau-Jehle *et al.*, 2012). In fact, similar to this denitrifying mesophile, most of the genes implicated in indole-3-acetyl-CoA degradation in *F. placidus* are clustered

in the genome. First, in *Aromatoleum aromaticum*, a molybdoenzyme of the xanthine dehydrogenase family composed of three subunits (*iaaIJK*) may oxidize indole-3-acetate to 2-oxoindole-3-acetate. Whilst the *F. placidus* genome does not contain homologues for these genes, there are four uncharacterized molybdopterin-binding oxidoreductases that are not aldehyde:ferredoxin oxidoreductases (Ferp_0094, Ferp_0122–0124, Ferp_1005–1007 and Ferp_1100). One of these enzymes might oxidize indole-3-acetyl-CoA to 2-oxoindole-3-acetyl-CoA.

Cleavage of the indole ring is a critical step in the tryptophan pathway. In *Aromatoleum aromaticum* EbN1, this is proposed to be accomplished by an ATP-dependent hydantoinase (*iaaCE*) (Ebenau-Jehle *et al.*, 2012). Similar to strain EbN1, the *F. placidus* genome contains genes that code for both subunits of an ATP-dependent hydantoinase/oxoprolinase domain protein (Ferp_2326–2327),

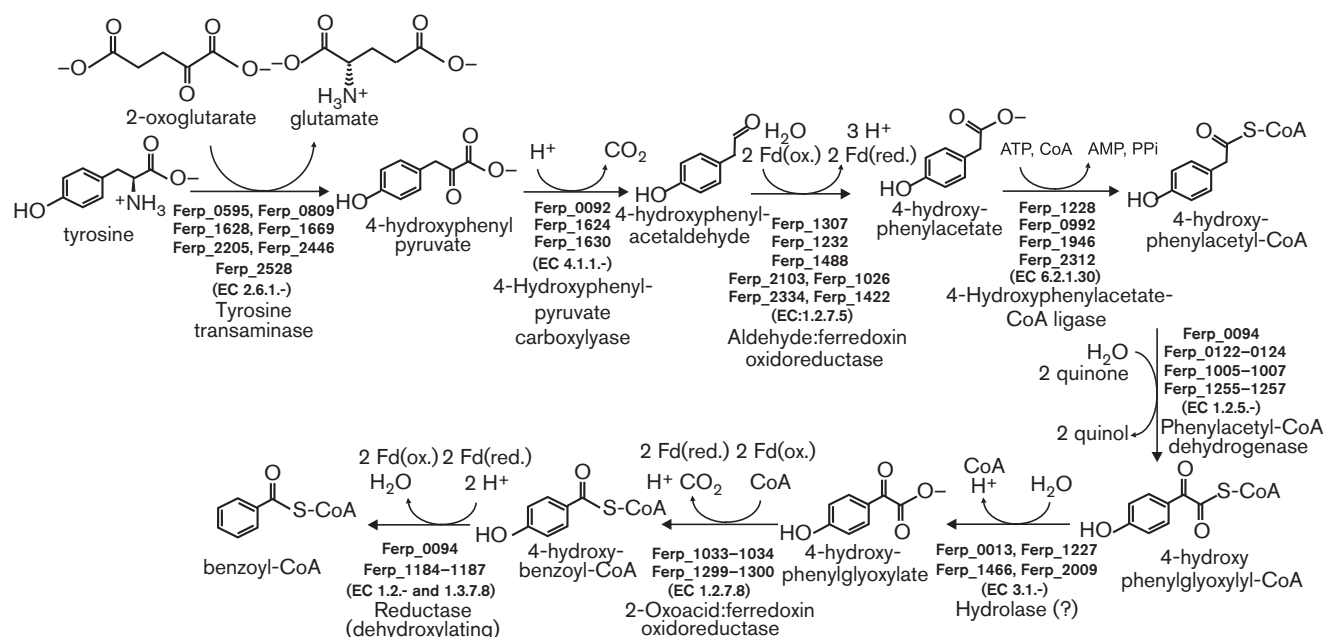


Fig. 5. Proposed pathway utilized by *F. placidus* to oxidize tyrosine to benzoyl-CoA.

which might hydrolyse 2-oxoindole-3-acetyl-CoA to 3-(2'-aminophenyl)succinyl-CoA. At this point, the predicted pathway in *Aromatoleum aromaticum* includes a thioesterification reaction to produce 2-(2'-aminophenyl)succinyl-CoA, but it is also possible that the intermediate is 3-(2'-aminophenyl)succinyl-CoA in both species. In the next step, 3-(2'-aminophenyl)succinyl-CoA could be rearranged to form 2-aminobenzylmalonyl-CoA by a coenzyme B12-dependent mutase, which may be encoded by Ferp_2330 and Ferp_2331. Oxidative decarboxylation by an acyl-CoA dehydrogenase protein (Ferp_2329) would produce 3-(2'-aminophenyl)acrylyl-CoA. The next two steps, i.e. hydration of the double bond and oxidation of the hydroxyl group, may be catalysed by a bifunctional protein (Ferp_1035), forming 3-(2'-aminophenyl)-3-oxopropanoyl-CoA. This compound would be split by a thiolase (Ferp_2323 or Ferp_1036) to form acetyl-CoA plus 2-aminobenzoyl-CoA.

In the final step, the amino group needs to be removed to form benzoyl-CoA, which can then enter the benzoyl-CoA reductase pathway for further degradation. Ferp_2318 encodes a flavin-binding NADPH-dependent oxidoreductase that could potentially remove the amino group via reductive deamination.

Genes of the benzoyl-CoA reductase pathway are upregulated during growth on phenylacetate

In *F. placidus*, the predicted catabolic pathways for three aromatic amino acids (phenylalanine, tyrosine and tryptophan) converge at benzoyl-CoA – a key metabolite formed during anaerobic degradation of all known benzene ring-containing aromatic compounds. Once benzoyl-CoA is

formed, it is further metabolized to glutaryl-CoA by the benzoyl-CoA reductase pathway, for which genes have been identified in previous studies (Holmes *et al.*, 2012). As expected, all of these genes were upregulated in phenylacetate-grown cells compared with acetate-grown cells (Table S12).

Glutaryl-CoA degradation pathway

Although the enzymes involved in the benzoyl-CoA reductase pathway were elucidated previously, the subsequent pathway of glutaryl-CoA degradation (Fig. 8) has not yet been described in *F. placidus*. Ferp_1566, which was previously speculated to encode pimelyl-CoA dehydrogenase, is homologous to the decarboxylating glutaryl-CoA dehydrogenase of *Pseudomonas putida*, which produces crotonyl-CoA directly (Härtel *et al.*, 1993). The other possibility, i.e. a non-decarboxylating glutaryl-CoA dehydrogenase, is unlikely because no candidate for an (*E*)-glutaconyl-CoA decarboxylase to produce crotonyl-CoA was found in the *F. placidus* genome. Ferp_1566 was upregulated 4.72- and 8.04-fold in phenylacetate-grown cells according to microarray and qRT-PCR analyses, respectively, making this the most likely candidate for this step.

In the next step of the pathway, a water molecule is added to crotonyl-CoA by an enoyl-CoA hydratase, forming (*S*)-3-hydroxybutanoyl-CoA. There are five enoyl-CoA hydratase proteins that could fulfil this role (Table S13). Two of these genes were upregulated in phenylacetate-grown cells, Ferp_1035 and Ferp_1031, but Ferp_1031 has homology to cyclohex-1-enecarbonyl-CoA hydratase and is predicted to

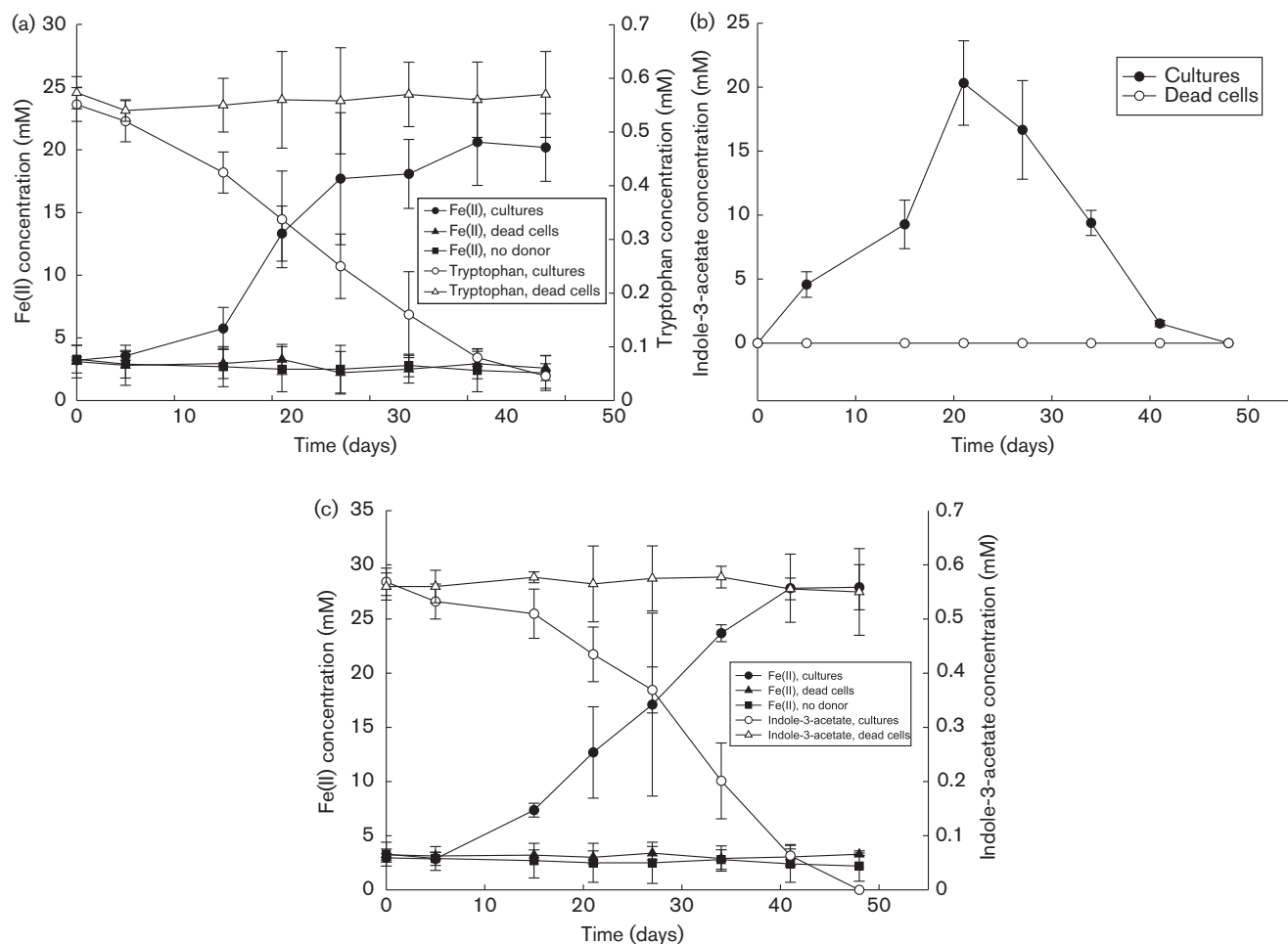


Fig. 6. (a) Fe(III) reduction and tryptophan consumption when *F. placidus* was grown with tryptophan (0.5 mM) as the electron donor and Fe(III) oxide (100 mM) as the electron acceptor. (b) Formation of indole-3-acetate as an intermediate in cultures grown with tryptophan as electron donor. (c) Fe(III) reduction and indole-3-acetate consumption when *F. placidus* was grown with indole-3-acetate (0.5 mM) as the electron donor and Fe(III) oxide (100 mM) as the electron acceptor.

function in the benzoyl-CoA reductase pathway (Holmes *et al.*, 2012). Electrons are then transferred by 3-hydroxybutyryl-CoA dehydrogenase to NAD^+ , forming acetoacetyl-CoA. There are four genes potentially coding for 3-hydroxybutyryl-CoA dehydrogenase: Ferp_1002, Ferp_1035, Ferp_1942 and Ferp_2322; however, only Ferp_1035 and Ferp_2322 had more transcripts during growth with phenylacetate as the electron donor (4.76- and 10.59-fold and 2.56- and 6.32-fold according to transcriptomic analysis).

Finally, an acetyl group is transferred by acetoacetyl-CoA thiolase to form two molecules of acetyl-CoA, which can then enter the citric acid cycle or Wood-Ljungdahl pathway for further degradation. There are nine genes coding for thiolases; however, only three of these genes were differentially expressed: Ferp_1036, Ferp_1189 and Ferp_2323 (Table S14). Both Ferp_1036 and Ferp_1189 are located in clusters with genes from the benzoyl-CoA reductase pathway, whilst Ferp_2323 is found in an operon

with a 3-hydroxybutyryl-CoA dehydrogenase protein (Ferp_2322). Therefore, Ferp_2323 is the most likely candidate for this step.

DISCUSSION

It is apparent from these results that anaerobic amino acid degradation pathways are relatively conserved across the domains Bacteria and Archaea. Although the majority of studies have focused on partial oxidation pathways in such genera as *Clostridium* and *Fusobacterium* (Barker, 1981; Kim *et al.*, 2004; Russell *et al.*, 2013), several isolates have been shown to oxidize amino acids completely to carbon dioxide with a variety of compounds as electron acceptors.

F. placidus is a representative of this group of prokaryotes that can couple the complete oxidation of an amino acid with cellular respiration, it also appears to utilize more amino acids as electron donors than any other prokaryote

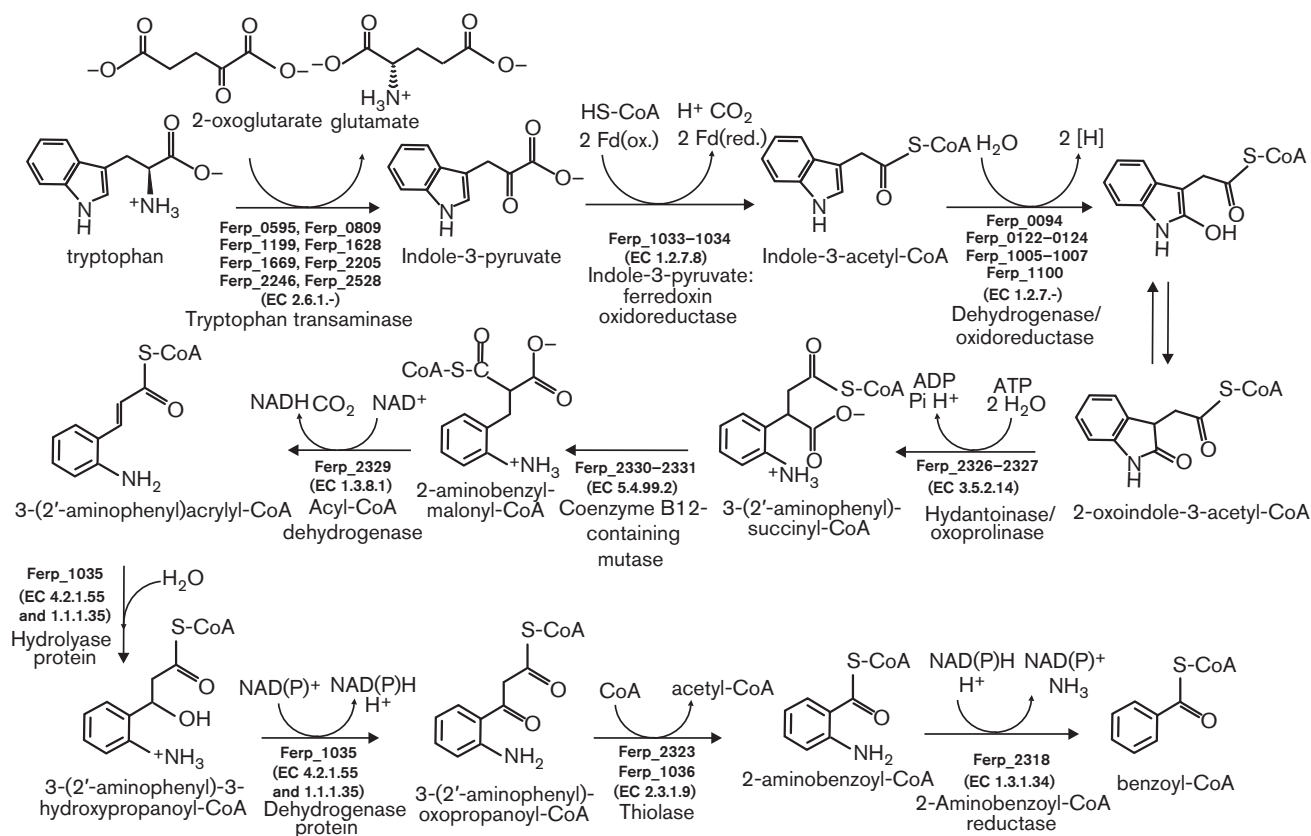


Fig. 7. Proposed pathway utilized by *F. placidus* to oxidize tryptophan to benzoyl-CoA.

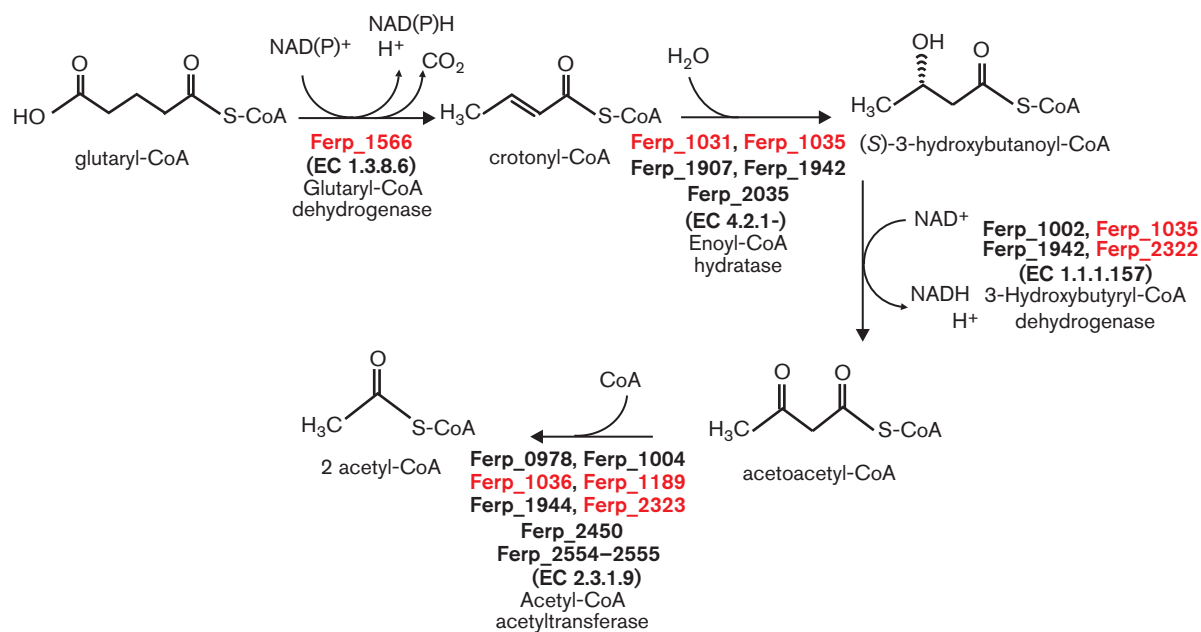


Fig. 8. Proposed pathway utilized by *F. placidus* to oxidize glutaryl-CoA to acetyl-CoA. Genes highlighted in red were upregulated in phenylacetate-grown cells.

with this type of metabolism. Whilst *Desulfobacula* and *Desulfotignum* species can convert phenylalanine into carbon dioxide with sulfate and/or thiosulfate as an electron acceptor, they cannot utilize the other aromatic amino acids, tyrosine or tryptophan, as electron donors (Bak & Widdel, 1986; Kuever *et al.*, 2001), and *Geopsychrobacter electrodiphilus* and *Geoglobus ahangari* can only utilize a handful of amino acids (none of them aromatic) as their sole carbon source (Holmes *et al.*, 2004b; Kashefi *et al.*, 2002). *F. placidus*, however, can anaerobically mineralize 15 out of the 20 proteinogenic amino acids. This wide range of amino acids includes polar, non-polar, positively charged, negatively charged and, most notably, all of the aromatic amino acids except histidine.

The pathways utilized by *F. placidus* for amino acid degradation are rather eclectic. The genome of this organism has genes that encode proteins that are homologous to enzymes from both partial and complete oxidation pathways. For example, *F. placidus* has several genes that code for enzymes found in the *Thermococcales* α -amino acid partial oxidation pathway (Table S2), and it possesses homologues for subunits from the 2-hydroxyacyl-CoA dehydratase complex HgdAB (Ferp_1042–1043) involved in amino acid fermentation by *Clostridia*, *Fusobacteria* and *Archaeoglobus* species (Kim *et al.*, 2004; Parthasarathy *et al.*, 2013). Meanwhile, this organism's genome also appears to contain genes that code for enzymes from aromatic amino acid degradation pathways associated with mesophilic nitrate-reducing, sulfate-reducing and phototrophic bacteria (DiDonato *et al.*, 2010; Ebenau-Jehle *et al.*, 2012; Fuchs *et al.*, 2011; Jiang *et al.*, 2012; Larimer *et al.*, 2004; Rabus *et al.*, 2005; Schneider *et al.*, 1997). Furthermore, these genes are arranged in clusters within the *F. placidus* genome – another feature shared with mesophilic aromatic amino-acid-degrading bacteria.

This functional conservation between a hyperthermophilic archaeon and mesophilic bacteria is intriguing. Based on GC content alone, it is unlikely that *F. placidus* acquired many of these genes through lateral gene transfer. The mean GC content of the *F. placidus* genome is 44 % and all of the genes from these pathways fall within 7 % of this percentage. In addition, relatively few mobile genetic elements were present (Table S15) and no plasmid or proviral sequences [scanned by PHAST (Zhou *et al.*, 2011) and Prophinder (<http://aclame.ulb.ac.be/Tools/Prophinder/>) software] were detected.

It is also extremely unlikely that these organisms were ever in direct contact as they inhabit completely different environments: *F. placidus* was isolated from hydrothermal vent marine sediments near Vulcano Island, Italy (Hafenbradl *et al.*, 1996), strain NaphS2 was isolated from marine sediments along the North Sea coastline, Germany (Galushko *et al.*, 1999), *Thauera aromatica* was isolated from sludge collected from a municipal sewage plant located in Konstanz, Germany (Anders *et al.*, 1995), *Aromatoleum aromaticum* was isolated from ethylbenzene-contaminated

freshwater sediments in Bremen, Germany (Rabus & Widdel, 1995), and *Rhodospseudomonas palustris* has been isolated from various mesophilic soil and sediment samples (Larimer *et al.*, 2004).

Not only do these organisms share homologous proteins involved in aromatic amino acid degradation, but also they utilize similar pathways for degradation of other aromatic compounds, such as ethylbenzene, benzoate and phenol (Anderson *et al.*, 2011; Breese *et al.*, 1998; Carmona *et al.*, 2009; Eglund *et al.*, 1997; Harrison & Harwood, 2005; Holmes *et al.*, 2012; Kube *et al.*, 2004; Rabus *et al.*, 2002). As predicted for the aromatic amino acids tryptophan, tyrosine and phenylalanine, benzoyl-CoA is formed as an intermediate during anaerobic degradation of these other aromatic compounds. The conservation of these pathways across different domains of life is probably the result of convergent evolution. Evidence of this comes from the fact that several of the predicted proteins involved in degradation of these aromatic compounds are quite different.

For example, whilst many of the mesophilic enzymes utilize NAD(P)^+ as electron carriers, NAD(P)^+ is rarely seen in the reactions catalysed by *F. placidus* that are described here. Rather, many of the oxidoreductase proteins of *F. placidus* transfer their electrons to a ferredoxin protein. Adenine dinucleotide coenzymes such as NAD(P)^+ are structurally complex (Gazzaniga *et al.*, 2009; Huang *et al.*, 2000), whilst 4Fe–4S clusters are amongst the simplest electron transfer groups. They are thought to have been amongst the earliest redox-active molecules to have emerged on the primitive Earth where conditions were most similar to those encountered in environments that hyperthermophilic archaea occupy (Kim *et al.*, 2012). These proteins are also extremely thermostable and can withstand high temperatures, such as those found in environments where organisms like *F. placidus* thrive. Other hyperthermophiles also have ferredoxin-dependent enzymes that carry out the same role as NAD-dependent enzymes in mesophilic bacteria. For example, many mesophilic bacteria use a pyruvate dehydrogenase complex to decarboxylate pyruvate to acetyl-CoA and transfer their electrons to NAD^+ , whilst *Ferroglobus*, *Archaeoglobus* and *Thermococcales* species all appear to use a ferredoxin oxidoreductase to catalyse this reaction (Blamey & Adams, 1993; Eram *et al.*, 2014; Kunow *et al.*, 1995).

Another striking feature of *F. placidus* is the fact that candidate genes of the aromatic amino acid degradation pathways in *F. placidus* appear to encode both molybdoenzymes and tungstoenzymes, whilst most of the mesophilic bacteria use only molybdoenzymes. Although *Aromatoleum aromaticum* uses both a molybdenum- and tungsten-containing enzyme for oxidation of phenylacetaldehyde to phenylacetate (Debnar-Daumler *et al.*, 2014), it is possible that *Aromatoleum aromaticum* acquired this gene from an archaeon as it has high genome plasticity and evidence of extensive lateral gene transfer events over its evolution (Rabus *et al.*, 2005). Tungstoenzymes are not

very common amongst mesophilic bacterial species, but are frequently associated with hyperthermophilic archaea (Bever *et al.*, 2009).

It does not seem too surprising that such a diversity of micro-organisms have acquired the ability to degrade aromatic amino acids anaerobically. They are abundant carbon sources in many oxygen-limiting environments and organisms that can degrade these compounds will have a competitive advantage. Prior to this study, no other hyperthermophilic archaeon had been shown to oxidize any of the aromatic amino acids completely. Therefore, *F. placidus* is likely to occupy a niche within hyperthermophilic environments that deserves more attention and studies of micro-organisms such as *F. placidus* will provide invaluable insight into the ecology of hot environments where conditions are similar to those of the early Earth.

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