Supplementary information for:

One-megadalton metalloenzyme complex involved in benzene ring reduction beyond the biological redox window

Simona G. Huwiler, Claudia Löffler, Sebastian E. L. Anselmann, Hans-Joachim Stärk, Martin von Bergen, Jennifer Flechsler, Reinhard Rachel, and Matthias Boll

Corresponding author: Matthias Boll
Email: matthias.boll@biologie.uni-freiburg.de

This PDF file includes:

www.pnas.org/cgi/doi/10.1073/pnas.1819636116

Supplementary Materials and Methods

Cultivation of cells. *E. coli* strains were grown in LB medium with 100 μg mL^{-1} kanamycin sulfate or 100 μg mL^{-1} ampicillin sodium salt and incubated over night at 37 °C. *G. metallireducens* GS-15 (DSMZ 7210) wild type and *G. metallireducens* GS-15 pGM2087e1 were anaerobically cultivated in a 200-l fermenter at 30°C or 28°C in a mineral salt medium (1) with benzoate (1-5 mM) as sole carbon source and nitrate (3-15 mM) as electron acceptor. Cells were harvested by centrifugation (20,000×*g*) in the exponential growth phase. *G. metallireducens* GS-15 pGM2087e10 was grown in a 1.8 L volume in the same media. The genetically modified *G. metallireducens* GS-15 strains were grown in the same media with 50 μg ml^{-1} kanamycin sulfate in the presence of a fivefold concentration of selenium and tungsten (0.114 μM Na_2SeO_3 and 0.121 μM Na_2WO_4). *G. metallireducens* cells were stored in liquid nitrogen.

Homologous expression of single and Twin-Strep-tagged BamB. General molecular biology techniques were performed according to standard protocols (2). The genomic DNA of *G. metallireducens* GS-15 was obtained by chlorophorm/phenol/isoamylalcohol extraction as described in (3). For homologous expression of single and Twin-Strep-tagged BamB expression plasmids pGM2087e1 and pGM2087e10 were generated (Table S9). All PCR-amplification reactions from genomic or plasmid DNA were performed with Pfu-polymerase. For construction of pGM2087e1 the 198-bp upstream region of *bamB* and *bamB* (gene locus: Gmet_2087 (former), GMET_RS10525 (new)) were amplified from *G. metallireducens* GS-15 genomic DNA with a primer-encoded C-terminal Strep-tag II ® using primers bamB_22_for (5'- ATCCCCCTCGAGCGGCAGCGCGCCCGGTG -3') and bamB_3_rev (5'- TCTAGAACTAGTTCATTTTTCGAACTGCGGGTGGCTCCAACCGGCCGACAGAATCCCG -3'). In primer sequences restriction sites are underlined. For generation of pGM2087e10 *bamB* was amplified from genomic DNA of *G. metallireducens* GS-15 with primer pair pPR_B_69_for (5'- GAATTCGAGCTCTGACCTAAGGAGGTAAATAATGAGGTATGCAGAGACCGGA -3') and pPR_B_70_rev (5'- GTCGACCTCGAGACCGGCCGACAGAATCCCG -3') for insertion into pPR-IBA101, resulting in pPR-GM2087e10. The later plasmid was used as a template for amplification with primers bamB_84_for (5'- TGCCGCTCAAGCTTTCCATG -3') and bamB_85_rev (5'- GGCCGCTCTAGATTATTATTTTTCGAACTGCGGGT -3') to clone the resulting *HindIII* / *XbaI* PCR fragment, containing the last 659 bp of *bamB* with attached sequence encoding a C-terminal linker LEVDLQGDHGLSA and a Twin-Step-tag® into pGM2087e10. PCR products, cut insert and according source plasmid were purified either by precipitation or by the Wizard® SV gel and PCR clean-up system (Promega, Mannheim, D) or the QIAquick® gel extraction kit (QIAGEN GmbH, Hilden, D) according to

manufacturer's instructions. Insert PCR products and source plasmids were digested with the respective restriction enzymes (New England BioLabs® Inc. (NEB), Ipswitch, Massachusetts, USA). The cut vectors were dephosphorylated with Antarctic phosphatase (NEB). Ligation was performed with T4 DNA ligase (fermentas). Ligation mixtures were transformed into *E. coli* JM109 or TOP10 by heat shock or electroporation and cell mixture was plated on LB agar plates with appropriate antibiotics. Insert length of target plasmids were verified by a PCR using biomass of single colonies with GoTaq Green Master Mix (Promega) and appropriate primers. Plasmids were purified from the according *E. coli* strains with the Wizard® *Plus* SV Minipreps DNA purification system (Promega) or the QIAprep® Spin Miniprep kit (Qiagen) according to manufacturer's instructions. Insert and adjoining vector regions were verified by Sanger sequencing. Electroporation of *G. metallireducens* GS-15 was performed according to (4) whereas mineral medium with benzoate as carbon source was used in all steps. For preparation of electrocompetent *G. metallireducens* GS-15 harvesting and washing of the cells occurred at ~2,900×*g*. For transformation at 17 kV cm^{-1} 100 ng of pGM2087e1 or 250 ng of pGM2087e10 was used. Additional control PCRs on cell mass were performed to check for the presence of the plasmid insert of pGM2087e1 or pGM2087e10 in the cultures itself with appropriate primers and GoTaq Green Master Mix (Promega). All enrichments were performed at 4-17°C in an anaerobic glove box (N_2:H_2, 95:5 by volume).

Enrichment of Strep-tagged BamB. Bam($B_{Strep}C)_2$ was purified using either a Strep-Tactin® Superflow® high capacity column followed by a Source 15Q anionic exchange column (GE Healthcare, Munich, D), and used for determination of K_m–values or ICP-MS analysis. Alternatively, it was obtained as a side product from the Bam[($B_{Strep}C)_2$DEF]$_2$ subcomplex purification by collecting and concentrating a peak eluting between 62-72 ml during size exclusion chromatography (for all other analyses, see section 'Enrichment of Bam[($B_{Strep}C)_2$DEF]$_2$'). Between 25-90 g cells (wet weight) of *G. metallireducens* GS-15 pGM2087e1 were suspended 1:2 (w/v) in cell disruption buffer (20 mM triethanolamine (TEA)/NaOH, 5 mM $MgCl_2$, 500 mM KCl, pH 7.8) containing 0.02 mg mL^{-1} DNaseI, 0.05 mg ml^{-1} DTE and 1 mg mL^{-1} lysozyme. After ultracentrifugation (optima L-60, rotor 60 TI, Beckman Coulter GmbH, Krefeld, D) for 1 h at 100,000×*g* the supernatant was removed and the membrane fraction was washed with wash buffer (20 mM TEA/NaOH, 5 mM $MgCl_2$, 150 mM NaCl, pH 7.8). After the second centrifugation for 1 h at 100,000×*g* both supernatants were pooled and centrifuged again for 10 min at 2,900-3,800×*g*. The supernatant of the later centrifugation was loaded with 0.5-1 mL min^{-1} onto a wash buffer pre-equilibrated 25-70 ml Strep-Tactin® Superflow® high capacity column. The column was washed with wash buffer for 2-3 column volumes at 0.5-1 mL min^{-1} before elution with wash buffer containing 5 mM D-desthiobiotin. The elution peak fraction was further purified by a 5-mL or 15-mL Source 15Q column, pre-equilibrated with wash buffer. The elution fraction of the Strep-Tactin column was applied with a

50 mL super loop. The loading and purification on the column was performed at 2 or 4-5 mL min^{-1}. After washing with one column volume with wash buffer, a linear gradient over fifty column volumes was applied from 150-300 mM NaCl. The $Bam(B_{Strep}C)_2$-containing fraction eluted at ~175 mM NaCl, and was concentrated in 30-kDa microconcentrators (Vivaspin 6, Sartorius, Göttingen, D) by centrifugation at ~2,900×*g*. $Bam(B_{Strep}C)_2$ was anaerobically stored at –80°C or in liquid nitrogen after addition of 5 % (w/v) polyethylene glycol 4000 (final concentration).

Enrichment of Twin-Strep-tagged $BamB_{2xStrep}C$. $BamB_{2xStrep}C$ was purified from 1.3 g of *G. metallireducens* GS-15 pGM2087e10 wet cells with the same protocol as used for $BamB_{Strep}C$ with a Strep-Tactin® column. A 1-mL Strep-Tactin® Superflow® high capacity column was used and washed for 12 column volumes after loading. A different cell disruption buffer (100 mM Tris/HCl, 500 mM NaCl, 5 mM $MgCl_2$, pH 8.0) and wash buffer (100 mM Tris/HCl, 500 mM NaCl, pH 8.0) were used.

Enrichment of $Bam(BC)_2$. Purification of $Bam(BC)_2$ from *G. metallireducens* GS-15 wild type for comparison was performed as described (5).

Synthesis and analysis of CoA esters. Benzoyl-CoA was synthesized from benzoic acid anhydride and CoA (6). 1,5-Dienoyl-CoA was synthesized enzymatically with enriched BCR from *Thauera aromatica* from benzoyl-CoA and the product was purified by preparative HPLC as described earlier (7). UPLC-ESI-Q-TOF analyses of benzoyl-CoA and 1,5-dienoyl-CoA was carried out as recently described (7).

DCO and NOR activity assays. The 1,5-dienoyl-CoA:methyl viologen oxidoreductase (DCO) activity was determined as described earlier with 0.1 or 0.2 mM 1,5-dienoyl-CoA and 0.5 mM methyl viologen at pH 6.8 (5). The time dependent reduction of methyl viologen was followed at 730 nm ($\Delta\varepsilon$= 2,400 M^{-1} cm^{-1}). The typical assay contained 150 mM Mops/KOH (pH 7.2), 15 mM $MgCl_2$, 150 mM NaCl, 2 mg mL^{-1} BSA, 1 mM methyl viologen, 0.1 mM 1,5-dienoyl-CoA, and 0.7-3 µg enzyme sample. Analogously, the NAD(P)H:benzyl viologen oxidoreductase (NOR) activity was determined by measuring the absorbance increase caused by dye reduction ($\Delta\varepsilon_{600}$ = 7,400 M^{-1} cm^{-1}) in the presence of 0.5 mM NAD(P)H and 1 mM benzyl viologen. K_m-value for NADH was determined by altering NADH concentrations from 15-480 µM and by fitting the initial rates to Michaelis-Menten curves using the Prism software package (GraphPad, San Diego, CA). In discontinuous assays substrate consumption and product formation was analyzed by HPLC. Such assays were carried out in reaction tubes in a glove box at 30°C under a N_2/H_2 atmosphere (95 % /5 %). Samples were taken at different time points, stopped with 2 volumes methanol and analyzed by HPLC as described (8). K_m for DCO activity of $Bam(B_{Strep}C)_2$ was determined in a

slightly modified reaction buffer (175 mM MOPS/NaOH, 3.5 µM $MgCl_2$, 150 mM NaCl, 1.75 mg ml^{-1} BSA, pH 6.8) with 1 mM methyl viologen and 1,5-dienoyl-CoA concentrations ranging from 10–100 µM. Two to three measurements of DCOR activity at one specific 1,5-dienoyl-CoA concentration were made. Evaluation was carried out with Prism as indicated above.

Preparation of crude extracts for purification the class II benzoyl-CoA reductase complex $Bam[(BC)_2DEFGHI]_2$ from wild type *G. metallireducens* and subcomplex $Bam[(B_{Strep}C)_2DEF]_2$. For the preparation of crude extract 2-50 g cells (wet weight) were anaerobically suspended in buffer A (50 mM potassium phosphate, 5 mM $MgCl_2$), pH 6.5-7.5 (1-2 ml g^{-1} cells) containing 100 mM KCl, 10 mM dithioerythriol, 1-20 mg DNAse I; for enrichment of Strep-tagged components of *G. metallireducens* GS-15 pGM2087e1 by affinity chromatography 2.4 % Triton X-100 (w/v) was additionally added. Cell lysis was accomplished by stirring at 4° C for 1 h using 2-50 mg lysozyme. After centrifugation with 100,000×*g* (1 h at 4°C) the supernatant was used for further studies. In case of purification with help of $BamB_{Strep}$, the pellet was washed in buffer A containing 100 mM KCl, pH 7.5 and centrifuged again (100,000*g*, 1 h at 4°C). The supernatants were combined.

Enrichment of $Bam[(B_{Strep}C)_2DEF]_2$. The 100,000×*g* supernatant of 30 g cells (wet mass) of *G. metallireducens* GS-15 pGM2087e1, producing $BamB_{Strep}$, was applied to a Strep-Tactin Superflow high capacity column (25 ml volume, IBA), which had been equilibrated with buffer B (50 mM potassium phosphate, 5 mM $MgCl_2$, 100 mM KCl, pH 7.5). After washing the column at a flow rate of 1 mL min^{-1} with 2 column volumes of buffer B the protein was eluted with 5 mM desthiobiotin in buffer B. The 30 mL eluate of the affinity column was concentrated in microconcentrators (10-kDa exclusion limit, Vivaspin 6, Sartorius, Göttingen, D) by centrifugation to 1.5 ml and applied in two runs to a Superdex 200 column (1 cm diameter, 120 mL column volume, 0.5 mL min^{-1} flow rate, GE Healthcare), which had been equilibrated with buffer B. The $Bam[(B_{Strep}C)_2DEF]_2$ complex eluted between 47-60 ml, $Bam(B_{Strep}C)_2$ eluted between 62-72 mL. The enzymes were concentrated to 12-25 mg mL^{-1} and stored in anaerobic glass vials at –80°C.

Enrichment of $Bam[(BC)_2DEFGHI]_2$ from wild type. After centrifugation with 100,000×*g* the supernatant of 50 g wild type cells (wet weight) was applied step-by-step to a DEAE Sepharose column (Fast Flow, 35 ml volume, 2.6 cm diameter, GE Healthcare) which had been equilibrated with buffer A, pH 6. Each 10-ml supernatant volume was applied and the column was washed with 10 ml buffer A with 200 mM KCl at pH 6. After the last application step the column was washed with 2 column volumes buffer A with 200 mM KCl at pH 6. A step gradient with one column volume of buffer A with 225, 250,

275, 300 and 325 mM KCl was used for protein elution. The fractions containing DCO and NOR activity eluted from 275-325 mM KCl within 76 ml. The fractions were concentrated in microconcentrators (10 kDa exclusion limit) by centrifugation to 1 ml and applied in two fractions to a Superdex 200 (1 cm diameter, 120 ml column volume, 0.5 ml min^{-1} flow rate), which had been equilibrated with buffer A with 100 mM KCl at pH 6. The DCO and NOR activity eluted between 45-51 mL. The enzymes were concentrated to 10 mg mL^{-1} and stored in anaerobic glass vials at –80°C. The purification was also performed by addition of 5 µM FAD, 5 µM FMN and 2 mM DTE in each purification buffer.

Enrichment of ferredoxin from *G. metallireducens*. To purify ferredoxin (Fd) from *G. metallireducens* the 100,000×*g* supernatant of 20 g wild type cells (wet weight) was applied to a DEAE Sepharose column (35 ml volume, 2.6 cm diameter), which had been equilibrated with buffer A at pH 7.2. The column was washed at a flow rate of 1 mL min^{-1} with one column volume of buffer A at pH 7.2, followed by one column volume buffer A at pH 7.2 with 100 and 200 mM KCl each. The brownish fraction was eluted in a third step gradient at 300 mM KCl in buffer A at pH 7.2 within 24 ml. The fraction was concentrated in microconcentrators (10-kDa exclusion limit) by centrifugation to 1 mL and applied to a Superdex 200 (1 cm diameter, 120 ml column volume, 0.5 ml min^{-1} flow rate), which had been equilibrated with 100 mM KCl in buffer A at pH 7.2. The low molecular weight fraction with a dark brownish color eluted in a volume of 12 mL. This fraction was applied to a MonoQ 5/50 column (1 ml volume, 0.5 ml min^{-1} flow rate, GE Healthcare) which was washed with 5 column volumes of buffer A. Fd eluted in a linear gradient from 0-500 mM KCl in buffer A at pH 7.2 over 30 column volumes between 280-410 mM KCl. The concentration of purified Fd was determined by using the molar extinction coefficient of ε_{390nm} = 30,000 M^{-1} cm^{-1} (9). The 2-oxoglutarate:Fd oxidoreductase from *T. aromatica* was enriched as described (10).

Enzyme assays with enriched class II BCR complexes other than DCO and NOR activity. 1) Reduction of Fd by 1,5-dienoyl-CoA: The assay was carried out in anaerobic quartz cuvettes (100% N_2) at room temperature. The mixture contained buffer A (pH 6), 2 mg mL^{-1} BSA, 0.1 mM 1,5-dienoyl-CoA, 10-50 µM Fd and 1.5-5 µg class II BCR. Reduction of Fd was followed by the absorbance decrease at 390 nm. 2) Reduction of NAD^+ by 1,5-dienoyl-CoA: The assay was carried out in reaction tubes in the glove box at 30°C under a N_2/H_2 atmosphere (96% /4%). The mixture contained buffer A (pH 6), 2 mg mL^{-1} BSA, 0.1 mM 1,5-dienoyl-CoA, 0.5 µM NAD^+, 20 µM FAD and 1.5-2.5 µg class II BCR. 3) Reduction of NAD^+ with reduced Fd: The assay was carried out in reaction tubes in the glove box at 30°C under a N_2/H_2 atmosphere (96% /4%). The mixture contained buffer A (pH 6), 2 mg mL^{-1} BSA, 10 µM Fd, 5 mM 2-oxoglutarate, 2.5 mM CoA, 0.5 U $KGOR_{Taro}$, 20 µM FAD, 0.5 µM NAD^+ and 1.5-2.5 µg

class II BCR. 4) Reduction of benzoyl-CoA and NAD^+ with reduced Fd (electron bifurcation): In comparison to (3) this assays additionally contained 0.2 mM benzoyl-CoA. Formation of NADH and/or 1,5-dienoyl-CoA was analyzed by UPLC® (see below). The setup of all other electron bifurcation assays is shown in Table S7.

UPLC analysis of CoA esters. CoA esters analysis was carried out using an Acquity UPLC® (ultra high performance liquid chromatography, H-Class, Waters GmbH, Eschborn, D). The CoA esters were applied to a C_{18} reversed-phase column (2.1 x 100 mm, Acquity UPLC BEH C18, 1.7 µm particle size, 130 Å pore size, Waters, using a VanGuard pre-column, 2.1 x 5 mm). The column was equilibrated with 12% acetonitrile (v/v) in 50 mM potassium phosphate buffer pH 6.8 at 0.2 mL^{-1} min^{-1}. The applied gradient was 0-0.01 min, 12-15% acetonitrile; 0.01-2.8 min, 15-30% acetonitrile: 2.8-5 min 12% acetonitrile.

Determination of molecular mass. The native molecular masses of the class II BCR complexes were determined by analytical gel filtration via Superdex 200 (1 cm diameter, 120 mL column volume, GE Healthcare 0.5 ml min^{-1} flow rate) using 50 mM potassium phosphate buffer with 5 mM $MgCl_2$ and 100 mM KCl at pH 6 or pH 7.5. The column was calibrated with thyroglobulin (670 kDa), apoferritin (443 kDa), catalase (245 kDa), and BSA (130 and 67 kDa).

Densitometrical analysis. The stoichiometric ration of the subunits was determined by densitometry in Coomassie-stained SDS/PAGE gels at 302 nm (ChemiDoc™ XRS+, Bio-Rad Laboratories GmbH). The band intensity was normalized according to molecular weights. Different SDS/PAGE conditions, protein preparations and applied protein amounts were used to determine the proportion of the subunits.

Identification of peptides by MS analysis. Excised SDS/PAGE protein gel bands of interest were digested by trypsin, whereas the Fd was digested in solution. Peptides were analyzed by UPLC-LTQ Orbitrap-MS/MS according to Bastida (2010) (11). A nanoAcquity column was used (C_{18}, 75 µm × 10 cm, 1.75 µm, Waters). Using a MASCOT platform, the best peptide matches were obtained.

Determination of metal cofactors and flavin. The content of iron was determined colorimetrically according to Lovenberg (1964) (12) for $Bam[(B_{Strep}C)_2DEF]_2$ and $Bam[(BC)_2DEFGHI]_2$. W, Zn, Se and Fe for $Bam(B_{Strep}C)_2$ were determined by ICP-MS analysis. Samples for ICP-MS were prepared and measured as described for W and Zn (5). For Fe and Se an analogous procedure was utilized. The following isotopes were used for ICP-MS measurements: ^{56}Fe, ^{66}Zn, ^{77}Se and ^{184}W. ^{103}Rh was applied as internal standard for ^{56}Fe, ^{66}Zn and ^{77}Se, ^{185}Re was applied as internal standard for ^{184}W. For the elements

Fe, Se and W mass resolution 4000 and for Zn mass resolution 10000 were used (Element XR, Thermo Fisher Scientific, Bremen). Buffer without protein was used as a reference. Three technical replicates for ICP-MS measurement were made three times. Sample preparation, including purification and ICP-MS measurement, was conducted two times independently for the $Bam[(BC)_2DEFGHI]_2$ and $Bam[(B_{Strep}C)_2DEF]_2$ complexes of *G. metallireducens.* Sample purification, preparation and ICP-MS measurement for $Bam(B_{Strep}C)_2$ was conducted once. To analyze the flavin cofactors the enzyme was precipitated with 25 mM H_2SO_4 (pH 2-2.5) and incubated 15 min in the dark at 0°C. The 13.000*g* pellet was washed once with H_2SO_4. Both supernatants were combined and analyzed by HPLC. The spectra and retention times were compared with standards flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin.

UV/vis spectroscopy. UV/vis spectra of approximately 1 µM purified enzyme complexes in buffer A, pH 6 containing 2 mg ml^{-1} BSA were recorded with a UV-1650PC Shimadzu spectrophotometer in a gas-tight quartz cuvette under anaerobic conditions. The enzymes were reduced with 50 µM 1,5-dienoyl-CoA, 50 µM sodium dithionite, 25 µM NADH or by 50 µM 2-oxoglutarate, 50 µM coenzyme A, 0.5 µM Fd from *G. metallireducens*, and 0.2 U KGOR from *T. aromatica*.

Phylogenetic analysis of BamE. The BamE sequence of *G. metallireducens* (Gmet_2084) was used as query sequence to identify BamE-like enzymes using Protein-BLAST. The chosen 172 sequences showed >40 % amino acid sequence identities and were predicted to contain at least one FAD binding site. The alignment of the sequences was calculated by using the MUSCLE algorithm (13). The replication setting was 1000. All ambiguous positions were removed for each sequence pair. The phylogenetic tree was created with the MEGA5 software package (Neighbor joining method) (14).

Western blot analysis. For Western blot analysis of BamF the membrane and soluble fraction of *G. metallireducens* cells grown on benzoate and acetate were transferred (transfer buffer: 50 mM tris, 40 mM glycin, 20% MeOH) from an SDS/PAGE-gel to a nitrocellulose membrane using the semi-dry blotting method. After the transfer, the membrane was incubated with blocking buffer (PBST: 290 mM Na_2HPO_4, 85 mM NaH_2PO_4, 340 mM NaCl, 0.1% Tween 20, 5% (w/v) BSA, pH 7.3) for 2 h at room temperature. The membrane was subsequently incubated with 0.5 µg mL^{-1} primary polyclonal BamF antibody (Davids Biotechnologie, Regensburg, D) in PBS (290 mM Na_2HPO_4, 85 mM NaH_2PO_4, 340 mM NaCl, pH 7.3) at 4° C overnight. Thereafter the membrane was incubated with 0.5 µg mL^{-1} secondary antibody (Thermo Fisher Scientific, Karlsruhe, D) (in PBS) which was conjugated with alkaline phosphatase. NBT (nitro blue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolyl

phosphate) were used for the detection. Between each incubation step, the membrane was washed three times (PBS, PBST, PBS, each 5 minutes).

Electron microscopy and immunogold labeling. For electron microscopy analysis, freshly grown cells were concentrated via filtration through a polycarbonate membrane with a pore size of 0.2 µm. Afterwards, cells were immediately high-pressure-frozen (Leica EM Pact2, Leica Microsystems GmbH, Wetzlar, D) and freeze-substituted (Leica AFS 2, Leica Microsystems GmbH, Wetzlar, D) in acetone containing 0.5 % (v/v) glutardialdehyde, 0.25 % (w/v) uranyl acetate and 5 % (v/v) water according to the following protocol: –90° C for 8 h, heating to –60° C within 1 h, –60° C for 6 h, heating to –40° C within 1 h, –40°C for 7 h. After washing cells three times with acetone, cells were infiltrated with Lowicryl HM20 and polymerized under UV light at –40° C. Immunogold labeling was performed according to Rachel *et al*., 2010 (15). For this purpose, ultrathin sections of 50-70 nm were prepared in a Leica UC7 ultramicrotome using a diamond knife. Polyclonal primary antisera directed against BamF was used in dilution of 1:50. For detection, goat-anti-rabbit coupled to ultrasmall gold particles were used. Immunogold labeling was followed by silver enhancement. Transmission EM analyses were performed on a 120 kV Philips CM12 (FEI, Hilsboro, OR, USA) equipped with a slow-scan CCD camera (TVIPS0124, 1k x 1k pixels, TVIPS GmbH, Gauting, D). For digital documentation, the software EM-Menu 4.0 (TVIPS, Gauting, D) was used.

Further determinations. Proteins were stained using SimplyBlue SafeStain (Invitrogen, Darmstadt, D). Protein concentrations were routinely determined by the Bradford method using BSA as standard. The protein concentration of samples including Triton X-100 was measured with the BCA kit (Interchim, Montluçon, F).

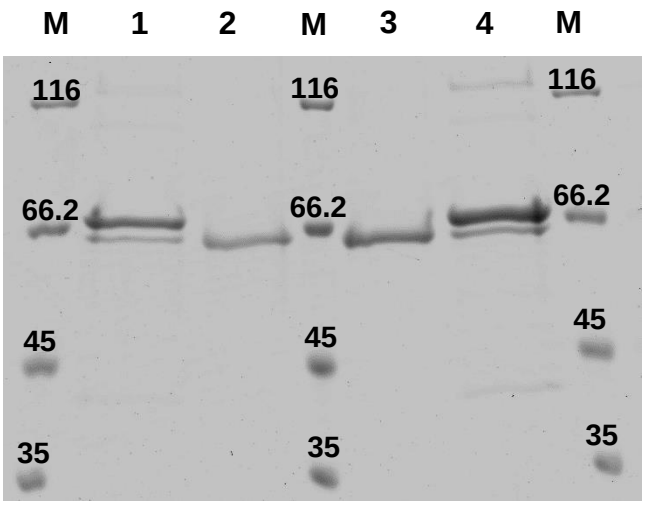

Fig. S1. Exchange of plasmid-encoded homologously produced $BamB_{2\times Strep}$ with genomically encoded BamB in $Bam(BC)_2$ complexes**.** SDS/PAGE analysis (8%) of enriched BamB derived from homologously produced $Bam(B_{2xStrep}C)_2$ complexes (lanes 1,4) and from wild-type $Bam(BC)_2$ complexes (lanes 2,3). Lane M = molecular weight marker (masses in kDa) . Lanes 1,2: 1.5 µg protein; lanes 3,4: 3 µg protein. The two bands at approximately 66.2 kDa correspond to $BamB_{2\times Strep}$ (upper band, ≈76% of total BamB) and wild type BamB (lower band, ≈24% of total BamB). Subscript '2×Strep' refers to a C-terminal Twin-Strep-tag®.

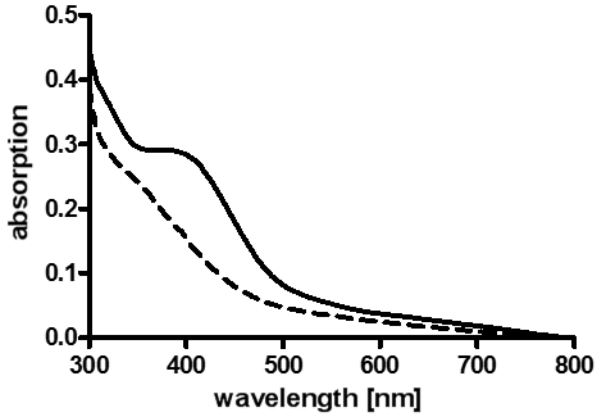

Fig. S2. UV/vis spectra of enriched ferredoxin from *G. metallireducens*. 10 µM oxidized ferredoxin as isolated (solid line) and after reduction (dashed line) by 2-oxoglutarate (0.1 mM), coenzyme A (0.1 mM) and enriched $KGOR_{Taro}$ (0.1 U) for 5 min.

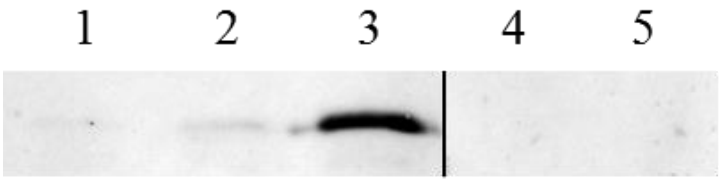

Fig. S3. Western blot analysis using polyclonal antibodies against BamF. Cell extract (lanes 1,4) and membrane protein fraction (lanes 2,5) of *G. metallireducens* cells grown on benzoate (lanes 1,2) and acetate (lanes 4,5); lane 3, isolated Bam$[(BC)_2DEFGHI]_2$. In each lane 1.5 µg of protein was applied.

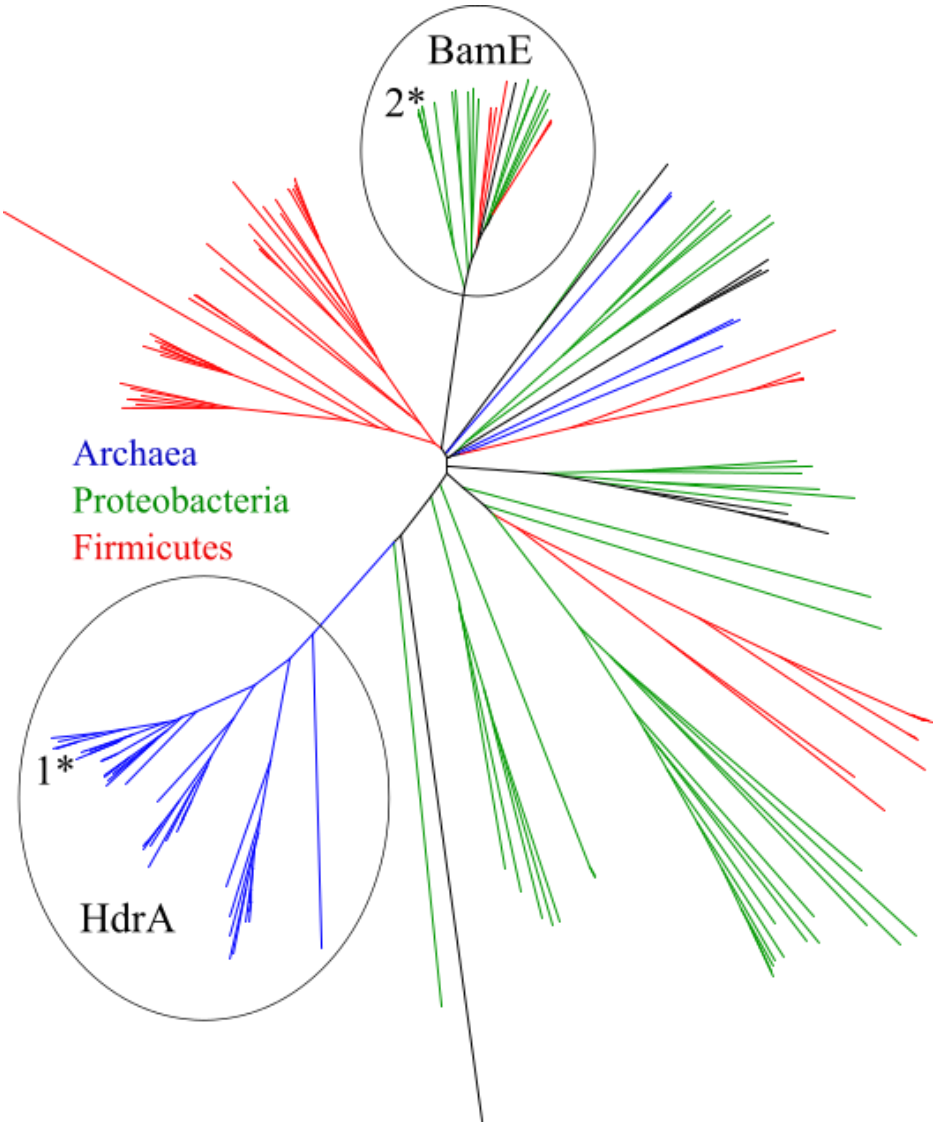

Fig. S4. Phylogenetic tree of HdrA-like components harboring at least one characteristic binding motif for electron bifurcating FAD. Color code: archaeal (blue), protobacterial (green), firmicutal (red) and other (black) HdrA-like components. The clusters comprising true HdrAs from methanogens and BamEs from aromatic compound degrading obligately anaerobic bacteria are encircled. 1*, HdrA from *M. thermolithotrophicus*; 2*, BamE from *G. metallireducens*. The phylogenetic tree was generated based on 172 amino acid sequences using the neighbor-joining method.

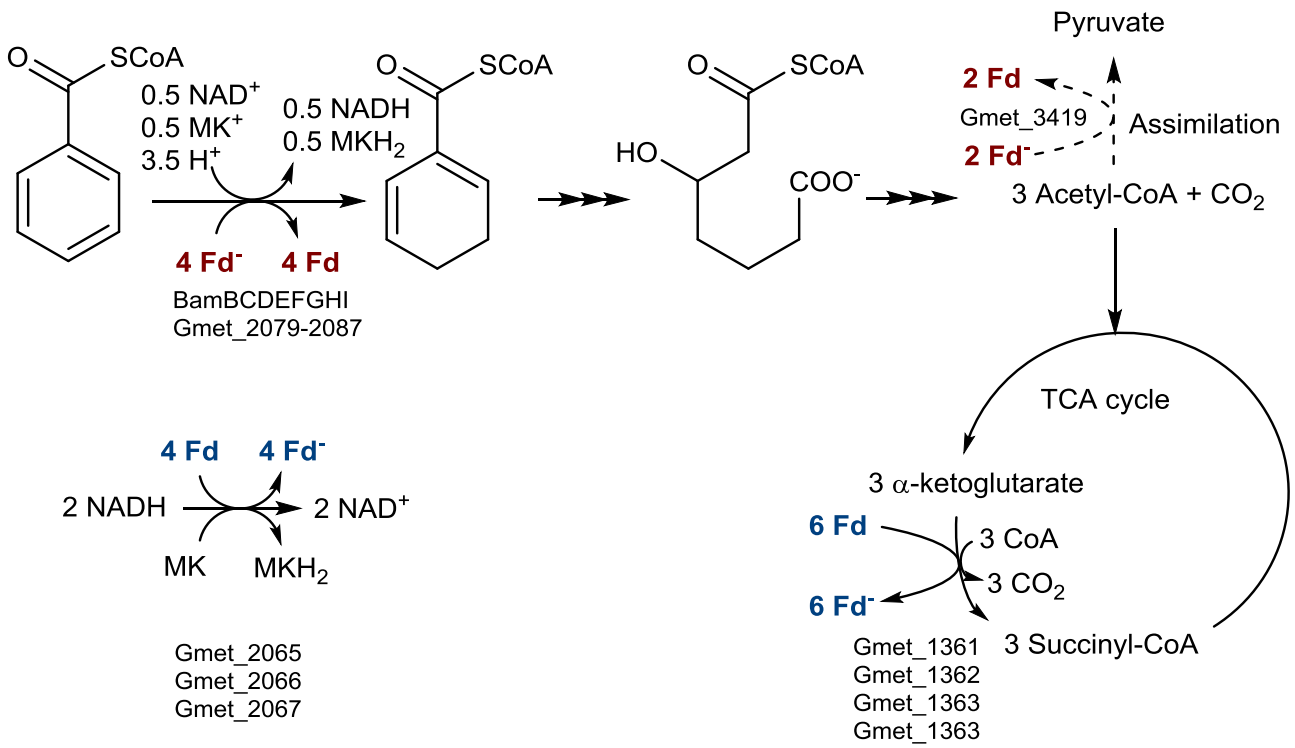

Fig. S5. Predicted ferredoxin reducing (blue) and oxidizing (red) reactions in *G. metallireducens* during growth with aromatic compounds. Per benzoyl-CoA reduced 4 Fd^- are oxidized assuming a double flavin-based electron bifurcation with MK and NAD^+ serving as high-potential acceptors. Three acetyl-CoA are formed from benzoyl-CoA in the benzoyl-CoA degradation pathway that are completely oxidized in the TCA cycle. The gene products annotated as α-ketoglutarate:ferredoxin oxidoreductase (KGOR) and pyruvate synthase have not been experimentally verified. Gmet_2065-2067 are proposed to be involved in endergonic ferredoxin reduction by NADH coupled to the exergonic reduction of MK by the same donor. Gmet_2066/Gmet_2067 code for putative electron bifurcating ETF αβ-subunits that harbor a Fd-domain with two predicted [4Fe-4S] clusters; Gmet_2065 codes for a putative membrane bound ETF:MK oxidoreductase. The combined action of KGOR and electron bifurcating ETF guarantees a high Fd^-/Fd ratio required for unidirectional benzoyl-CoA reduction.

Table S1. UPLC-LTQ Orbitrap-MS/MS analysis of protein bands obtained during the enrichments of Bam[$(BC)_2$DEFGHI]$_2$ and Bam[$(B_{Strep}C)_2$DEF]$_2$ complexes from *G. metallireducens* as shown in Fig. 2 in the main article. The numbering refers to the migration order of bands obtained during Bam[$(BC)_2$DEFGHI]$_2$ enrichment on the SDS gel (from top to bottom). Analysis of bands obtained during Bam[$(B_{Strep}C)_2$DEF]$_2$ enrichment identified the corresponding proteins I-V with highly similar scores; the BamGHI subunits were not identified during Bam[$(B_{Strep}C)_2$DEF]$_2$ enrichment.

#	Accession	Coverage	PSMs	Peptides	aa	MW [kDa]	Score	Description
I	Gmet_2084	46.84	58	38	1014	111.5	1740.22	**BamE**
	Gmet_1802	10.21	5	5	656	74.6	182.14	BamB-2; Benzoyl-CoA reductase, putative
II	Gmet_2080	64.09	126	44	635	68.6	2597.42	**BamH**; Benzoyl-CoA reductase, putative
	Gmet_2087	41.19	76	30	653	73.8	2119.01	BamB; Benzoyl-CoA reductase, putative
	Gmet_1802	32.32	54	21	656	74.6	1235.25	BamB-2; Benzoyl-CoA reductase, putative
III	Gmet_2087	63.86	105	50	653	73.8	3067.47	**BamB**; Benzoyl-CoA reductase, putative
	Gmet_2080	28.19	16	14	635	68.6	500.96	BamH; Benzoyl-CoA reductase electron transfer protein, putative
IV	Gmet_2085	70.03	76	40	387	43.2	1602.76	**BamD**; Iron-sulfur cluster-binding oxidoreductase, CCG domain pair-containing, putative benzoyl-CoA reductase electron transfer protein
	Gmet_2080	38.11	29	18	635	68.6	618.35	BamH; Benzoyl-CoA reductase electron transfer protein, putative
	Gmet_2675	47.66	36	18	384	43.2	547.02	YhaM; 3'-to-5' exonuclease
V	Gmet_2083	44.34	20	14	212	23.9	488.96	**BamF**; Benzoyl-CoA reductase electron transfer protein, selenocysteine-containing, putative
	Gmet_2079	28.90	8	6	218	23.7	287.46	BamI; Iron-sulfur cluster-binding protein, putative
	Gmet_2059	34.41	8	8	247	26.3	267.03	Oxidoreductase, short-chain dehydrogenase/reduc. family
VI	Gmet_2079	43.58	50	11	218	23.7	1126.23	**BamI**; Iron-sulfur cluster-binding protein, putative
	Gmet_2086	48.60	17	9	179	20.1	491.15	BamC; Iron-sulfur cluster-binding oxidoreductase, putative benzoyl-CoA reductase electron transfer protein
VII	Gmet_2086	89.39	51	25	179	20.1	931.69	**BamC**; Iron-sulfur cluster-binding oxidoreductase, putative benzoyl-CoA reductase electron transfer protein
	Gmet_0983	37.74	8	6	159	17.1	285.71	Biotin carboxyl carrier protein of acetyl-CoA carboxylase
VIII	Gmet_2081	49.33	30	9	150	16.5	759.33	**BamG**; Benzoyl-CoA reductase electron transfer protein
	Gmet_2086	26.26	5	4	179	20.1	153.33	BamC; Iron-sulfur cluster-binding oxidoreductase, putative benzoyl-CoA reductase electron transfer protein
	Gmet_1627	28.39	4	4	155	16.5	127.99	6,7-dimethyl-8-ribityllumazine synthase

Table S2. Conserved noncubane [4Fe-4S] cluster-binding sequence motif in HdrB from heterodisulfide reductases from methanogenic archaea and BamD from aromatic compound degrading obligately anaerobic bacteria.

Organism	Protein	Sequence motif
Methanothermococcus thermolithotrophicus	HdrB	$CX_{31}CCX_{35}CXXCX_{71}CX_{39}CCGAGGGX_{31}CXXC$
Methanothermobacter marburgensis	HdrB	$CX_{31}CCX_{35}CXXCX_{71}CX_{39}CCGAGGGX_{31}CXXC$
Geobacter metallireducens	BamD	$CX_{34}CCX_{35}SXXCX_{48}CX_{35}CCGGGGGX_{31}CXXC$
Delsulfosarcina cetonica	BamD	$CX_{34}CCX_{35}SXXCX_{48}CX_{35}CCGGGGGX_{31}CXXC$
Syntrophus aciditrophicus	BamD	$CX_{34}CCX_{35}SXXCX_{48}CX_{35}CCGGGGGX_{31}CXXC$

Table S3. Conserved [Fe-S] cluster binding motifs in HdrA and BamE. Assignment of cysteines was based on the structural characterization of heterodisulfide/hydrogenase complex from *Methanothermococcus thermolithotrophicus* (16).

Organism	Gene product	[Fe-S] cluster	Sequence motif
Methanothermococcus thermolithotrophicus	HdrA	HA1	$CX_2CX_2CX_{37}C$
		HA2	$CX_{22}CX_2CX_2C$
		HA3	$CXCX_{32}CX_{24}CX_{125}C$
		HA4	$CX_{12}CX_3CC$
		HA5	$CX_2CX_2CX_{50}C$
		HA6	$CX_{36}CX_2CX_2C$
Geobacter metallireducens	BamE	BE1	$CX_2CX_2CX_{37}C$
		BE2	$CX_{16}CX_{22}CX_5C$
		BE4	$CX_{13}CX_3CC$
		$BE4_2$	$CX_2CX_7CX_3CC$
		BE5	$CX_2CX_2CX_{35}C$
		BE6	$CX_{36}CX_7CX_2C$

Table S4. Conserved motifs involved in binding the two putative electron-bifurcating FADs in BamE from various obligately anaerobic, aromatic compound degrading bacteria. The highlighted glutamates (E226 and E729) and lysines (K280 and K771, notification for BamE from *G. metallireducens*), are conserved residues involved in binding electron bifurcating FADs in HdrA-like modules (16).

Organism	Gene locus	FAD I	$Glu_{(Gmet226)}$ I	$Lys_{(Gmet280)}$ I	FAD II	$Glu_{(Gmet729)}$ II	$Lys_{(Gmet771)}$ II
Geobacter metallireducens	Gmet_2084	IVGGGISGIQAA	TSIDFERILCA	CAYSQKQVILA	VVGGGVAGMTAA	TQLELEGEIVS	CSQAIKNAHKL
Geobacter pickeringii	GPICK_09410	IVGGGISGIQAA	TSIDFERILCA	CAYSQKQVILA	VVGGGVAGMTAA	TQLELEGEIVS	CSQAIKNAHKL
Geobacter daltonii	Geob_0215	IVGGGISGIQAA	TSIDYERILCA	CAYSQKQVILA	VVGGGVAGMTAA	TQLELEGEIVD	CSQAIKNAHKL
	bamE-2	VVGGGISGIQAA	TSIDYERILSS	CAYTQKQVILT	VVGGGIAGMTSA	TSLELEERIAK	CSQSIKNALKL
Geobacter bemidjiensis	Gbem_1447	IVGGGISGIQAA	TSIDFERILCA	CAYSQKQVILA	VVGGGVAGMTAA	TQLELEGEIVA	CSQAIKNALKL
Geobacter pelophilus	GPEL0_01f0241	IVGGGISGIQAA	TSIDFERILCA	CAYSQKQVILA	VVGGGVAGMTAA	TQLELEGEIVA	CSQAIKNALKL
Dethiosulfatarculus sandiegensis	X474_01300	IVGGGISGIQAA	TSMDYERLLCA	CTYTQKQVILT	VVGGGMAGMTAS	TNLEFEEKLAG	CSHSIKNALKL
Desulfomonile tiedjei	Desti_1915	IVGGGVSGIQAS	TSMDYERLLSS	CTYTQKQVILT	VVGGGIAGMNCA	THLELEEGIAR	CSESVKNALKL
Desulfobacula toluolica	hdlA9	IVGGGISGIQAS	TSIDYERLLCS	CTYTQKQVILT	VVGGGLAGMTGS	TQLDLEENIFK	CTHAIKNALKL
	Desgi_0481	IVGGGISGIQAS	TSMDFERLLSA	CTYAQKQVILT	VVGGGIAGMNCA	THLELEEKTAK	CSESIKNALKL
	Desgi_1112	IVGGGISGIQAS	TSMDYERLLCS	CTYTQKQVILT	VVGGGLAGMISA	THLELEEQIAK	CSESIKNALKL
Desulfotomaulum gibsoniae	Desgi_1980	IVGGGISGIQAS	TSMDYERLLCS	CTYTQKQVILT	VVGGGLAGMISA	THLELEEQIAK	CSESIKNALKL
	Desgi_1974	IVGGGISGIQAS	TSMDFERLLSS	CTYAQKHVILT	VVGGGIAGMNCA	THLELEEQIAG	CSESIKNALKL
	Desgi_3841	IVGGGISGIQAS	TSMDFERLLSS	CTYAQKQVILT	VVGGGIAGMNCA	THLELEEKTAK	CSESIKNALKL
Desulfotigum phosphitoxidans	hdrA4	IVGGGISGIQAS	TSIDYERLLCA	CTYTQKQVILT	VVGGGVAGMTSA	TQLALEEKLSS	CTHAVKNALAL
Desulfococcus multivorans	dsmv_2376	VVGGGISGVQAS	TSIQFERILSA	CMYATKQAVLA	VVGGGIAGMTSA	THLELEDRIAK	CGQSVKHALKL
Desulfosarcina cetonica	n.a.	VVGGGISGIQAA	TSIDFERILCS	CAYTQKQVILA	VVGGGIAGMTSA	TSIELEEMIAG	CSHSIKNALKL
Desulfocarbo indianensis	AAU61_09850	VVGGGISGIQAS	TSMDYERLLSS	CTYTQKQVILT	VVGGGIAGMTCA	TQVELEEKIVN	CGHAIKNALKL
Syntrophus aciditrophicus	SYN_00626	IVGGGISGIQAA	TGIDYERLLCA	CTYTQKQVILT	VVGGGIAGMISA	TQLELEERIAR	CSHAIKNALKL
	SYN_01645	VVGGGISGIQAS	TSMDFERLLSA	CTYNQKQVILT	VVGGGIAGMTCA	TQLELEERIAK	CSHAVKNALKL
	SYN_02423	VVGGGISGIQAS	TSMDFERLLSA	CTYNQKQVILT	VVGGGVAGMTCS	TQLDLEEWIAE	CTHAIKNALKL
	SYN_03215	VVGGGISGIQAS	TSMDFERLLSA	CTYSQKHAILT	IVGGGVAGMVCA	TQLELEEKIAR	CSHAVKNALKL

n.a. = not available

Table S5. Conserved [2Fe-2S] cluster-binding sequence motif in MvhD and BamF.

Organism	Enzyme	Sequence motif
Methanothermococcus thermolithotrophicus	MvhD	$CX_2\mathbf{C}X_{25}CX_{24}CX_4C$
Methanothermobacter marburgensis	MvhD	$CX_2\mathbf{C}X_{25}CX_{24}CX_4C$
Geobacter metallireducens	BamF	$CX_2\mathbf{U}X_{25}CX_{24}CX_4C$
Desulfosarcina cetonica	BamF	$CX_2\mathbf{U}X_{25}CX_{24}CX_4C$
Syntrophus aciditrophicus	BamF	$CX_2\mathbf{U}X_{25}CX_{24}CX_4C$
C: involved in [2Fe-2S] cluster coordination **C/U**: not involved in [2Fe-2S] cluster coordination		

Table S6. Extinction coefficients determined in different Bam complexes under varying conditions.

Condition	**Complex/extinction coefficient [M^{-1} cm^{-1}]**			
	$(BC)_2$	$(B_{Strep}C)_2$	$[(B_{Strep}C)_2DEF]_2$	$[(BC)_2DEFGHI]_2$
Oxidized as isolated	ε_{398nm}= 41,400	n.d.	ε_{400nm}= 103,800	ε_{401nm}= 156,400
Oxidized minus reduced by				
- 1,5-dienoyl-CoA	$\Delta\varepsilon_{409nm}$= 28,700	n.d.	$\Delta\varepsilon_{409nm}$= 49,700	$\Delta\varepsilon_{409nm}$= 94,400
- sodium dithionite	$\Delta\varepsilon_{409nm}$= 10,600	n.d.	$\Delta\varepsilon_{409nm}$= 37,600	$\Delta\varepsilon_{409nm}$= 78,000
- reduced ferredoxin	$\Delta\varepsilon_{409nm}$= 3,700	n.d.	$\Delta\varepsilon_{409nm}$= 30,300	$\Delta\varepsilon_{409nm}$= 56,300
- NADH	n.d.	n.d.	$\Delta\varepsilon_{409nm}$ = 7,300	$\Delta\varepsilon_{409nm}$= 10,900
n.d. not determined				

Table S7. Combination of enzyme fractions, electron donors/acceptors used in assays monitoring FBEB-driven benzoyl-CoA reduction. DCPIP: 2,6-Dichlorophenolindophenol; PMSF: phenylmethylsulfonyl fluoride; Ferrocenium = ferrocenium tetrafluoroborate and hexafluorophosphate.

Enzyme fraction	Electron donor (mM)	High-potential electron acceptor (mM)
Crude extract (soluble and membrane fraction)	Fd^- (0.5, $KGOR_{Taro}$ reduced)	$NAD(P)^+$ (1-2), Menadione (1-2), Glutathione (1-2), Cystine (1-2), Potassium ferrocyanide (2), Nitrate (5-10), Nitrite (5-10), Ferrocenium (0,5), Fumarate (1-2)
	Ti(III)-citrate (5)	$NAD(P)^+$ (1-2), Menadione (1-2), Cystine (1-2), Nitrate (5-10), Nitrite (5-10)
	Formate (10)	$NAD(P)^+$ (1-2), Menadione (1-2), Glutathione (1-2), Cystine (1-2)
	NAD(P)H (1-2)	Menadione (1-2), Glutathione (1-2), Cystine (1-2), Potassium ferrocyanide (2), Nitrate (10), Nitrite (10), DCPIP (2), PMSF (2), Ferrocenium (0.5)
$Bam[(B_{Strep}C)_2DEF]_2$	Ferredoxin (0.5)	$NAD(P)^+$ (1-2), Menadione (1-2), Glutathione disulfide (1-2), Cystine (1-2), Potassium ferrocyanide (2), DCPIP (2), Ferrocenium (0.5)
$Bam[(BC)_2DEFGHI]_2$	Fd^- (0.5, $KGOR_{Taro}$ reduced)	$NAD(P)^+$ (1-2), Menadione (1-2), Glutathione (1-2), Cystine (1-2), Potassium ferrocyanide (2), DCPIP (2), Ferrocenium (0.5)
	Ti(III)-citrate (5)	$NAD(P)^+$ (1-2), 1,5-dienoyl-CoA (1-2)
	NAD(P)H (1-2)	$NAD(P)^+$ (1-2), Glutathione (1-2), Cystine (1-2), DCPIP (2), PMSF (2), Ferrocenium (0.5)

Table S8. Distribution of the BamB-specific DCO activity in differentially lysed *G. metallireducens* cells grown with benzoate.

Lysis method	Activity in membrane fraction [%]	Activity in soluble fraction [%]
French-Press	30	60
Thawing and freezing (3x)	45	55
Lysozyme	42	54
French-Press + 0.5 M KCl	16	81
Thawing and freezing (3x) + 0.5 M KCl	2	95
Lysozyme + 0.5 M KCl	3	92

Table S9: Plasmids used.

Plasmids	Relevant characteristics	Source/reference
pBBR1MCS-2	Km^r, broad-host-range cloning vector	Kovach *et al.* 1995 (17)
pGM2087e1	Km^r, pBBR1MCS-2 (source plasmid) with sequence 198 bp upstream of *bamB* and encoding BamB with a C-terminal Strep-tag II® ($BamB_{Strep}$) on a 2190-bp *XhoI/SpeI* fragment	this work
pPR-IBA101	Amp^r, MCS with C-terminally encoded Twin-Strep-tag®	IBA GmbH, Göttingen, D
pPR-GM2087e10	Amp^r, pPR-IBA101(source plasmid), used for combination of sequence encoding BamB with Twin-Step-tag®	this work
pGM2087e10	Km^r, pPR-GM2087e10 and pGM2087e1 (source plasmids), same plasmid as pGM2087e1 but sequence encoding BamB with C-terminal Twin-Strep-tag® ($BamB_{2xStrep}$)	this work

Abbreviations: ampicillin resistance (Amp^r), kanamycin resistance (Km^r), subscript 'Strep' refers to a C-terminal Strep-tag II®, subscript '2xStrep' refers to a C-terminal Twin-Strep-tag®.

References

1. Lovely DR, Giovannoni SJ, White DC, Champine JE, Phillips EJP, Gorby YA. & Goodwin, S. (1993) *Geobacter metallireducens* gen. 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.
2. Green MR & Sambrook J (2012) Molecular cloning: a laboratory manual, p. 1890. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA
3. Cheng HR and Jiang N (2006) Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnol Lett* 28:55-59.
4. Oberender J, Kung JW, Seifert J, von Bergen M & Boll M (2012) Identification and characterization of a succinyl-coenzyme A (CoA):benzoate CoA transferase in *Geobacter metallireducens*. *J Bacteriol* 194:2501-2508.
5. Kung JW *et al*. (2009) Identification and characterization of the tungsten-containing class of benzoyl-coenzyme A reductases. *Proc Natl Acad Sci USA* 106:17687-92.
6. Schächter D & Taggert JV (1953) Benzoyl coenzyme A and hippurate synthesis. *J Biol Chem* 203:925-934.
7. Boll M, Laempe D, Eisenreich W, Mittelberger T, Heinze J, Bacher A & Fuchs G (2000) Non-aromatic products from anoxic conversion of benzoyl-CoA reductase and dienoyl-CoA hydratase. *J Biol Chem* 275:21889-21895.
8. Tiedt O, Fuchs J, Eisenreich W & Boll M (2018) A catalytically versatile benzoyl-CoA reductase, key enzyme in the degradation of methyl- and halobenzoates in denitrifying bacteria. *J Biol Chem* 294:10264-10274.
9. Schönheit P, Wäscher C & Thauer RK (1978) A rapid procedure for the purification of ferredoxin from clostridia using polyethylenemine. *FEBS Lett* 89:219-222.
10. Dörner E & Boll M (2002) Properties of 2-oxoglutarate:ferredoxin oxidoreductase from *Thauera aromatica* and its role in enzymatic reduction of the aromatic ring. *J Bacteriol* 184:3975-3983.
11. Bastida F *et al*. (2010) Elucidating MTBE degradation in a mixed consortium using a multidisciplinary approach. *FEMS Microbiol Ecol* 73:370-384.
12. Lovenberg W, Buchanan BB & Rabinowitz JC (1963) Studies on the chemical nature of clostridial ferredoxin. *J Biol Chem* 238:3899-3913.
13. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792-1797.
14. Saitu N & Nei M (1987) The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol Biol Evol* 4:406-425.
15. Rachel R et al. (2010) Analysis of the Ultrastructure of Archaea by Electron Microscopy. *Methods Cell Biol* 96:47-69.
16. Wagner T, Koch J, Ermler U & Shima S. (2017) Methanogenic heterodisulfide reductase (HdrABC-MvhAGD) uses two noncubane [4Fe-4S] clusters for reduction. *Science* 357:699-703.
17. Kovach ME et al. (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175-176.