A catalytically versatile benzoyl-CoA reductase, key enzyme in the degradation of methyl- and halobenzoates in denitrifying bacteria

Oliver Tiedt¹, Jonathan Fuchs¹, Wolfgang Eisenreich², and Matthias Boll¹⁺*

From the ¹Fakultät für Biologie – Mikrobiologie, Albert-Ludwigs-Universität Freiburg, Germany, ²Lehrstuhl für Biochemie, Technische Universität München, Germany

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*To whom correspondence should be addressed: Prof. Matthias Boll, Faculty of Biology, Institute of Biology II, Schänzlestr. 1, 79104 Freiburg, Germany Telephone: +49 761 2032649; FAX: +49 761 2032626; E-mail: matthias.boll@biologie.uni-freiburg.de

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ABSTRACT

Class I benzoyl-coenzyme A (BzCoA) reductases (BCRs) are key enzymes in the anaerobic degradation of aromatic compounds. They catalyze the ATP-dependent reduction of the central BzCoA intermediate and analogues of it to conjugated cyclic 1,5-dienoyl-CoAs probably by a radical-based, Birch-like reduction mechanism. Discovered in 1995, the enzyme from the denitrifying bacterium Thauera aromatica (BCRₜₐ) has so far remained the only isolated and biochemically accessible BCR, mainly because BCRs are extremely labile and their heterologous production has largely failed, so far. Here, we describe a platform for the heterologous expression of the four structural genes encoding a designated 3-methylbenzoyl-CoA reductase from the related denitrifying species Thauera chlorobenzoica (MBRₜₐ) in Escherichia coli. This reductase represents the prototype of a distinct subclass of ATP-dependent BCRs that were proposed to be involved in the degradation of methyl-substituted BzCoA analogues. The recombinant MBRₜₐ had an αβγδ-subunit architecture, contained three low-potential [4Fe-4S] clusters, and was highly oxygen-labile. It catalyzed the ATP-dependent reductive dearomatization of BzCoA with 2.3–2.8 ATPs hydrolyzed per two electrons transferred, and preferentially dearomatized methyl-and chloro-substituted analogues in meta- and para-position. NMR analyses revealed that 3-methylbenzoyl-CoA is regioselectively reduced to 3-methyl-1,5-dienoyl-CoA. The unprecedented reductive dechlorination of 4-chloro-BzCoA to BzCoA probably via HCl elimination from a reduced intermediate allowed for the previously unreported growth of T. chlorobenzoica on 4-chloro-benzoate. The heterologous expression platform established in this work enables the production, isolation and characterization of bacterial and archaeal BCR- and BCR-like radical enzymes, for many of which the function has remained unknown.

Aromatic compounds are the second most abundant class of naturally occurring organic molecules that are predominantly produced by plants and microorganisms. Aromatic hydrocarbons of fossil oil reservoirs serve as solvents and precursors of plastics, dyes, resins, insecticides, herbicides and pharmaceuticals, many of which are harmful to the environment and human health (1). The complete degradation of aromatic compounds by aerobic microorganisms heavily depends on oxygenases and has been studied in detail since more than six decades (2, 3). Such a strategy is not an option at anoxic sites, e.g., at marine or freshwater sediments, aquifers with high carbon loads or rice-fields. In these environments, anaerobic bacteria channel monocyclic aromatic growth substrates into the central intermediate benzoyl-coenzyme A (BzCoA). It serves as substrate for dearomatizing benzoyl-CoA reductases (BCRs) that dearomatize their substrate to cyclohexa-1,5-diene-1-carboxyl-
The redox potential of the BCR substrate/product couple is with \( E'_0 = -622 \text{ mV} \) among the lowest in biology, and electron transfer from any physiological reductant to BzCoA, such as reduced ferredoxins (\( E' \approx -500 \text{ mV} \)), has to be coupled to an exergonic reaction (5).

The analogous reaction in organic synthesis, referred to as the Birch reduction, involves the formation of radical intermediates at extremely low redox potentials with the first electron transfer yielding a radical anion being rate-limiting (\( E'_0 = -1.9 \text{ V} \) for a benzoic acid thioester 6). The Birch reduction uses solvated electrons as electron donors that are generated by dissolving alkali metals in ammonia; an alcohol serves as proton source (7). Kinetic data with BCR favor a Birch-like mechanism (8, 9).

There are two totally non-related classes of BCRs suggesting they have independently evolved in nature. Class I BCRs predominantly occur in facultative anaerobic bacteria and couple BzCoA reduction to a stoichiometric ATP hydrolysis (two ATP hydrolyzed to ADP + P\(_i\) per BzCoA reduced). So far, a class I BCR has exclusively been isolated from the denitrifying *Thauera aromatica* (BCR\(_{Tar}\)) more than 20 years ago (10). BCR\(_{Tar}\) is a 170 kDa heterotetramer with an \( \alpha \beta \gamma \delta \) -architecture, encoded by the \( bcrABCD \) genes (Fig. 1) (11). Class II BCRs are found in obligate anaerobes (12, 13). The active site of class II BCRs harbors a tungstopterin cofactor (14), and endergonic electron transfer to the aromatic ring is suggested to be driven by a flavin-based electron bifurcation (4).

Class I BCRs belong to the BCR/HAD (2-hydroxyacyl-CoA dehydratase) radical enzyme family, that are all composed of two functional modules (16). The dimeric electron-activating module (BcrAD or \( \alpha \delta \)-subunits in BCR\(_{Tar}\)) harbors a ATP-binding site in each subunit and contains a bridging [4Fe-4S] cluster (6). After reduction of the cluster by a reduced ferredoxin, ATP hydrolysis results in conformational changes initiating low-potential electron transfer to the two [4Fe-4S] clusters of the CoA-ester binding module (Fig. 1). The CoA-ester-binding module (BcrBC or \( \beta \gamma \) subunits in BCR\(_{Tar}\)) catalyzes electron transfer to BzCoA. While class I BCRs transfer two electrons and protons to the aromatic ring of BzCoA yielding the reduced 1,5-dienoyl-CoA product (Fig. 1), electron transfer in the redox-neutral dehydration of 2-hydroxyacyl-CoAs to enoyl-CoAs by HADs is only catalytically required (17,18). The crystal structure of a 2-hydroxyacyl-CoA dehydratase revealed that the CoA ester substrate is directly ligated to an active site cluster via the carboxyl oxygen atom of the thioester substrate, suggesting an inner-sphere electron transfer (19). A similar mode of binding to an active-site [4Fe-4S] cluster seems plausible in the catalytic BcrB-subunit.

Based on amino acid sequence similarities and subunit sizes, the *Thauera* and the *Azooaracus* subclasses of class I BCRs are distinguished. Recently, a phylogenetically distinct third BCR subclass has been proposed that comprises BCRs that are upregulated during complete anaerobic degradation of 4- and 3-methylbenzoates (20, 21). Though no BCR of this third subclass has been isolated and characterized, so far, it was suggested that MBR-like enzymes are especially optimized for the conversion of para- and meta-substituted BzCoA analogues. In contrast, BCR\(_{Tar}\) exhibits a decreased activity with meta-positioned substrates (10, 22, 23), whereas analogues with para-substituents generally do not serve as substrates for BCR\(_{Tar}\) (22). The only recently reported exception is BCR-catalyzed deflorination of 4-fluoro-BzCoA to BzCoA (24).

The BCR/HAD family comprises a large number of related enzymes, of which only a few can unambiguously be assigned to true BCRs or HADs (16). Heterologous production allowed for the study of a number of HADs promoting the elucidation of their structure and function (19, 25). Attempts to establish a comparable tool for BCRs were less successful. A first promising step was achieved with the BCR from the hyperthermophilic Euryarchaeon *Ferroglobus placidus*. Heterologous production of all four subunits in *E. coli*, yielded a recombinant BCR (BCR\(_{Fpl}\)) that exhibited BzCoA reducing activity, albeit four orders of magnitude below that of wild type BCR\(_{Tar}\). This low activity was assigned to the >99% loss of the active site subunits (26).

Recently, functional heterologous expression of the ATP-binding module of BCR\(_{Fpl}\) was achieved in the absence of the active site subunits (27).

In this work, we developed a general platform for the heterologous production of the four subunits of class I BCRs and related enzymes. Using this tool, we produced recombinant BCR\(_{Tar}\) as a proof of principle. Moreover, an archetypical MBR was heterologously produced and characterized as a class I BCR with a largely extended substrate preference. This enzyme allowed *Thauera chlorobenzoica* for the previously unknown growth with 4-
chlorobenzoate under denitrifying conditions.

RESULTS

Phylogenetic analysis of BCR/HAD family enzymes — The recent discovery of a new subfamily of ATP-dependent BCRs putatively specific for the conversion of 3- or 4-methyl-BzCoA (20, 21) motivated us for an updated phylogenetic analysis of the designated active site subunits of class I BCRs (referred to as BcrB or BzdO). Among the candidates identified in databases, only those were considered that derived from gene clusters containing all four structural genes of BCRs. We assessed the diversity by aligning the amino acid sequences from 64 putative BCRs and two HADs (Table S1). In agreement with earlier studies, the computation of evolutionary distances revealed that MBR-like enzymes do not affiliate with Thauera and Azoarcus subclass BCRs (20, 21). Instead, they group with a separated cluster of class I BCRs from αβδ-Proteobacteria, but also from a number of distinct phyla (Fig. 2, Table S1); this phylogenetic cluster is henceforth referred to as MBR subclass of ATP-dependent BCRs.

The analysis identified a putative MBR from Thauera chlorobenzoica next to the putative 4-methylbenzoyl-CoA reductase from Magnetospirillum sp. pMbN1 (20), and the 3-methylbenzoyl-CoA reductase from Azoarcus sp. CIB (21). Similar as Magnetospirillum sp. pMbN1 and Azoarcus sp. CIB, T. chlorobenzoica contains two different class I BCRs, one of the Thauera subclass (BCRTcl, gi no. 1125920966, 1126144411–13) and one of the MBR subclass (MBRTcl, gi no. 1126145460–62, 1126145791). The former is highly similar to BCRtar (BcrABCD, 98–99% amino acid sequence identity), the latter to MBR from Azoarcus sp. CIB (MBRAsc, MbrNOPQ, 96–98% amino acid sequence identity).

Heterologous production and isolation of BCRtar and MBRTcl — For the development of a heterologous expression platform, the BCRtar encoding genes bcrABCD and the MBRTcl encoding genes mbrONPQ (also referred to as mbdONPQ in Azoarcus sp. CIB (21)) were chosen. The corresponding DNA sequences were cloned into the mid-copy plasmid pOT1 constructed in a recent work (28). This plasmid permits inducible gene expression at moderate levels in E. coli to minimize misfolding of proteins. A C-terminal Strep-tag II was fused to the individual δ-subunits. After gene expression in E. coli under anaerobic growth conditions, the proteins produced were largely enriched by affinity chromatography (Fig. 3). The yields were 0.4–0.6 mg protein per gram of cells.

The four subunits of MBRTcl had an almost perfect 1:1:1:1 stoichiometry (Fig. 3A), whereas in preparations of BCRtar, the β- and γ-subunits of the CoA-ester-binding module were frequently less represented than the α- and δ-subunits of the electron-activating module (Fig. 3B). This finding may result from stronger contacts between the modules in MBRTcl than in BCRtar under the enrichment conditions.

ATP-dependent BzCoA dearomatizing activities of recombinant BCRtar and MBRTcl — Heterologously produced BCRtar and MBRTcl both catalyzed the Ti(III)-citrate-dependent (5 mM) reduction of BzCoA to 1,5-dienoyl-CoA, as evidenced by co-elution with authentic standards and typical ultraviolet-visible (UV-Vis) spectra. BzCoA reduction of both enzymes strictly depended on the presence of MgATP (5 mM); representative diagrams from ultra performance liquid chromatography (UPLC) based assays for MBRTcl are shown in Fig. 4.

BCRtar dearomatized BzCoA to 1,5-dienoyl-CoA at a rate of 93 mU min⁻¹ mg⁻¹ (1 mU = 1 nmol min⁻¹ mg⁻¹). This value is three to four times slower than reported for the wild type enzyme (10), which corroborates the corresponding loss of the active subunit during the enrichment. The specific activity of MBRTcl was with 212 mU mg⁻¹ substantially higher, fitting to the almost stoichiometric presence of all four subunits. Reduced methyl viologen, that routinely served as electron donor for wild type BCRtar in continuous spectrophotometric assays was also used by recombinant BCRtar at rates comparable to that of Ti(III) citrate. However, it barely served as electron donor for MBRTcl (1.5% of the rate with Ti(III) citrate). Addition of 0.5 mM methyl viologen to an assay with 5 mM Ti(III) citrate as electron donor enhanced BzCoA reduction rate by 10%, indicating that methyl viologen acts as an electron carrier between Ti(III) citrate and MBRTcl. Sodium dithionite (5 mM) served as an alternative electron donor at 100% (BCRtar) and 53% (MBRTcl) of the rate observed with Ti(III) citrate. With dithionite as electron donor, UPLC analyses revealed a different product pattern, an observation that was also reported for the conversion of BzCoA by wild type BCRtar (29). This altered pattern results from artificial effects of
dithionite and its oxidized product sulfite, yielding physiologically irrelevant CoA ester products that were not further analyzed in this work. NAD(P)H did not serve as electron donor for MBR\text{Tcl}. Based on the results obtained, Ti(III) citrate was routinely used as electron donor in UPLC-based, discontinuous MBR\text{Tcl} assays.

**Native molecular mass, cofactor content, and spectral properties of MBR\text{Tcl}** — The partial loss of the β- and γ-subunits precluded a detailed analysis of the molecular and kinetic properties of BCRT\text{Tar}, and they have already been described in detail for wild type BCRT\text{Tar} (10). The molecular mass of MBR\text{Tcl} was 140±1 kDa (mean value ± standard deviation of three biological replicates) as determined by size exclusion chromatography suggesting a heterotetrameric composition. This value is only slightly lower than that deduced from the amino acid sequences (152 kDa) (25).

The Fe content was 11.6±1.0 Fe per MBR\text{Tcl} as determined by a spectrophotometric assay suggesting the presence of three \([4Fe-4S]\) clusters, as described for BCRT\text{Tar} (30). Supernatants of acid precipitated MBR\text{Tcl} were analyzed by UPLC coupled to UV/vis absorbance detection. The results obtained excluded the presence of a flavin or other organic cofactors to a significant extent (<0.02 mol per mol enzyme).

The UV/vis absorbance spectrum of oxidized MBR\text{Tcl} showed a major maximum at 407 nm and a minor one at 317 nm (Fig. 5). The spectrum was bleached in the visible region upon reduction by sodium dithionite at pH 8.3, where \(E^\prime_0\) of dithionite is below -600 mV (31). Titration of MBR\text{Tcl} with dithionite at this pH revealed an almost linear course of absorbance decrease at 407 nm up to the addition of one electron equivalent, suggesting that one \([4Fe-4S]\)\textsuperscript{2+\textUILT}} cluster was readily reduced (Fig. 5C). In this range, the difference spectrum of oxidized minus dithionite reduced enzyme revealed a difference absorbance coefficient of \(\Delta\varepsilon_{407} \approx 5.0\) mM\textsuperscript{-1} cm\textsuperscript{-1} (Fig. 5B). Further addition of dithionite did not result in a further stoichiometric reduction of the spectrum at 407 nm, indicating that a redox equilibrium was approached (Fig. 5C). Upon the addition of 300-fold excess of dithionite vs enzyme, the maximal difference in absorbance was reached, with \(\Delta\varepsilon_{407\text{max}} = 9.6\) mM\textsuperscript{-1} cm\textsuperscript{-1} indicating the reduction of two of the three \([4Fe-4S]\)\textsuperscript{2+\textUILT}} clusters. The difference absorbance at 317 nm could not be resolved accurately due to the absorbance of accumulating dithionite.

**Catalytically versatile methylbenzoyl-CoA reductase** — Similar to BCRT\text{Tar}, MBR\text{Tcl} activity was highest at pH ≈ 7.3 (Fig. S1A). Whereas BCRT\text{Tar} activity was only measurable in a rather narrow pH range between 6 and 9 (10), MBR\text{Tcl} still exhibited 68% residual activity at pH 6.3 and 88% at pH 8.8.

**Sensitivity of MBR\text{Tcl} towards oxygen** — MBR\text{Tcl} was incubated in air for different time spans before the reaction was started with BzCoA under anaerobic conditions. The enzyme was severely susceptible for loss of activity upon contact to oxygen with a half-life time in air of around 30 seconds (Fig. S1B). This value is in the same range as half-life times of wild-type BCRT\text{Tar} and the activator module of HAD from *Acidaminococcus fermentans*, which are 20 s and 10 s, respectively (10, 32).

**Substrate preference of MBR\text{Tcl}** — The substrate preference of MBR\text{Tcl} was investigated and compared with reported values for wild type BCRT\text{Tar} in UPLC-based discontinuous assays at 0.25 mM thioester concentrations (Table 1). For selected substrates, the \(K_m\) values were determined. Most products were additionally subjected to electrospray-ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) analysis (Fig. S2–S4).

MBR\text{Tcl} preferentially dearomatized meta-substituted BzCoA-analogues containing methyl-, chloro- or hydroxy-functionalities as indicated by the highest specificity constants determined. With these substrates, relative specific activities compared to BzCoA were substantially higher with MBR\text{Tcl} than with BCRT\text{Tar}. Remarkably, MBR\text{Tcl} also converted para-substituted halo- and methyl-BzCoA analogues that were not converted by BCRT\text{Tar}. Exceptions were 3-fluoro- and 4-fluoro-benzoyl-CoA that served as substrates for both enzymes. In summary, MBR\text{Tcl} appears to be less sensitive to steric effects of meta- and para-positioned substituents as BCRT\text{Tar}, even 4-bromo-BzCoA was readily converted. Neither of the enzyme converted 4-hydroxy-BzCoA. In contrast, ortho-substituted BzCoA analogues were generally accepted by both enzymes, with BCRT\text{Tar} showing higher relative activities than MBR\text{Tcl}. Neither of the two enzymes reduced heterocyclic nicotinoyl-CoA.

ESI-Q-TOF-MS analysis of the products formed from various ortho-substituted BzCoA analogues by MBR\text{Tcl} revealed the formation of the corresponding two-electron-reduced 1,5-dienoyl-CoA analogues (Fig. S2). Notably, two different isomers with the substituent in 2- or 6-position of the 1,5-dienoyl-CoA products can be formed that
cannot be distinguished by MS analysis. The potential 2-hydroxy- or 6-hydroxy-1,5-dienoyl-CoA isomers formed from 2-hydroxy-BzCoA, both spontaneously tautomerase to 6-oxo-cyclohex-1-enoyl-CoA (6-oxo-1-enoyl-CoA). In the case of the reduction of 3-methyl, 3-fluoro, and 3-hydroxy-BzCoA analogues, again the corresponding 1,5-dienoyl-CoA products were identified, with the 3-hydroxy-1,5-dienoyl-CoA product, most probably again tautomeraere to 5-oxo-1-enoyl-CoA. However, 3-chloro- or 3-bromo-BzCoA were dehalogenated to BzCoA that was then further reduced to 1,5-dienoyl-CoA (Fig. S3). This finding can be explained by the spontaneous elimination of HCl/HBr from halogenated 1,5-dienoyl-CoA intermediates as reported earlier for BCR

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ATPase activity of MBR

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Oxygen-sensitive MBR

Oxygen-sensitive MBR was routinely stored in the presence of 50 µM dithionite. Under anoxic conditions, the enzyme could reversibly be oxidized with 10-fold excess of thionine with virtually no loss of activity. In the thionine-oxidized state, CoA ester independent ATPase activity of MBR was greatly diminished to < 1% of the rate observed in the presence of BzCoA and Ti(III) citrate. Re-reduction of thionine-oxidized enzyme by excess Ti(III) citrate fully restored ATPase activity to the level before thionine oxidation. This redox dependence of the thioester substrate independent ATPase activity was also observed for BCR but not for the isolated electron-activating module of the Azoarcus subclass BCR from F. placidus

Complete degradation of 3-methyl- and 4-chlorobenzoate by T. chlorobenzoica — The MBR subclass of ATP-dependent BCRs was originally deduced from upregulated bcr-like genes during anaerobic growth of Magnetospirillum sp. pMbN1 with 4-methylbenzoate (20) and Azoarcus sp. CIB with 3-methylbenzoate (21). The dearomatizing activities of MBR with 3- or 4-methyl-/chloro-

Discussion

By establishing a first heterologous production platform for functional class I BCRs, the recombinant MBR prototype of an only recently defined BCR subclass was isolated and biochemically characterized in this work. The
properties are summarized and compared with the only other described BCR\textsubscript{T\textregistered\textsubscript{a}r}, isolated more than two decades ago (Table 2). General properties such as subunit architecture, cofactor content, ATP-dependence/ATPase activity, and oxygen-sensitivity are similar in t members of different BCR subclasses. But there are marked differences between the two enzymes with regard to the substrate specificity. While BCR\textsubscript{T\textregistered\textsubscript{a}r} predominantly converts BzCoA and ortho-substituted analogues, MBR\textsubscript{T\textregistered\textsubscript{c}h} exhibits a clearly extended substrate preference towards meta- and para-substituted BzCoA analogues with methyl, chlorine, and bromine substituents. In particular the para-position has previously been suggested as critical position for class I BCRs (22), and so far, only the stericly irrelevant fluorine atom was accepted as para-substituent by BCR\textsubscript{T\textregistered\textsubscript{a}r}, albeit at drastically reduced rates (24).

The observed differential substrate preference of the BCR and MBR subclasses suggests structural variations with regard to the electronic settings of the benzene binding pocket and the nature, position and pK\textsubscript{a}-values of amino acid residues involved in C3 and C4 protonation. The proposed radical anion intermediate exhibits highest electron density at the para-position (22). For this reason, any substituent that adds additional electron density at this position should have a counterproductive effect on BCR activity, as demonstrated by the inability of all BCRs to reduce 4-hydroxy-BzCoA containing a para-substituent of a marked positive mesomeric effect. The positive inductive effect of alkyl substituents is less pronounced, and as a result, MBR\textsubscript{T\textregistered\textsubscript{c}h} deaeromatises 4-methyl-BzCoA, albeit at a clearly reduced rate compared to BzCoA. In contrast, BCR\textsubscript{T\textregistered\textsubscript{a}r} shows no activity with 4-methyl-BzCoA, which is most likely due to steric effects of the relatively bulky methyl moiety. The proposed steric hindrance of BCR\textsubscript{T\textregistered\textsubscript{a}r} by bulky para-substituents is further corroborated by the ability to defluorinate 4-fluoro-BzCoA but not the the bulky 4-chloro- and 4-bromo-BzCoA analogues with good leaving groups. In contrast, MBR\textsubscript{T\textregistered\textsubscript{c}h} converts para-substituted BzCoA analogues in the order Br > Cl > F, as expected from the order of bond dissociation energies (37).

The reductive dehalogenation of 4-chloro-/4-bromo-BzCoA to BzCoA and HCl has previously not been reported for a BCR and accounts for further ATP-dependent dehalogenation reaction of BCRs, next to the dehalogenation of 3-chloro-/3-bromo-BzCoA (23), and 4-fluoro-BzCoA (24). The dehalogenation reactions most likely proceed via reduced 4-chloro-/4-bromo-1,5-dienoyl-CoA intermediates that similar to 3-chloro-1,5-dienoyl-CoA will spontaneously eliminate HCl/HBr, driven by rearomatization to BzCoA. This assumption is supported by the determination of traces of a compound by ESI-Q-TOF-MS with ion spectra fitting to a chloro-1,5-dienoyl-CoA intermediate. As an alternative, dehalogenation may occur on the level of the one-electron reduced aryl radical anion intermediate. Such a S\textsubscript{RN}1 mechanism has recently been proposed for aminofutalosine synthase during debromination of a substrate analogue (38).

The preferred substrate of MBR\textsubscript{T\textregistered\textsubscript{c}h} 3-methyl-BzCoA, was predominantly reduced to the 3-methyl-1,5-dienoyl-CoA isomer demonstrating the regioselectivity of the enzyme towards the meta-positioned substituent. This finding is in full accordance with the observed complete reductive dehalogenation of 3-chloro-BzCoA to BzCoA and HCl in both MBR\textsubscript{T\textregistered\textsubscript{c}h} and BCR\textsubscript{T\textregistered\textsubscript{a}r} (23). The latter reaction can only occur via a 3-chloro-1,5-dienoyl-CoA but not via a 5-chloro-1,5-dienoyl-CoA intermediate. No regioselectivity was observed for the conversion of 3-fluoro-BzCoA, where both 3- and 5-fluoro-1,5-dienoyl-CoA products were identified in a recent study (28). Therefore, obviously steric effects govern the regioselectivity of BCRs towards meta-positioned functionalities.

This work demonstrates that the previously unknown capability of a BCR to reduce 4-chloro-BzCoA to BzCoA and HCl allows for growth with 4-chlorobenzoate under denitrifying conditions. Growth with this halobenzoate in aerobic bacteria has been demonstrated already 25 years ago (39–42). Interestingly, the aerobic degradation also proceeds via 4-chloro-BzCoA, but in this case, dehalogenation is achieved by hydrolysis to 4-hydroxy-BzCoA rather than by reduction, as demonstrated in this work. The 4-hydroxy-BzCoA dehalogenase involved catalyzes a nucleophilic substitution reaction via a Meisenheimer complex, and its structure and function has been studied on the molecular level (43–45). Under anaerobic conditions, complete mineralization of 4-chloro-benzoate has so far only been described for enrichment cultures, often comprising \(\beta\)-Proteobacteria (46–48, 36, 49). Thus, \textit{T. chlorobenzoica} appears to be the first pure culture described with the capability to use 4-chlorobenzoate anaerobically as growth substrate.

Dechlorination of 4-chloro-BzCoA via ATP-dependent MBR, instead of using a 4-chloro-
BzCoA hydrolase, appears at first sight as an unnecessary consumption of ATP. However, MBR converts 4-chloro-BzCoA directly to the central intermediate BzCoA, and circumvents the biosynthesis of 4-hydroxy-BzCoA reductase that would be required for reductive dihydroxylation. This complex enzyme of the xanthine oxidase family depends on numerous redox cofactors, such as molybdopterin, FAD, and three Fe/S clusters (50, 11, 51, 52). Finally, the expense of two ATP for the reductive dehalogenation appears to be rather marginal relative to the overall high ATP yield achieved by the oxidation of benzoate analogues under denitrifying conditions (53).

The capability of MBR<sup>Td</sup> to reduce 4-methyl-BzCoA suggests that <i>T. chlorobenzoica</i> is also capable of using 4-methylbenzoate as growth substrate as reported for <i>Magnetospirillum</i> sp. pMbN1 (20). However, growth of <i>T. chlorobenzoica</i> with 4-methylbenzoate was not observed, probably due to the lack of a 4-methylbenzoate CoA ligase and/or downstream enzymes of the 4-methylbenzoate degradation pathway.

In contrast to BCR<sub>Tar</sub>, MBR<sup>Td</sup> did not accept reduced methyl viologen as an electron donor, which prevented the employment of a continuous spectrophotometric assay monitoring the oxidation of colored reduced methyl viologen. The rational for this finding could be the redox potential of reduced viologen (<i>E<sub>0</sub><sup>′</sup> = −446 mV</i> [31]) that might be too positive to donate electrons to MBR<sup>Td</sup>. Methyl viologen did not act as an inhibitor, as the reaction catalyzed by MBR<sup>Td</sup> with Ti(III) citrate as electron donor was stimulated by 0.5 mM methyl viologen.

The stoichiometry of ATP hydrolyzed per electrons transferred is controversial for members of the BCR/HAD family. For 2-hydroxyacyl-CoA dehydratases a stoichiometry of two ATP hydrolyzed per one electron transferred has been suggested (17). In contrast, for BCR<sub>Tar</sub>, 2.1–2.2 ATP hydrolyzed per BzCoA reduced were determined (33), and for MBR<sup>Td</sup> this study revealed a slightly higher stoichiometry (2.3–2.8 ATP hydrolyzed per BzCoA reduced). Taken together, experimental data point to rather one than two ATP hydrolyzed per electron transferred in class I BCRs, assuming that each electron is transferred in an ATP hydrolysis dependent manner. The question rises, why the stoichiometries determined for BCRs are always slightly higher than two ATP/BzCoA? One possible explanation could be that ATP hydrolysis is not 100% coupled to BzCoA reduction and that a parallel futile background ATP hydrolysis activity exists. Such a scenario is in line with the first electron transfer to the substrate being rate determining in overall BCR<sub>Tar</sub> reaction, rather than ATP hydrolysis (22); it would theoretically allow for a non-coupled ATP hydrolysis to some extent. As an alternative explanation, a portion of the active site module of BCR might be inactive, e.g. due to partial cluster degradation. In such a scenario, the apparent ratio of ATP hydrolysis uncoupled vs coupled to BzCoA reduction would be artificially increased.

The expression platform for genes encoding BCRs established in this work paves the way for the heterologous production of many other BCR- and BCR-like enzymes within the BCR/HAD radical enzyme family. Many BCR-like proteins are present in bacteria that do not grow with aromatic compounds under anaerobic conditions (16), the function of which has remained unknown. Very recently, the crystal structure of an ATP-dependent, BCR-like enzyme has been solved that contained two [4Fe-4S] clusters, bridged by an inorganic sulfur ligand. The enzyme did not contain a CoA-ester-binding pocket but instead reduced a number of small molecules such as acetylene or azide (54). Thus, the catalytic versatility of BCRs and related enzymes may be much bigger than originally anticipated.

**EXPERIMENTAL PROCEDURES**

*Phylogenetic analyses* — Amino acid sequences of 66 Bcr<sub>Tar</sub> homologues of the BCR/HAD family (Table S1) were aligned using MUSCLE (55) in MEGA 6.0.6 software. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (56). The tree with the highest log likelihood (−16445.6661) was depicted. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. No branch swap filter was applied. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA 7.0 software.

*Growth of bacterial cells* —
T. chlorobenzoica was cultivated and the concentrations of growth substrates were determined as described for T. aromatica in a recent work (24). The initial concentrations of growth substrates and nitrate were 1.3 and 6.0 mM, respectively.

**Heterologous gene expression in E. coli**

— Primers for the amplification of the bcrABCD and the mbrONQP gene clusters were derived from partial genome sequences of T. aromatica and T. chlorobenzoica (gi no. 19571177 and 1341820151, respectively) (Table S3). Reverse primers were elongated for C-terminal fusions of the δ-subunits with Strep-tag II (SA–WSHPQFEK). Cloning of DNA sequences using pOT1 as expression vector was conducted as recently described (28).

Gene expression was carried out in E. coli MC4100 (57) growing anaerobically in 2 L TB medium (89 mM KH₂PO₄/K₂HPO₄ at pH 7.5, 12 g L⁻¹ tryptone, 24 g L⁻¹ yeast extract, 0.4% glycerol [v/v], 5 mM NaNO₃, 50 mM fumarate, 0.2 g L⁻¹ Fe(III) citrate; 75 µg mL⁻¹ kanamycin A). After inoculation, flasks were sealed with rubber stoppers and air in the headspace was replaced by flushing with N₂ gas. Cells were incubated at 37°C until reaching an optical density of 0.4–0.6 whereupon gene expression was induced with 1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG) and growth medium was supplemented with 1 mL L⁻¹ of vitamin solution VL-7 (58), trace element solution SL9 (59), and 0.8 mM MgSO₄. Cells were incubated for another 10–12 h at 30°C while they were agitated at 120 rpm. The pH was monitored and frequently adjusted to values >7.0. Cells were harvested anaerobically by centrifugation at 4,500xg (4°C), and were immediately further processed or stored in liquid nitrogen until use.

**Purification of recombinant BCRs**

Purification procedures were carried out under anaerobic conditions (95% N₂/5% H₂) at 4°C. Cells were resuspended (1.5 mL buffer per 1 g of cells) in a 20 mM HEPES/NaOH standard buffer (pH 7.8) containing 200 mM KCl, 4 mM MgCl₂, 50 mM L-arginine, 50 mM L-glutamate, 10% glycerol (v/v), 1 mM dithioerythritol (DTE), 200 µM dithionite and ~0.1 mg L⁻¹ DNase I. Cell suspensions were passed twice through a French pressure cell at 120 MPa. The cell lysate was centrifuged at 150,000xg for 1 h prior to applying the supernatant onto a Strep-Tactin Superflow High Capacity column (IBA) according to manufacturer's instructions. After washing with DNase-free buffer containing 100 mM KCl and 50 µM dithionite, proteins were eluted at pH 7.3 with 3 mM desthiobiotin. Proteins were concentrated using Vivaship Turbo 4 centrifugal concentrators (30K MWCO) (Sartorius) to final concentrations of 5–30 mg mL⁻¹ and stored anaerobically at −80°C.

**Estimation of the molecular mass of MBRₜᵣₐₜ**

— The molecular mass was estimated by size exclusion chromatography applying 100 µL of a 3 mg mL⁻¹ protein solution to a 24 mL Superdex 200 Increase 10/300 GL column (GE Healthcare, Freiburg, Germany), in 20 mM HEPES buffer with 150 mM NaCl, 4 mM MgCl₂, 1 mM DTE and 50 µM dithionite. Calibration was performed using 100 µL standard solutions containing thyroglobulin (bovine thyroid) (Mᵣ = 669,000), alcohol dehydrogenase (S. cerveisia) (Mᵣ = 150,000), carbonic anhydrase (Mᵣ = 29,000) and cytochrome c (equine heart) (Mᵣ = 12,400), 3 mg mL⁻¹ of each.

**Synthesis of CoA thioesters**

— BzCoA was synthesized from benzoic acid anhydride and CoA as described (60). Substituted BzCoA analogues were synthesized from the corresponding acids via their succinimidyl esters as described (61).

**Determination of protein and iron content**

— Protein concentrations were routinely determined by the method of Bradford using bovine serum albumin solutions as standard. The iron content was determined as described recently (27). Amounts of the heterotetrameric protein applied for determination were 0.5–3 nmol.

**UV/vis spectroscopy**

— UV/vis spectra were recorded under anaerobic conditions in a glove box. MBRₜᵣₐₜ as isolated was pretreated as a mixture of 25.5 µM enzyme plus 125 µM thionine acetate as mild oxidant in 20 mM HEPES buffer, containing 4 mM MgCl₂ and 10% glycerol (v/v). Thionine was then removed by applying a PD MinTrap G-25 desalting column (GE Healthcare) according to manufacturer’s instructions. During the recording of UV/vis spectra, 1 mL MBRₜᵣₐₜ diluted to 4.2 µM was reduced by addition of dithionite from a 0.5 mM stock solution in 2.5 µL steps. After the addition of 22.5 µL, the steps were raised to 10 µL and after the total addition of 72.5 µL, a 5 mM stock solution was used, instead.

**Determination of BCR/MBR activities**

— Enzyme activities were determined and CoA ester intermediates were identified in a discontinuous HPLC/UPLC (Waters) based assay as described earlier, using routinely 5 mM Ti(III) citrate as artificial electron donor (24, 29). Dithionite, NADH and dithionite-reduced methyl viologen as...
alternative electron donors were applied at concentrations of 5 mM, 1 mM and 1 mM, respectively. For the determination of the pH-dependence of enzyme activities, 3-(N-morpholino)propanesulfonic acid (MOPS) as standard reaction buffer was substituted by 2-(N-morpholino)ethanesulfonic acid (MES; pH 6.3), 2-[(1,3-dihydroxy-2-(hydroxymethyl)-2-propanyl]amino]ethanesulfonic acid (TES; pH 7.8) or [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS; pH 8.3/8.8). Studies of oxygen sensitivity were conducted by aerobic incubation of enzyme solutions for different periods in shaking 1.5 mL Eppendorf tubes before the immediate start of reactions under anoxic conditions. Reactions were stopped by two volumes of methanol, and centrifuged supernatants were subjected to isocratic HPLC (Waters). For the detection of lower CoA ester concentrations during Kcat-value determinations, reactions were stopped by the addition of 0.08 volumes of 1% H2SO4. For determination of catalytic constants, the initial rates determined at different substrate concentrations were fitted to Michaelis-Menten curves using the Prism 6 software package (GraphPad). Activity of BCR was also determined in a continuous spectrophotometric assay with dithionite reduced methyl viologen serving as the electron donor as described before (10).

Determination of ATPase activities and stoichiometry of ATP hydrolysis/CoA reduction — MBRrTc, oxidized as described above for UV/vis spectroscopy, was incubated at a final concentration of 2 µM with 5 mM ATP and 15 mM MgCl2 in a 100 mM MOPS buffer (pH 7.3) at 30 °C. Samples were taken at different time points from the running assays and stopped by the addition of two volumes of a 1 M HCl solution containing 10% acetonitrile. For determination of the stoichiometry of ATP hydrolysis and BzCoA reduction, BzCoA was present in the assays. Samples for BzCoA determination were stopped in 66% methanol (v/v, final concentration).

Analysis of ATP/ADP and BzCoA by UPLC was performed using a Waters C18 HSS T3 column (2.1 mm × 100 mm, 1.8 µm particle size) at 25°C. ATP/ADP were separated isocratically in 10 mM potassium phosphate buffer (pH 6.5) as the sole component in the mobile phase, at a flow rate of 0.2 mL min⁻¹ for 5 min. For detection of BzCoA, the acetonitrile content in a 10 mM potassium phosphate buffer (pH 7) was gradually increased at a flow rate of 0.21 mL min⁻¹ from 2 to 10% within 1.5 min followed by an increase from 20% up to 30% within 1.6 min. Products were identified by comparing retention times and UV/vis absorption spectra with standards and/or additionally by subsequent MS analysis (see below).

Analysis of CoA esters by mass spectrometry – LC/MS analysis of CoA esters was performed with a Waters Acquity UPLC using standard reaction buffer was substituted by 2-(N-morpholino)ethanesulfonic acid (MES; pH 6.3), 2-[(1,3-dihydroxy-2-(hydroxymethyl)-2-propanyl]amino]ethanesulfonic acid (TES; pH 7.8) or [tris(hydroxymethyl)methylamino]propanesulfonic acid (TAPS; pH 8.3/8.8). For separation, a 6 min linear gradient from 2 to 30% acetonitrile in 10 mM ammonium acetate (pH 6.8) at a flow rate of 0.35 mL min⁻¹ was applied. The mass spectrometer was operated in MS negative or positive mode with a capillary voltage of 2.5 kV or 3.0 kV, 120°C or 150°C source temperature, 400°C or 450°C desolvation temperature and 750 L h⁻¹ or 1000 L h⁻¹ N2 desolvation gas flow, respectively. In MS negative mode, additionally cone gas flow was applied at a rate of 100 L h⁻¹.

Analysis of products formed from 3-methylbenzoyl-CoA by NMR spectroscopy – The assay for NMR spectroscopic analyses comprised 0.5 mM 3-methyl-BzCoA, 5 mM Ti(III) citrate, 5 mM MgATP and 25 µM MBBrTc in 100 mM MOPS buffer (pH 7.3) with 15 mM MgCl2 in 95% D2O, and the reaction mixture had a total volume of 22.7 mL. Reactions were stopped with by addition of 66% of methanol (v/v, final content), which was removed after subsequent centrifugation from the supernatant by flash evaporation at 40°C and before freeze-drying of the residual solution. CoA thioesters were purified by reversed-phase HPLC following previously described procedures (61). Purified compounds were desalted by solid phase extraction as described earlier (62).

Prior to analysis, the compounds were dissolved in 0.5 mL deuterated water. 1H NMR and 13C NMR spectra were recorded at 500 MHz and 126 MHz, respectively, with Avance-HD 500 spectrometers operating at 27°C. 1H-Detected experiments including two-dimensional COSY, NOESY, HSQC and HMBC were measured with an inverse 1H/13C probe head; direct 13C measurements were performed with a QNP 13C/1H/P/19F/59Si cryprobe. All experiments were done in full automation using standard parameter sets of the TOPSPIN software package (Bruker). 13C NMR spectra were recorded in proton-decoupled mode. Data processing was
typically done with the MestreNova software.

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**Author contributions:** OT planned and conducted most of the experiments, analyzed the results, and wrote the paper; JF carried out enzyme purification and ATP-stoichiometry experiments; WE performed NMR spectroscopic analyses; MB conceived the idea for the project, and wrote the paper.
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Abbreviations used are: BzCoA, benzoyl-coenzyme A; BCR, BzCoA reductase; MBR, methyl-BzCoA reductase; HAD, 2-hydroxyacyl-CoA dehydratase; UPLC, ultra performance liquid chromatography; UV-Vis, ultraviolet-visible; ESI-Q-TOF-MS, electrospray quadrupole time-of-flight mass spectrometry; NMR, nuclear magnetic resonance.
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Table 1. Substrate preference of MBR_{Tcl} and BCR_{Tar} and identification of the products formed. The relative activities were derived from specific activities determined in assays with 0.25 mM CoA ester substrate and are referenced to those obtained with BzCoA as substrate (100%). Specific activity of MBR_{Tcl} with BzCoA was 212 mU mg\(^{-1}\). Activity values of recombinant BCR_{Tar} were determined in this work, or are referred to wild type BCR_{Tar} taken from (§) (16), (†) (22), (#) (23) and (‡) (24). Reaction products were identified by UPLC analysis and, in case of unknown retention times and/or UV/vis absorbance spectra, coupled to ESI-QTOF-MS detection (Fig. S4–S7). After prolonged incubation with Ti(III) citrate, 1,5-dienoyl-CoA as well as the fluorinated and methylated analogues were further reduced to corresponding monoenoyl-CoAs as reported earlier for BCR_{Tar} (29). – not determined.

| Substrate | Relative activities (%) | Kinetic properties of MBR_{Tcl} | Products identified |
|-----------|-------------------------|--------------------------------|---------------------|
|           | MBR_{Tcl} | BCR_{Tar} | \(K_m\) (\(\mu\)M) | \(k_{cat}\) (s\(^{-1}\)) | \(k_{cat}/K_m\) (s\(^{-1}\) M\(^{-1}\)) | |
| BzCoA     | 100       | 100       | 10                  | 0.55               | 5.5×10\(^4\) | 1,5-dienoyl-CoA |
| 2-CH\(_3\)-BzCoA | 45    | 119       | –                   | –                  | –           | 2- or 6-CH\(_3\)-1,5-dienoyl-CoA |
| 3-CH\(_3\)-BzCoA | 196   | 97        | \(\leq 5\)          | 1.06               | \(\geq 2.1\times10^5\) | 3-CH\(_3\)-1,5-dienoyl-CoA* |
| 4-CH\(_3\)-BzCoA | 15    | < 0.1     | 92                  | 0.10               | 1.1×10\(^3\) | 4-CH\(_3\)-1,5-dienoyl-CoA |
| 4-CH\(_3\)-CH\(_2\)-BzCoA | 31   | –         | –                   | –                  | –           | 4-CH\(_3\)-CH\(_2\)-1,5-dienoyl-CoA |
| 2-F-BzCoA  | 102       | 145\(^\dagger\) | 66                  | 0.56               | 8.5×10\(^3\) | 2- and 6-F-1,5-dienoyl-CoA |
| 3-F-BzCoA  | 12        | 31\(^\dagger\) | –                   | –                  | –           | 3- and 5-F-1,5-dienoyl-CoA |
| 4-F-BzCoA  | 7         | 6\(^\dagger\) | –                   | –                  | –           | 1,5-dienoyl-CoA |
| 2-Cl-BzCoA | 37        | 106\(^\dagger\) | –                   | –                  | –           | 2- and/or 6-Cl-1,5-dienoyl-CoA |
| 3-Cl-BzCoA | 145       | 29        | \(\leq 2\)          | 0.89               | \(\geq 4.4\times10^5\) | BzCoA |
| 4-Cl-BzCoA | 18        | < 0.1     | 32                  | 0.10               | 3.1×10\(^3\) | BzCoA |
| 2-Br-BzCoA | 19        | –         | –                   | –                  | –           | 2- and/or 6-Br-1,5-dienoyl-CoA |
| 3-Br-BzCoA | 63        | 34        | –                   | –                  | –           | BzCoA |
| 4-Br-BzCoA | 94        | < 0.1     | –                   | –                  | –           | BzCoA |
| 2-OH-BzCoA | 18        | –         | –                   | –                  | –           | 2- and/or 6-OH-1,5-dienoyl-CoA |
| 3-OH-BzCoA | 173       | 12\(^\dagger\) | –                   | –                  | –           | 3- and/or 5-OH-1,5-dienoyl-CoA |
| 4-OH-BzCoA | < 0.1     | < 0.1     | –                   | –                  | –           | – |

*Product was identified by NMR in this study.
### Table 2. Comparison of MBR_{Tcl} and BCR_{Tar}

| Property                                | MBR_{Tcl}                          | BCR_{Tar}                          |
|-----------------------------------------|-------------------------------------|------------------------------------|
| Reaction catalyzed                      | R–BzCoA -> R–1,5-dienoyl-CoA        | R–BzCoA -> R–1,5-dienoyl-CoA        |
| Estimated molecular size                | 140 ±1 kDa                         | 168 ±1.3 kDa                       |
| Subunit composition                     | αβγδ                               | αβγδ                               |
| Absorption maxima (λ)                   | 317/407 nm                         | 324/410 nm                         |
| Reducibility with dithionite pH 7.3/ 8.3| −/+                                | +/ n.d.                            |
| Metal cofactor                          | 11.6 ±1 Fe/ enzyme                 | 12 Fe/ enzyme                      |
| Half-life time in air                   | 30 s                               | 20 s                               |
| pH optimum                              | 7.3                                | 7.3                                |
| Relative activity at pH 6 / pH 9        | ≈60% / ≈90%                        | <5% / <2%                          |
| Reduction of *para*-substituted BzCoA analogues | 4-F-/Cl-/Br-/CH₃-BzCoA | 4-F -BzCoA                         |
| Artificial electron donors, Ti(III) citrate/ dithionite/ methyl viologen | 100%/ 53%/ 1.5% | 100%/ 100%/ 100% |
Figure 1. Subunit architecture, cofactors and electron transfer of BCR$_{Tet}$. The electron-activating module is depicted in grey, the BzCoA reducing module in white. The electron transfer from the donor reduced ferredoxin (15) to BzCoA is schematically indicated by arrows. Similarities with 2-hydroxyacyl-CoA dehydratases suggest that the cluster in BcrB binds BzCoA. AH = proton donor for BzCoA reduction.
Figure 2. Phylogenetic tree of the BCR/HAD family of radical enzymes. Evolutionary distances of active site β-subunits calculated by the Maximum Likelihood method are illustrated by branch lengths measured in the number of substitutions per site (scale shown as bar). Phylogenetic assignments correspond to the source organisms. Numbers 1–66 refer to strains listed in Table S1. The asterisk marks MBR from T. chlorobenoica.
Figure 3. SDS PAGE of heterologously produced A, MBR$_{Tcl}$ and B, BCR$_{Tar}$ after enrichment by Strep-Tactin affinity chromatography. Lanes are labeled corresponding to protein fractions obtained during the enrichment: M = protein molecular weight marker, CE = crude extract after cell lysis, MF = membrane fraction (150,000×g pellet), SF = soluble fraction (150,000×g supernatant), FL and EL = flow through and elution fraction of affinity chromatography, respectively. The subunits of the enriched enzymes are labeled by Greek letters. The α-subunit of BCR$_{Tar}$ is significantly larger than that of MBR$_{Tcl}$. 
Catalytically versatile methylbenzoyl-CoA reductase

Figure 4. Conversion of BzCoA to 1,5-dienoyl-CoA by MBR in the absence and the presence of MgATP. UPLC analyses of samples are shown that were taken at representative time; (1) BzCoA, (2) 1,5-dienoyl-CoA.
Figure 5. UV-Vis spectra of MBR\textsubscript{Tcl} and titration with dithionite. A, absorption spectrum of oxidized enzyme; the bleaching of the spectra after step-wise addition of dithionite is indicated by the arrow at 407 nm. The additionally observed decrease of a shoulder around 320 nm overlapped with the absorbance of dithionite in the course of the measurement. B, selected difference absorbance spectra of oxidized minus reduced MBR\textsubscript{Tcl} obtained during titration with dithionite. C, titration of MBR\textsubscript{Tcl} with dithionite. Up to the addition of one electron equivalent per protein molecule, an almost linear decrease was observed. Further bleaching required high excess of dithionite.
Figure 6. ATPase activities of MBR<sub>Tcl</sub>. ATPase activities were monitored by determination of time-dependently formed ADP, using UPLC analysis. Shown are: ATPase activities of MBR<sub>Tcl</sub> (○) in the thionine-oxidized state and the absence of BzCoA, (●) in the Ti(III)-citrate-reduced state and the absence of BzCoA, (▲) in the Ti(III)-citrate-reduced state and the presence of BzCoA.
Figure 7. Anaerobic growth of *T. chlorobenzoica* with 3-methyl-, 3-chloro-, and 4-chlorobenzoate. Growth curves as determined by determination of OD$_{578}$ of three biological replicates each are depicted as solid lines, substrate consumption curves as dashed lines. Symbols correspond to growth substrates as follows: ( –×–) 3-methylbenzoate, (–□–) 3-chlorobenzoate, (– ○ –) 4-chlorobenzoate.
A catalytically versatile benzoyl-CoA reductase, key enzyme in the degradation of methyl- and halobenzoates in denitrifying bacteria

Oliver Tiedt, Jonathan Fuchs, Wolfgang Eisenreich and Matthias Boll

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