**Finis tolueni**: a new type of thiolase with an integrated Zn-finger subunit catalyzes the final step of anaerobic toluene metabolism

Sina Weidenweber, Karola Schühle, Marie-Luise Lippert, Johanna Mock, Andreas Seubert, Ulrike Demmer, Ulrich Ermler, and Johann Heider

1 Max-Planck-Institut für Biophysik, Frankfurt am Main, Germany
2 Technische Hochschule Mittelhessen, Gießen, Germany
3 Laboratorium für Mikrobiologie, Fachbereich Biologie and SYNMIKRO Center, Philipps-Universität, Marburg, Germany
4 Analytische Chemie, Fachbereich Chemie, Philipps-Universität, Marburg, Germany

**Keywords**

beta-oxidation; half-of-the-sites reactivity; thiolase; toluene metabolism; Zn$^{2+}$ finger

**Correspondence**

U. Ermler, Max-Planck-Institut für Biophysik, Max-von-Laue-Str. 3, Frankfurt am Main 60438, Germany
Tel: +49 69 6303 1054
E-mail: ulrich.ermler@biophys.mpg.de

J. Heider, Laboratorium für Mikrobiologie, Fachbereich Biologie and SYNMIKRO-Center, Philipps-Universität, Marburg 35032, Germany
Tel: +49 6421 28 21527
E-mail: heider@biologie.uni-marburg.de
Website: https://www.uni-marburg.de/de/fb17/fachgebiete/mikrobiologie/ag-heider

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Anaerobic toluene degradation involves β-oxidation of the first intermediate (R)-2-benzylsuccinate to succinyl-CoA and benzoyl-CoA. Here, we characterize the last enzyme of this pathway, (S)-2-benzoylsuccinyl-CoA thiolase (BbsAB). Although benzoylsuccinyl-CoA is not available for enzyme assays, the recombinantly produced enzymes from two different species showed the reverse activity, benzoylsuccinyl-CoA formation from benzoyl-CoA and succinyl-CoA. Activity depended on the presence of both subunits, the thiolase family member BbsB and the Zn-finger protein BbsA, which is affiliated to the DUF35 family of unknown function. We determined the structure of BbsAB from *Geobacter metallireducens* with and without bound CoA at 1.7 and 2.0 Å resolution, respectively. CoA binding into the well-known thiolase cavity triggers an induced-fit movement of the highly disordered covering loop, resulting in its rigidification by forming multiple interactions to the outstretched CoA moiety. This event is coupled with an 8 Å movement of an adjacent hairpin loop of BbsB and the C-terminal domain of BbsA. Thereby, CoA is placed into a catalytically productive conformation, and a putative second CoA binding site involving BbsA and the partner BbsB subunit is simultaneously formed that also reaches the active center. Therefore, while maintaining the standard thioester-dependent Claisen-type mechanism, BbsAB represents a new type of thiolase.

**Introduction**

The four harmful hydrocarbons benzene, toluene, ethylbenzene and xylene, summarized as BTEX, are frequently used in industrial manufacturing processes and therefore occur often as environmental contaminants. Despite their chemical inertness, these pollutants are degraded by specific microorganisms under aerobic or anaerobic conditions [1,2]. The universally conserved pathway of anaerobic toluene degradation (Fig. 1) is

**Abbreviations**

- ACP, acyl carrier protein; ATase, acetyltransferase; bbs, β-oxidation of benzylsuccinate; BbsAB, (S)-2-benzoylsuccinyl-CoA thiolase; BP, basic buffer; BSS/bbs, benzylsuccinate synthase; CHS, chalcone synthase; CoA, coenzyme A; CV, column volume; DUF, domain of unknown function; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HOPC, 17-hydroxy-3-oxo-4-pregnene-20-carboxy-CoA; ICP-OES, inductively coupled plasma optical emission spectroscopy; KAS, ketoacyl-ACP synthase; KCS, ketoacyl-CoA synthase; NAD, nicotinamide adenine dinucleotide; PKS, polyketide synthase; SAD/MAD, single/multiple-wavelength anomalous diffraction; SCP, sterol-carrier-protein.
initiated by adding the methyl group to the double bond of a fumarate cosubstrate, catalyzed by the glycyl radical enzyme benzylsuccinate synthase [3–5]. The product formed, (R)-benzylsuccinate, is further metabolized to benzoyl-CoA via a modified β-oxidation pathway consisting of five enzymatic steps [6]. It is first activated to 2-(R)-benzylsuccinyl-CoA by the CoA-transferase BbsEF [7,8], then oxidized to (E)-benzylidenesuccinyl-CoA by the acyl-CoA dehydrogenase BbsG [9]. Subsequently, water is added at the double bond by enoyl-CoA hydratase BbsH to generate the β-hydroxyacyl compound (S, R)-2-(α-hydroxybenzyl)succinyl-CoA, which is further oxidized to (S)-2-benzoylsuccinyl-CoA by the short-chain dehydrogenase BbsCD [10]. The final step of the pathway is a thiolytic cleavage of (S)-2-benzoylsuccinyl-CoA to succinyl-CoA and benzoyl-CoA [6] (Fig. 1). The product benzoyl-CoA is one of the central metabolites of anaerobic degradation of aromatic compounds and is further degraded via ring reduction [1].

The biochemistry of the first four enzymes of the benzylsuccinate degradation pathway has already been investigated [8–10]. To complete the understanding of the pathway and the enzymatic reactions involved, we focus in this study on the biochemical and structural properties of the last enzyme of the pathway, benzoylsuccinyl-CoA thiolase (EC 2.3.1.-). All known bbs operons, as well as the operons involved in degrading other methyl-aromatic compounds such as p-cresol, p-cymene or 2-methylnaphthalene, contain a gene homologous to bbsB, which codes for a 41 kDa protein affiliated to the thiolase superfamily. This superfamily is subdivided into decarboxylating and non-decarboxylating β-ketothiolases [11,12]. The former catalyze β-condensation reactions and consist of three ketoacyl-ACP synthase subfamilies (KAS1-3), polyketide synthase modules (PKS) and the chalcone synthase subfamily (CHS), which use malonyl-acyl carrier protein (ACP), as well as the ketoacyl-CoA synthase subfamily (KCS), which uses malonyl-CoA. The enzymes affiliated to the non-decarboxylating subfamilies use acetyl-CoA or other CoA thioesters in either β-oxidation or β-condensation reactions and include degradative type I-thiolases, biosynthetic type II-thiolases, 3-hydroxy-3-methylglutaryl-CoA synthases (HMGS) and sterol-carrier-protein (SCP2)-type thiolases. The latter are named after the first identified member, a mammalian peroxisomal thiolase for methyl-branched acyl-CoAs, which is fused to a sterol carrier protein [13]. Judging by sequence alignment, the BbsB-like thiolases, which are all involved in arylsuccinate degradation pathways, form a subgroup of the SCP2-type β-ketothiolases [6], although they show only low identities with the currently characterized members of the subfamily (maximum sequence identities ca. 35%).

Various members of all thiolase subfamilies were already studied and compared on the biochemical and structural levels [12,14,15]. Thiolases are normally homodimers or homotetramers with a characteristic three-domain architecture [16–18]. They catalyze the
The genes encoding BbsB-like proteins are always accompanied by directly neighbouring bbsA-like genes in the known operons related to arylsuccinate degradation. The 17 kDa protein BbsA belongs to the DUF35 protein family, and almost all genes coding for DUF35-like proteins are located directly adjacent to genes for thiolas in bacteria or archaea. Moreover, DUF35 proteins have recently been reported to occur together with thiola-like subunits as constituents of enzyme complexes harbouring C–C forming or cleaving functionalities, such as an archaeal thiola/HMGS complex [19], an acyltransferase involved in diacetylpolarolucinol synthesis in Pseudomonas protegens (ATase) [20], or a complex 17-hydroxy-3-oxo-4-pregnene-20-carboxy-CoA (HOPC-CoA) aldolase of Thermomonospora curvata, where the thiola-like subunit has evolved to an aldolase functionality [21]. In addition, some additional solitary DUF35 proteins have been characterized as potential regulators [22–25]. The solitary SSO2064 protein of Sulfolobus solfatarius (31RB) [24] has long been the only structurally studied DUF35 family member, and only recently more structures of DUF35 family members have been reported [22,23]. In case of BbsA, we have proposed earlier that it may be an additional subunit of benzoysuccinyl-CoA thiolase or otherwise associated with the enzyme [6]. Therefore, our aim was to find out the relation of the bbsA gene product with benzoysuccinyl-CoA thiola activity. In this report, we analyse the benzoysuccinyl-CoA thiola (BbsB) complex biochemically and structurally and thereby confirm that both BbsA and BbsB are essential for its enzymatic functionality. This dependency on DUF35-type subunits is likely shared by most thiolas of the largely uncharacterized SCP2 subfamily, unveiling a novel catalytic role of Zn-finger proteins and further expanding the variety of the vast and important thiola superfamily.

Results

Production of recombinant benzoysuccinyl-CoA thiolas BbsAB

We set out to characterize benzoysuccinyl-CoA thiola by expressing the bbsAB genes from Thauera aromatica [6] in recombinant Escherichia coli cells (protein accession numbers AVR89970 and AVR89971). During the first purification step, chromatography on DEAE-sepharose, the jointly produced BbsB proteins were separated into three distinct pools: the first, eluted at 80 mM KCl, contained both subunits, the second, eluted at 300 mM, contained only BbsA and the third, eluted at 500 mM, contained only BbsB. The final yields were 2 mg of BbsAB, 8 mg of BbsA and 9 mg of BbsB from 9.3 g wet cell mass (765 mg total soluble protein). Because of the low amount of the retrieved BbsAB complex, only the separated BbsA and BbsB proteins were further purified by chromatography on ceramic hydroxyapatite to 80–90% homogeneity, as evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 2A). For the production of an intact BbsAB complex in higher yield, the bbsAB genes from T. aromatica were cloned into an anhydrotetracyclin-inducible broad host range vector (described in Ref. [5]) with a 5’ strep-tag fusion sequence added to the bbsA gene. The same was done for the related bbsAB genes from the toluene-degrading Fe3+-reducing bacterium Geobacter metallireducens whose genome contains orthologues of all enzymes involved in benzoysuccinate metabolism (Fig. 1; protein accession numbers ABB31762 and ABB31763) [26]. As the protein yields of these BbsAB orthologues in E. coli expression strains (DH5α or Arctic) were still rather poor (5–15 mg/10 g wet cell mass), the plasmids were transformed into various other potential bacterial host species using electroporation, resulting in a much better production yield of BbsAB in Pseudomonas stutzeri (up to 60 mg/10 g wet cell mass). The tagged BbsAB complexes from either T. aromatica or G. metallireducens were purified in one step via strep-tag affinity chromatography without apparent splitting up into the respective subunits, yielding 50–60 mg pure protein per 10 g wet cell mass. The proteins intended for crystallization were further purified by size exclusion chromatography to remove the last few contaminations (Fig. 2B).

Activity of the proteins as benzoysuccinyl-CoA thiola was determined by measuring the reverse reaction via high performance liquid chromatography (HPLC) analysis, namely the condensation of benzoyl-CoA and succinyl-CoA to benzoysuccinyl-CoA, because benzoysuccinyl-CoA is neither commercially available nor easily accessible via chemical synthesis. No activity was recorded in recombinant cell extracts or DEAE fractions because thioesterase activity leads to rapid hydrolysis of benzoysuccinyl-CoA [10]. Likewise, purified BbsB or BbsA alone did not show measurable activity, but equimolar mixtures of both proteins showed benzoysuccinyl-CoA synthesis with specific activities of 6–11 µmol·min⁻¹·mg⁻¹, indicating that benzoysuccinyl-
CoA thiolase is indeed the first confirmed example of a thiolase which depends on the presence of a DUF35 family protein for activity. In case of the tagged BbsAB preparations, specific activities of the purified proteins were in the range of 12–14 µmol·min⁻¹·mg⁻¹ with no significant difference between the T. aromatica or G. metallireducens versions or the preparations with or without the size exclusion step.
Functional and molecular properties of BbsAB

The optimum pH for benzoysuccinyl-CoA formation from benzoyl-CoA and succinyl-CoA was at pH 6.4, and HPLC analyses confirmed that BbsAB catalyzed the time-dependent decrease of succinyl-CoA and benzoyl-CoA with a simultaneous increase of a new compound, which comigrated with benzoysuccinyl-CoA obtained in another study (Fig. 2C) [10]. The chemical nature of this product as a ketone-containing compound was further supported by converting it to a hydrazone by adding phenylhydrazine, resulting in a shift of the retention time (Fig. 2C). The native molecular masses of BbsA, BbsB and BbsAB were determined by size exclusion chromatography, indicating a heterotetrameric α2β2 structure of the holoenzyme (125 ± 8 kDa) as well as homodimeric structures of the BbsA and BbsB subcomplexes (31 ± 4 and 91 ± 7 kDa, respectively). The high affinity between BbsA and BbsB was also documented by the finding that tagged BbsA and BbsB always eluted together in a strict 1:1 stoichiometry although only BbsA was equipped with an N-terminal strep-tag fusion. UV–Vis spectroscopy revealed no apparent light-absorbing cofactors in either protein.

As the deduced amino acid sequence of BbsA predicted the presence of a zinc-finger motif (CxxC-X10-CxxC), we performed the metal analysis with the purified proteins. The presence of Zn was indeed confirmed: a chemical determination using zincon reagent [27] showed 0.7–1.3 mol zinc per mol BbsA or BbsAB protomer but no zinc in purified BbsB, and an additional metal analysis using inductively coupled plasma–optical emission spectroscopy (ICP-OES) revealed 1.1 zinc per BbsA and no further metals in significant amounts.

Kinetic properties of BbsAB

The kinetic properties of the reverse reaction of BbsAB were determined with the reconstituted untagged enzyme from T. aromatica in a direct photometric assay by coupling the formation of benzoysuccinyl-CoA to condensation with phenylhydrazine and following the reaction by recording the absorption increase at 312 nm due to hydrazone formation (Fig. 2D). Preincubated assays with constant amounts of benzoyl-CoA (50, 100 and 250 μM, respectively) were started with varying concentrations of succinyl-CoA (0–500 μM) and vice-versa. The obtained data (substrate concentrations versus specific activity) showed hyperbolic curve progressions, consistent with Michaelis–Menten kinetics without cooperative effects (Fig. 2E). Since two-substrate reactions usually follow either ping-pong (= substituted enzyme) or ternary complex mechanisms [28], the kinetic behaviour of BbsAB was analyzed by non-linear curve fitting against the equations describing either kinetic mechanism. Surprisingly, we obtained high R² correlation coefficients for either type of mechanism, which showed only a small preference for a ternary-complex mechanism (Table 1, Fig. 2E).

The structure of the Bbs(AB)₂

Unfortunately, we did not get good crystals for the tagged or untagged versions of BbsAB from T. aromatica, and therefore switched to a strep-tagged BbsAB complex from G. metallireducens for crystallization studies. Due to the high sequence similarity between the orthologues (BbsA: 69% identical amino acids; BbsB: 77% identical amino acids) and the same physiological roles, we expect comparable characteristics of both enzymes and have not repeated the detailed biochemical assays. The crystal structure at 2.0 Å resolution revealed the enzyme as heterotetrameric Bbs(AB)₂ complex in line with the biochemical data in solution. The four subunits are assembled in a compact and interconnected manner with a BbsB dimer forming the core and one BbsA attached to each BbsB (Fig. 3A). This overall architecture corresponds

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<th>Kinetic model</th>
<th>Ternary complex</th>
<th>Ping-Pong</th>
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<tr>
<td>Variable substrate</td>
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<td>Benzoyl-CoA</td>
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<tr>
<td>Parameters</td>
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<td>Kᵣₘ benzoyl-CoA (μM)</td>
<td>114 ± 30</td>
<td>172 ± 54</td>
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<td>Kₛₐₜ (μM)</td>
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<td>R²</td>
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Table 1. Calculated kinetic parameters for the two-substrate reaction of BbsAB, using either succinyl-CoA or benzoyl-CoA as variable substrate, and assuming either a ternary complex or a ping-pong mechanism. Standard deviations are given as obtained from the respective curve fitting. R² indicates the correlation coefficient of the respective fit. 1 U = 1 μmol-min⁻¹; n.a., not applicable.
to those of the corresponding submodules in the multisu
bunit complexes of thiolase/HMGS of Methanothermococcus thermolithotrophicus [19], ATase of P. protegens [20], or HOPC-CoA aldolase of T. curvata [21].

The structure of the subunit BbsB revealed a classi
cal thiolase fold with an N-terminal domain (residues 1–113 and 209–233), an insertion domain (residues 114–208) and a C-terminal domain (residues 234–391) (Fig. 3A). The close structural relationship between the N- and C-terminal domains, which are both endowed with a typical \( \alpha \beta \alpha \beta \beta \beta \) thiolase topology, is reflected in an rmsd value of 2.3 \( \AA \) between them (84% of the C\( \alpha \)-atoms used for calculation). The insertion domain, which is usually the most variable region between different thiolases, is located between the \( \beta \)-strands 106:114 and 210:219 of the N-terminal domain [12]. As previously reported for several other thiolases, a short segment within this domain (residues 119–129) is highly flexible or disordered [16,18,29]. This segment has been identified as a covering loop for the bound CoA in thiolases, but its amino acid sequence is only conserved among BbsB orthologues and not similar to that of any other group of thiolases. The closest structurally characterized relatives to BbsB are a peroxisomal SCP2-type thiolase from zebrafish (6HRV) [30], and the thiolase modules embedded in the more complex thiolase/HMGS from M. thermolithotrophicus (6ESQ) [19] and ATase from P. protegens (5M3K) [20]. They are all affiliated to the SCP2 subfamily of thiolases and exhibit sequence identities of 34%, 31% and 22% as well as rmsd values of 1.9, 2.1 and 2.3 \( \AA \) (94%, 95% and 92% of the C\( \alpha \)-atoms used), respectively [31].

BbsA is a member of the DUF35 family [25] and is architecturally composed of an N-terminal rubredoxin-like Zn-finger domain (residues 16–70) and an oligonucleotide/oligosaccharide binding (OB) C-terminal domain (residues 71–160) (Fig. 3A). The OB domain is built up of five long antiparallel \( \beta \)-strands (residues 72–80, 91–97, 102–109, 135–143 and 122–131) in a \( \beta \)-barrel-like arrangement. The Zn-finger domain consists of an N-terminal extension (residues 1–30) and a globular Zn binding segment (residues 31–70) with a Znribbon fold [25] characterized by a three-stranded anti
parallel \( \beta \)-sheet (residues 35–42, 46–49 and 63–68) (Fig. 3A). It contains a conserved Zn-binding
signature motif with four cysteines (C_{42}XXC_{45}-10X-C_{55}XXC_{58}). The position of the Zn was identified by an anomalous peak (40σ) obtained from a synchrotron data set collected at the Zn absorption edge (Table 2). In the last years, several DUF35 family members were structurally characterized. The closest relationship was calculated between BbsA and the DUF35 subunits of thiolase/HMGs from *M. thermmolitotrophicus* (6ESQ), HOPC-CoA aldolase of *T. curvata* (6OK1) and the multicomponent ATase from *P. protegens* (5M3K) [31], resulting in an rmsd values of 2.4, 2.9, and 2.9 Å (representing 93–83% of the C\textsubscript{a}-atoms) with sequence identities between 30% and 18%, respectively. Noticeable differences among the DUF35 family members are present in their N-terminal extensions, which mostly show helical folds in other DUF35 proteins while residues 1–16 of BbsA are disordered and residues 17–29 form two short β-strands interacting with β-strands 35 : 42 and 135 : 143 to a common β-sheet. The disordered strep-tag and the first 16 residues of the N-terminal arm of BbsA (dominated by acidic and basic amino acids) are not cleaved off by

<table>
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<th>Table 2. Statistics of the crystal structure analysis. rmsd, root mean square deviation.</th>
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<td><strong>Crystallization</strong></td>
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<td>16% PEG3350, 100 mM sodium-citrate, 100 mM Bis-Tris propane pH 6.5</td>
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<tr>
<td>100 mM Bis-Tris, pH 6.5, 2.5 mM K\textsubscript{2}OsO\textsubscript{4}</td>
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\textsuperscript{a}Values relative to the highest resolution shell are within parentheses.; \textsuperscript{b}R\text{free} was calculated as the R\text{work} for 5% of the reflections that were not included in the refinement.
proteases, based on mass-spectrometric data and the retention of the N-terminal strep-tag in the recombinant protein, but it is unknown whether they have any function. The first visible amino acid, Asp17, forms a salt bridge with Arg129 of BbsB, and the short distance between Asp17 and the bound CoA cofactor in BbsB (< 15 Å) may allow potential interactions of the unresolved N-terminal arm of BbsA and the active site(s) of the BbsB subunits.

The Bbs(AB)_2–CoA complex
The X-ray structure of the Bbs(AB)_2–CoA complex at 1.7 Å resolution indicates that one BbsAB protomer contains the substrate CoA in the position conserved in all thiolases (Fig. 3A,B) while the equivalent binding site in the second BbsAB protomer is empty (Fig. 3A). This CoA cofactor shows high occupancy and is embedded in a 20 Å deep cavity of BbsB and fixed by multiple interactions (Figs 3B and 4). Its binding cavity is predominantly formed by the segments between residues 194–208 and the covering loop (residues 119–129) of BbsB. Notably, both segments are highly conserved between different BbsB orthologues, but differ considerably from any other enzyme of the thiolase superfamily, including other enzymes of the SCP2-branch (see Discussion). The bound CoA cofactor exhibits an outstretched conformation from the thiol to the diphosphate. At the ribose moiety, positioned at the entrance of the cavity, the molecule is bent by 180° (Fig. 3B). This allows intramolecular hydrogen bonding within the CoA cofactor between the hydroxy group of the pantoic acid and the amine group of the adenine moiety (Fig. 4). The cavity is predominantly coated by hydrophobic residues, except for Lys19, Arg119 and Arg194, which interact with the diphosphate and 3'-phosphate groups of the tightly bound CoA. The conserved Cys206 within this region, which was initially considered as a candidate for acid/base catalysis, is involved in CoA binding (Fig. 4). Comparing the structures of the empty and CoA-bound BbsAB protomers of Bbs(AB)_2–CoA as well as CoA-free enzyme, it becomes evident that CoA binding induces considerable changes (Fig. 5). The covering loop of BbsB is converted from completely disordered in the CoA-free enzyme (Fig. 5B) into a rigid conformation when contacting the bound CoA cofactor in the CoA-binding protomer (Fig. 5C). It moves ca. 8 Å towards CoA and folds into a predominantly linear conformation parallel to the stretched CoA segment between the cysteamine and the diphosphate, which creates multiple mutual interactions (Figs 4 and 5C). Concomitantly, a hairpin-like loop between residues 128 and 141 of BbsB (directly following the covering loop) and the entire OB domain of the BbsA subunit rotate towards the bound CoA, while the globular Zn binding segment of BbsA remains in its original position (Fig. 5A). As a consequence, the linker between the prolonged strands 72 : 80 and 102 : 109 of the OB domain of BbsA gets displaced by 6 Å and interacts with various residues of the covering loop and the hairpin-like loop of BbsB. Therefore, positioning and trapping of the CoA in the active site are clearly dependent on a concerted action of BbsA and BbsB, consistent with the biochemical finding that both subunits are required for activity. BbsA even forms one direct interaction with CoA,
namely a van-der-Waals contact between Ile82 and the pantoic acid moiety (Fig. 4).

The rearrangement of the covering loop observed in the Bbs(AB)₂–CoA complex also implies the formation of a new cavity close to the tightly bound CoA, which is mainly lined up by the covering loops of both BbsB subunits and segments of one BbsA (Fig. 3B). This cavity contains an elongated ligand, which we interpret as a second, more weakly occupied CoA (Fig. 3A). The found electron density can be assigned to the segment between the thiol and amido groups (cysteamine/β-alanine), the phospho and partially to the diphospho groups of CoA (Fig. 3B). The adenosine part is essentially disordered. The negatively charged diphospho and phospho groups might interact with His118 and Arg119 of BbsB, as well as Arg129 of BbsB0. The cavity reaches a depth of ca. 15 Å and the thiol group of the putative CoA would approach Cys85 of the active center to ca. 5 Å (Fig. 3B). In comparison with the CoA-free Bbs(AB)₂ structure (Fig. 5B) the disordered covering loop of BbsB from (C) superimposed with CoA-free BbsB or BbsB₀ is drawn in line mode into (B) and (D) (grey C-atoms).

**Fig. 5.** Detail view of the first CoA binding site. (A) Polypeptide rearrangements induced by CoA binding. CoA is drawn as a stick model (C atoms in yellow). The coincident movements of the covering loop of BbsB (dark grey to red) and the OB domain of BbsA (light grey to green) upon CoA binding to the Bbs(AB)₂–CoA complex are inferred from comparing the empty and CoA-bound BbsAB protomers (marked by arrows). The four catalytic loops framing the active site of BbsB, highlighted in black (L1–L4), supply the required set of key amino acids and reaction compartments [11,12]. (B) Electron density (σ = 1.0) at 2.0 Å resolution of a covering loop of the Bbs(AB)₂ complex (light salmon) that contains no CoA. There is no electron density between Gln120 and Ala127. (C) Interactions of the tightly bound CoA-cofactor with the covering loop of the Bbs(AB)₂–CoA complex. The electron density at 1.7 Å resolution is contoured at 2.0 σ. (D) Covering loop of the BbsAB protomer of the Bbs(AB)₂–CoA complex that does not contain CoA (green C-atoms). Electron density is contoured at 1.0 σ (grey) and 2.0 σ (gold) to visualize that the covering loop is highly flexible but can still be modelled. Its partial rigidification compared to the CoA-free enzyme is presumably due to its interactions with the putative second CoA. For comparison, the CoA associated covering loop of BbsB from (C) superimposed with CoA-free BbsB or BbsB₀ is drawn in line mode into (B) and (D) (grey C-atoms).
(AB)₂-CoA structure (Fig. 5C). Its position and conformation significantly differ between BbsB' and BbsB. While the covering loop of BbsB is strongly bound to the first CoA (Fig. 5C), its counterpart in BbsB' substantially contributes in binding the second putative CoA at BbsB to form a productive active site with both substrates (Fig. 3B). Therefore, the covering loop of BbsB' is too far away to bind CoA in an equivalent fashion in its own binding site, which rationalizes the observed absence of the cofactor. Obviously, active site formation, primarily associated with one BbsAB protomer, is highly dependent on interactions with the second protomer (Figs 3B and 5A) such that only one active site is operative in the enzyme at a certain time.

The site of the thiolase reaction is located in front of the thiol groups of the two bound CoA cofactors and is essentially built up of four catalytic loops (residues 85–87, 281–284, 330–332 and 369–372; L1-4 in Fig. 5A), which are characterized by the fingerprint motifs CSA, HDAF, GHP and GGG, respectively. While loops 1 and 2 contain the strictly conserved catalytic key players Cys85 and His281, which are in direct contact to the thiol group of the tightly bound CoA, loops 3 and 4 constitute the oxyanion holes II and I, respectively, which are required for stabilizing the two o xoanion intermediates involved during the reaction mechanism (see below).

Locking the covering loop around the CoA cofactor also leads to a conformational change of the catalytic loop 4, by which a hydrogen bond is formed between the amido function of Ala128 and the carbonyl oxygen of Gly372 (Fig. 5C). Thus, the CoA binding induced conformational changes of BbsA and BbsB may directly affect the turnover process of BbsAB.

**Substrate binding in BbsAB**

BbsAB is expected to turn over (S)-2-benzylo succinyl-CoA as substrate, because this diastereomer was found to be produced by BbsCD, the enzyme preceding BbsAB in the pathway (Fig. 1) [10]. Therefore, (S)-2-benzylo succinyl-CoA was modelled based on the geometric/e lectrostatic properties of the active site of BbsAB thereby starting from the firmly bound CoA. Accordingly, the carboxymethyl group of (S)-2-benzylo succinyl-CoA group interacts with the hydroxy groups of Tyr144 and Thr369 and the δ-nitrogen of the imidazole ring of His378, which are all universally conserved in the BbsB clade, but not in other members of the thiolase superfamily (only a few other bacterial SCP2-members contain a His378 analogue). The benzoyl group of (S)-2-benzylo succinyl-CoA is flanked by three hydrophobic amino acids of BbsB (Ile333, Phe125 and Met59) and the thiol group of the putative second CoA, which has to evade ca. 1.5 Å when the first CoA is replaced by (S)-2-benzylo succinyl-CoA (Fig. 6A). In comparison to other thiolases, the special conformation of the covering loop allows providing enough space to accommodate either the bulky (S)-2-benzylo succinyl-CoA or succinyl-CoA together with an already bound benzoyl moiety at Cys85 (Fig. 6B).

**Classification of BbsAB within the thiolase superfamily**

Phylogenetic analysis shows that the BbsB orthologues, which are encoded in bbs-like operons and involved in anaerobic degradation of methyliaromatic compounds, form a monophyletic subbranch of the previously annotated SCP2-like thiolases within the thiolase superfamily (Fig. 7). Further members of SCP2-like thiolases are encoded in many bacteria, archaea or eukaryotes and form several additional subbranches (Fig. 7). These include the thiolase-like subunits of *M. thermolithotrophicus* HMGS and *P. protegens* ATase as well as some characterized eukaryotic thiolases from mouse, zebrafish and trypanosomatid species [17,30,32,33]. Note that only the vertebrate enzymes contain a C-terminally fused SCP domain [30,32], and only the eukaryotic SCP2-type thiolases have been reported to be active without an associated DUF35-type subunit. Members of the previously described thiolase subfamilies, namely type 1 and 2 thiolases, 3-ketoacyl-ACP or 3-ketoacyl-CoA synthases (KAS1-3; KCS), CHS, PKS and HMGS were included in the analysis and show a very similar branching pattern as previously reported [11,12].

**Discussion**

The biochemical and structural characterization of BbsAB represents the third solved enzyme of the SCP2-subfamily of thiolases with a Zn-finger protein as an additional small subunit, and the first one revealing the participation of this subunit in the catalytic mechanism. The two enzymes reported earlier, ATase and archaeal HMGS, are even more complex with three separate subunits, precluding conclusions on potential catalytic functions of the small subunits [19,20]. Other SCP2-thiolases with known structures are a few homodimeric eukaryotic enzymes, namely mitochondrial acetoacetyl-CoA thiolases of *Leishmania* and *Trypanosoma* species [17] and the original
SCP fusion enzymes from vertebrates, which have been characterized as peroxisomal thiolas involved in cholesterol degradation after their C-terminal sterol binding domains are removed by proteolysis [30,32]. The eukaryotic species do not contain recognizable genes for DUF35-like proteins and their SCP2-type thiolas are active without additional subunits [17,30]. In contrast, all bacterial and archaeal members of the SCP2 subfamily are encoded by directly adjacent gene pairs coding for a thiola and a DUF35-like subunit (in some cases even two genes each), suggesting that all of them may depend on both subunits for activity. Only one structure of a homodimeric archaeal SCP2 member is deposited (4XZO; WP_010978035; Fig. 7A), but it is unknown whether this enzyme is active in the absence of the correlated Zn-finger protein (WP_052846153).

**Special features of the SCP2 thiolase subunit**

BbsB and its close orthologues (named BbsB in Fig. 7) occur exclusively in anaerobic hydrocarbon degraders that also harbor the genes for a fumarate-adding enzyme and the other genes for succinyl-adduct degradation. Therefore, they are clearly the active-site subunits of 2-acylsuccinyl-CoA-cleaving thiolas. These proteins exhibit high amino acid identities in all regions related to catalysis as outlined above, but especially around positions 119–129 and 194–208, which are involved in tightly binding the first CoA cofactor. Although the location of the former motif is equivalent to that of general CoA-binding loops in other thiolas, the extensive conformation changes and final shapes of the two motifs in free and CoA-bound BbsB are unique and have not been observed in any other thiolas [12,34]. The classical homodi- or -tetrameric thiolas
normally use helical segments of varying orientation and totally different amino acid sequences as covering loops to bind CoA-thioesters into a pre-formed cavity [12,18], instead of the two highly mobile loop segments linked with BbsA. Their strong sequence conservation (>50% and >60% sequence identity within all BbsB orthologues) supports their expected roles in β-oxidation of bulky acylsuccinyl-CoA adducts (Fig. 7B). In case of the other SCP2 members, which are encoded next to bbsA-like genes, but not in entire bbs-like operons, the similarity of these motifs decreases with increasing distance from the BbsB orthologues: the enzymes from other bacterial groups still exhibit similar motifs (>50% similar, but <30% identical amino acids), and the enzymes from eukaryotes and archaea only show a few conserved residues in the 194–208 motif, but deviate completely in the 119 : 129 region. In contrast, the sequences of the four active site loop motifs show very high identity levels in all members of the SCP2-branch (Fig. 7C).

The function of BbsA

The observed dependence of benzoysuccinyl-CoA thiolase on the presence of BbsA and BbsB for catalytic activity can be rationalized on the basis of the structural analysis. Although the BbsA subunit does not directly participate in the actual catalytic process, it critically contributes to an induced-fit process for productive binding of a CoA substrate into the active site of BbsB (Fig. 5A). A firmly anchored CoA residue is a prerequisite for properly adjusting the conformation of the bulky benzoysuccinyl moiety of the substrate with the binding site of the polypeptide towards a productive pre-reaction geometry. BbsA essentially exerts its function by a rigid-body movement of its entire OB domain towards BbsB, which induces a reshaping and rigidification of the covering loop of BbsB into a quasi-linear conformation parallel to the CoA molecule, enabling a stable and enzymatically operative thiolase-substrate complex (Fig. 5A). Since BbsA is mandatory for enzyme activity, we assume this rearrangement process is too complex to be accomplished by BbsB on its own. The globular Zn binding segment of BbsA maintains the integrity of the heterotetrameric complex by interacting with the partner subunit BbsB' and thus anchors the more weakly attached OB domain during its rotation movements (Fig. 5A). Very similar interfaces between the BbsA- and BbsB-paralogues were reported for the structural analogues, HMGS and ATase, which both contain an additional HMGS-type subunit in heterohexameric complexes.

Fig. 7. Phylogeny and conserved sequence motifs. (A) Similarity tree representing all major branches of decarboxylating (KAS1-3, KCS, CHS) and non-decarboxylating members (HMGS, Thiolases, SCP2-like, BbsB) of the thiolase superfamily, including some thiolase domains of polyketide synthases (PKS) and ‘oxidized polyvinylalcohol hydratases’ (OPH). Structurally characterized enzymes are indicated by asterisks (PDB codes: SCOT, 4EGV, 6HSJ, 1PXT, 5AB7, 4YBV, 1HYF, 6HRD, 3WL4, 1KAS, 6DXA, 1XPM, 1U0M, 1U0V, 2H84, 2WYA, 3SQZ, 3VS9, 4M20, 4R5E, 4YLT, 5UC0, 5W80, 5AB4, 6DX7, 4RYB, 6DX8, 1ULQ, 5YO9, 6OKC, 3S3L, 1DD8, 4V2P, 3WLA, 5C1J, 2GP6, 5YD3, 6CON, 7CW4, 2IIK, 4C2K, 1WL4, 5LOT, 6PCC, 5CB0, 5F38, 2WUA, 4XL2, 6UBJ, 4YLT). Red asterisks indicate structures of complexes containing a DUF35-type subunit, and SCP2 subfamily members whose genes are located immediately next to genes for such subunits are highlighted by grey background shading. Bootstrapting confirmed the major branches with values of 100% or close to 100%; only the branches containing the archaeal and eukaryotic members of the SCP2-subfamily yielded values of less than 50%, casting some doubt on their branching pattern, but not on their subfamily affiliation (http://avermitilis.ls.kitasato-u.ac.jp/clustalo). Note that the overall branching pattern of the superfamily is highly similar to those shown in previous reports [11,12]. (B) Amino acid conservation of the covering loop (119–129) and the 194–208 segment involved in CoA-binding between BbsB orthologues. (C) Amino acid conservation of the active site loops 1–4 between all enzymes of the SCP2 subfamily.
A characteristic feature of the Bbs(AB)2 complex is that the catalytic reaction proceeds through a ternary complex, where the function of BbsA cannot be observed because the enzyme lost any thiolase functionality and evolves into a bifunctional thiolase and alcohol dehydrogenase [19,20]. No relative movements of the subunits were observed for these two protein complexes, but this may be explained by their higher complexity, the lower resolution of the respective structures and incomplete substrate binding. The same holds for the third known structural BbsAB analog, HOPC-CoA-aldolase [21], where the function of BbsA cannot be observed because the enzyme lost any thiolase functionality and evolved into a bifunctional thiolase and alcohol dehydrogenase [36].

The catalytic reaction

A characteristic feature of the Bbs(AB)2 complex is that each of the two active sites is reached by two elongated cavities. Both cavities are suitable for substrate binding; one CoA is confidently detected in the electron density, the identification of the second, which is more weakly bound and only partially resolved, is less obvious. The second cavity is partly built up from the covering loop of BbsB, such that binding of the second CoA to form one productive catalytic site of the Bbs(AB)2 complex prevents the formation of the second active site. Therefore, we suggest that the enzyme employs ‘half-of-the-sites’ activity cycles during the reaction, which may alternate between the two halves of the enzyme complex, thereby involving all subunits. This observation may explain the puzzling observation that BbsAB from T. aromatica did not follow the expected conventional ping-pong mechanism of normal thiolases in the two-substrate kinetic analysis of benzoylsuccinyl-CoA synthesis from succinyl-CoA and benzoyl-CoA, but rather showed a slight preference of a ternary complex mechanism. This may reflect the combination of binding of the second CoA in a ternary complex with the benzoyl moiety still forming a covalently bound intermediate with the enzyme. The BbsAB orthologues from T. aromatica and G. metallireducens are so similar that the same behaviour may be expected. More than one CoA-binding cavity has been previously postulated for the KAS3 subfamily member OleA [14] suggesting that this option may also be used by other thiolases.

Because BbsAB and all other SCP2-thiolases contain the typical four loops of CHH type thiolases around the active site (Fig. 7C) [14], they still appear to operate via the standard Claisen condensation mechanism via an enzyme-bound benzyol thioester intermediate at Cys85 (Fig. 6B). The mechanism is initiated by deprotonation of Cys85 (loop 1) by His331 (loop 3), which is compatible with the observed pH optimum of 6.4. The nucleophilic Cys85 thiolate then attacks the carbonyl carbon of (S)-benzoylsuccinyl-CoA and forms a tetrahedral oxyanion transition state intermediate. The negative charge of the benzoyl oxygen is neutralized by hydrogen bonds to the backbone NH group of Gly371 (Fig. 6A) in the oxyanion pocket I (loop 4). Subsequently the C–C bond is heterolytically cleaved, forming a carbanion of succinyl-CoA (in mesomeric equilibrium with the corresponding CoA enolate) in oxyanion pocket II. The conserved His331 (loop 3) of oxyanion pocket II probably stabilizes the negative charge of this intermediate (followed by protonation to the first product succinyl-CoA in the usual ping-pong mechanism). The mechanism then continues by deprotonation of the thiol group of an incoming CoA, which would conventionally be catalyzed by His281. However, in case of a ternary complex mechanism involving the second CoA-binding site, neither His281 nor His331 are close enough to deprotonate the thiol group (distances of 7.7 and 6.5 Å, respectively), therefore a direct proton transfer between the succinyl-CoA anion and the second CoA appears to be an attractive alternative (ca. 4.5 Å distance). In any case, the CoA thiolate then nucleophilically attacks the carbonyl carbon of the covalent enzyme-benzoyl thioester intermediate. The resulting short-living tetrahedral oxyanion intermediate is again stabilized by His331 in oxyanion pocket I (with potential support by Gly371, His281 and His378) and decomposes into the Cys85 thiolate and the second product benzyol-CoA.

The main mechanistic difference between ping-pong and ternary complex mechanisms relies on the timing between the release of the first product (succinyl-CoA) and binding of the second substrate (free CoA). Because of the two potential CoA-binding sites of BbsAB, we propose that its kinetic behavior may involve binding of the second CoA (or succinyl-CoA in synthetic direction) before the first product (from cleaving the first substrate) has left the enzyme. This would allow the combination of ternary-complex type
kinetics with a covalently bound thioester intermediate in the reaction mechanism. A similar case of enzymes exhibiting a ternary complex mechanism, while clear evidence of substituted enzyme intermediates is available, are the CoA transferases of family III, which include the benzylsuccinate:CoA transferase BbsEF (Fig. 1) [37,38].

Materials and methods

Cloning, overproduction and purification of BbsAB orthologues

The genes encoding the two subunits of benzoylsuccinyl-CoA thiolase, bbsA and bbsB, were amplified via PCR from chromosomal DNA of T. aromatica (primers for BspHI ggctcagagcggagaaaaaccgaacaag; rev_BamHI cggtgattccctgattccgtcctag) and cloned into the IPTG-inducible expression vector pTrc99A [39]. The plasmid was transformed into E. coli strain DH5α, and the bacteria were grown at 37 °C in LB medium containing ampicillin (100 µg·mL⁻¹). When the culture reached an OD₄₀₀ of 0.5, 1 mM IPTG was added to induce expression of the bbsAB genes and the culture was incubated at 37 °C for an additional 3–4 h. The cells were harvested by centrifugation [9] and 10 g of wet cell mass were suspended in 20 mL of buffer A (10 mM Tris/HCl pH 7.5 containing 0.05 mg·mL⁻¹ DNase I). Cell-free extract was prepared by passage through a French press cell and subsequent ultracentrifugation (100 000 g; 1 h). BbsA and BbsB were purified from the centrifugation supernatant by chromatography in two steps. First, 12 mL of the cell free extract were applied to a DEAE sepharose column (GE Healthcare, Freiburg, Germany; 30 mL volume) equilibrated with buffer B (10 mM Tris/HCl pH 8; 2 mM MgCl₂; 10% glycerol). The column was then washed with 6 column volumes (CV) buffer B containing 200 mM KCl, and a KCl gradient from 200 mM to 1 M was applied over 10 CV. During this step the BbsAB proteins were separated with BbsA eluting at 300 mM KCl and BbsB at 500 mM KCl. Subsequently, the fractions containing BbsA or BbsB, respectively, were purified further by applying them separately on a hydroxyapatite column (Macro-Prep Ceramic Hydroxyapatite Tye II, Biorad, Feldkirchen, Germany; 20 mL volume) equilibrated with buffer C (10 mM Tris/HCl pH 7.5; 10% glycerol) and applying a linear gradient of 0–400 mM potassium phosphate in buffer B over 10 CV. BbsA eluted at 80–120 mM K-phosphate, while BbsB eluted at 150–200 mM K-phosphate.

Because we did not obtain crystals with purified BbsA, BbsB or a mixture of both, we constructed additional expression plasmids for the bbsAB genes of T. aromatica and for those of the Fe³⁺-reducing toluene-degrading bacterium G. metallireducens (Fig. 1), which code for highly similar proteins (69% and 77% identity for BbsA and BbsB, respectively). The BbsAB genes were amplified via PCR from chromosomal DNA of T. aromatica (primers forward agctctcaagcggagaaaaaccgaacaag and reverse agctctcaagcggagaaaaaccgaacaag) and G. metallireducens (primers forward agctctcaagcggagaaaaaccgaacaag and reverse agctctcaagcggagaaaaaccgaacaag) and cloned via a modified Stargate cloning system (IBA Lifesciences, Goettingen, Germany): the PCR fragments were first cloned into plasmid pEntry-IBA according to the supplier’s manual, and subsequently into an anhydrotetracycline (AHT)-inducible broad host range vector based on pBRR-MCS1 [5,40]. The respective expression vector also provided a sequence coding for an in-frame strep-tag sequence fused to the 5′-end of bbsA. Neither of the constructed plasmids yielded high product yields in E. coli strain DH5α, but the genes were expressed much better after transforming the broad-host-range expression plasmids into a spontaneous streptomycin-resistant mutant of P. stutzeri via electroporation, yielding strep-tagged BbsAB from either species. The recombinant P. stutzeri clones were grown in LB media containing ampicillin (100 µg·mL⁻¹) and streptomycin (50 µg·mL⁻¹) at 25 °C, and expression of the bbsAB genes was induced by adding AHT (0.2 µg·mL⁻¹) when an OD400 of 0.5 was reached. The cultures were incubated further for 10 h, and the cells were harvested by centrifugation (6000 g; 15 min; 4 °C) and resuspended in 10 mM Tris/HCl pH 7.5 containing 0.05 mg·mL⁻¹ DNase I and 0.1 mg·mL⁻¹ lysozyme. Cell-free extracts were prepared by sonication on ice and ultracentrifugation (100 000 g; 1 h). Finally, the proteins were purified in two steps via strep-tag affinity chromatography as recommended by the supplier (IBA, Goettingen, Germany), and subsequent size exclusion chromatography (Superdex 200 PG, GE Healthcare; buffer 100 mM Tris/HCl pH 7.5 containing 100 mM KCl). Selenomethionine-substituted versions of BbsAB_Gm were produced as described in [41].

Synthesis of CoA thioesters

CoA thioesters of benzoate and succinate were prepared in two steps by chemical synthesis of the respective anhydrides and subsequent reaction with CoA as described previously [7,42]. The obtained thioesters were purified by HPLC on a C18 reversed-phase column (Sep-Pak Vac &cc; Waters, Eschborn, Germany) as described before [9]. Concentrations were determined photometrically at 260 nm, using a molar absorption coefficient of 16.4 mM⁻¹cm⁻¹.

Size determination and metal content

The native molecular masses of BbsA, BbsB and BbsAB were determined by size exclusion chromatography (Superdex 200 PG; GE Healthcare) after calibration of the column with standard proteins (Gel Filtration Calibration Kit HMW; GE Healthcare). The zinc content of purified proteins was determined colorimetrically by reaction with zinc (2-carboxy-2'-hydroxy-5-sulfoformazylbenzene) following the protocol in...
The zinc ions were extracted by adding HCl to adjust the pH to 2.5 and denature the protein. Precipitated protein was removed by centrifugation (18 000 g; 10 min), and the pH of the supernatant was readjusted to pH 8.5–9.0 by addition of NaOH before adding the zinc reagent. Zn contents were obtained by photometric determination of the respective complexes against a calibration curve. Additionally, the metal content of purified BbsAB was also by ICP-OES.

Enzymatic assay and HPLC analysis

The activity of benzoysuccinyl-CoA thiolase was assayed by following the condensation of benzoyl-CoA and succinyl-CoA to benzoysuccinyl-CoA, respectively its hydrazone with phenylhydrazine. The standard assays (0.5–1.0 mL total volume) contained 0.2–0.5 mg-mL⁻¹ of an equimolar mixture of BbsA and BbsB, 250–500 µM of benzoyl-CoA and succinyl-CoA and 750 µM phenylhydrazine in a buffer of 50 mM MES/NaOH (pH 6.2). Phenylhydrazine was omitted for assays intended for HPLC analysis of benzoysuccinyl-CoA formation, and lower concentrations of the substrates were applied for kinetic analysis (see below). The reaction was followed photometrically at 312 nm, which represents an absorption maximum of the substrates were applied for kinetic analysis (see below). The reaction was followed photometrically at 312 nm, which represents an absorption maximum of the hydrazone formed from phenylhydrazine and benzoysuccinyl-CoA. The relevant extinction coefficient at 312 nm (6.3 mM⁻¹ cm⁻¹) was calculated from the absorption properties at 260 nm are identical (21.1 mM⁻¹ cm⁻¹) since they mainly depend on the adenosyl moiety of CoA [43]. The reaction was started by the addition of the CoA thioesters. For product analysis via HPLC, samples were drawn at various time points (0–60 min) and acidified with 10% (v/v) NaHSO₄. Precipitated protein was removed by centrifugation (18 000 g; 10 min), and the supernatants were applied to an HPLC column (Chromolith® Performance RP18 end-capped, 100 × 4.6 mm, Merck, Darmstadt, Germany) equilibrated with 50 mM MES/NaOH pH 6.2 + 3% acetonitrile. CoA thioesters were separated by a linear gradient of 3–20% acetonitrile in 50 mM MES/NaOH pH 6.2 (30 min; 1 mL·min⁻¹) and detected at 260 nm (retention times: benzoysuccinyl-CoA 6.5 min; benzoysuccinyl-CoA hydrazone 22 min; benzoyl-CoA 17 min; succinyl-CoA 3 min).

Kinetic characterization

Enzyme kinetics of the reverse reaction was determined using the photometrical assay with succinyl-CoA and benzoyl-CoA as substrates. Specific activities were determined in triplicate assays using constant concentrations of benzoyl-CoA (50, 100 and 250 µM) with varying concentrations of succinyl-CoA (0–500 µM) and vice versa. The resulting data were analysed and fitted to two-substrate Michealis–Menten-type kinetic mechanisms (either ping-pong or ternary complex mechanisms) by non-linear regression using the program LEONORA [28,44] and internet resources (https://statpages.info/nfnonlin.html).

Crystallization and structure determination

For crystallization, purified BbsAB batches of G. metallireducens were subjected to ca. 2000 screening conditions. Needle-shaped crystals grew with the precipitant PEG3350, which diffracted non-reproductively between 30 and 2.5 Å in monoclinic, orthorhombic and tetragonal geometries (Table 2) and contained variable numbers of molecules in the asymmetric unit. Frequently, the crystals exhibited an inhomogeneous reflection profile and were merohedrally twinned. Data sets were collected at the SLS-PXII beamline in Villigen, Switzerland and processed with XDS [45] (Table 2). Phase determination failed with the method of molecular replacement, using the most related structurally solved thiolase Tha1 of Clostridium difficile (4DDS) or 3-ketoacyl-CoA thiolase of Saccharomyces cerevisiae (1AFW) for BbsB and the protein SSO2064 of S. solfataricus (3IRB) for BbsA. Therefore, selenomethionine (Se-Met) substituted BbsAB was produced by growing recombinant P. stutzeri cells in amino acid supplemented M9 minimal medium as described in [41], except that anhydrotetracycline (0.2 µg·mL⁻¹) was used for induction. Phasing also failed with the Se-Met-substituted enzyme, but after co-crystallizing it in the presence of 2.5 mM K₂OsO₄, a measurement at the Os (L-III) edge was successful, allowing SAD/MAD phasing (Table 2). After localization of the heavy atoms, phase determination and phase improvement with SHELXD [46], SHARP [47] and SOLOMON [48], three sets of four separate Os atoms were separated in the asymmetric unit. Threefold molecular averaging was performed with DM [49]. The model was partly built with Buccaneer [50], then completed manually with COOT [51]. The native structures of Bbs(AB)₂ and a Bbs(AB)₂–CoA complex were determined by molecular replacement using the obtained Bbs (AB)₂ model and refined with REFMAC [52], PHENIX [53] and BUSTER [54]. Refinement was performed using NCS and TLS parameters. Model superpositions were performed with DALI [31] or COOT and contact area calculations between subunits with PISA [55]. Figures 3, 5 and 6A were produced with PYMOL 2.1 (DeLano Scientific LLC, South San Francisco, CA, USA).

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Conflict of interest
The authors declare no conflict of interest.

Author contributions
SW solved and refined the crystal structure of BbsAB; KS, M-LL and JM provided the enzymes and performed biochemical experiments; AS determined elemental composition by ICP-OES, UD performed crystallization experiments, UE evaluated and interpreted the structural data and wrote the manuscript; JH designed the project, interpreted the data and wrote the manuscript.

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Data availability statement
The atomic coordinates and structure factors have been deposited in the protein data bank (ID codes 7PXP, 7PYT and 7YXM).

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