Succinyl-CoA:3-sulfinopropionate CoA-transferase
from Variovorax paradoxus strain TBEA6,
a novel member of the class III CoA-transferase family

Marc Schürmann, Beatrice Hirsch, Jan Hendrik Wübbeler, Nadine Stöveken,
and Alexander Steinbüchel

Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität
Münster, D-48149 Münster, Germany, and Environmental Sciences Department, King
Abdulaziz University, Jeddah, Saudi Arabia

Running title: Succinyl-CoA:3-sulfinopropionate CoA-transferase

*Corresponding author. Mailing address: Institut für Molekulare Mikrobiologie und
Biotechnologie, Westfälische Wilhelms-Universität, Corrensstrasse 3, D-48149 Münster,
Germany. Phone: +49-251-8339821. Fax: +49-251-8338388. E-mail: steinbu@uni-muenster.de.
Summary

The act gene of Variovorax paradoxus TBEA6 encodes a succinyl-CoA:3-sulfinopropionate CoA-transferase (2.8.3.x), which catalyzes the activation of 3-sulfinopropionate (3SP), an intermediate during 3,3'-thiodipropionate (TDP) degradation. In a previous study, accumulation of 3SP was observed in a Tn5::mob-induced mutant defective in growth on TDP. In contrast to the wild type and all other obtained mutants, this mutant showed no growth when 3SP was applied as a sole source of carbon and energy. The transposon Tn5::mob inserted in a gene showing high homology to class III CoA-transferases. In the current study, analyses of the translation product clearly allocated ActTBEA6 to this protein family. The predicted secondary structure indicates the lack of a C-terminal α-helix. ActTBEA6 was heterologously expressed in E. coli Lemo21 (DE3) and was then purified applying Ni-NTA affinity chromatography. Analytical size-exclusion chromatography revealed a homodimeric structure with a molecular mass of 96 kDa ± 3 kDa. Enzyme assays identified succinyl-CoA, itaconyl-CoA and glutaryl-CoA as potential CoA donors and unequivocally verified the conversion of 3SP to 3SP-CoA. Kinetic studies revealed an apparent $V_{\text{max}}$ of 44.6 µmol min$^{-1}$ mg$^{-1}$ for succinyl-CoA which corresponds to a turnover number of 36.0 s$^{-1}$ per subunit of ActTBEA6. For 3SP, the apparent $V_{\text{max}}$ was determined as 46.8 µmol min$^{-1}$ mg$^{-1}$ which corresponds to a turnover number of 37.7 s$^{-1}$ per subunit of ActTBEA6. The apparent $K_{\text{m}}$ values were 0.08 mM for succinyl-CoA and 5.9 mM for 3SP. Nonetheless, mutant V. paradoxus Δact did not reproduce the phenotype of the Tn5::mob induced mutant. This defined deletion mutant was able to utilize TDP or 3SP as sole carbon source like the wild type. Complementation of the Tn5::mob induced mutant with pBBR1MCS5::acdDPN7 partially restored growth on 3SP, which indicated a polar effect of the Tn5::mob transposon on acdTBEA6, located downstream of actTBEA6.
Keywords: CoA-transferase, succinyl-CoA, 3-sulfinopropionate, 3-sulfinopropionyl-CoA, 3,3’-thiodipropionate, *Variovorax paradoxus.*

Abbreviations: Act, acyl-CoA-transferase; Ap, ampicillin; CaiB, crotonobetainyl-CoA:carnitine CoA-transferase; DTNB, 5,5’-dithiobis(2-nitrobenzoic acid); Frc, formyl-CoA:oxalate CoA-transferase; GC/MS, gas chromatography / mass spectrometry; Gm, gentamycin; HPLC-ESI-MS, high performance liquid chromatography – electro spray ionisation mass spectrometry; IPTG, isopropyl β-D-1-thiogalactopyranoside; Km, kanamycin; LB, lysogeny broth medium; 3MP, 3-mercaptopropionate; MSM, mineral salt medium; ORF, open reading frame; PTE, polythioester; SEC, size-exclusion chromatography; 3SP, 3-sulfinopropionate; 3SP-CoA, 3-sulfinopropionyl-CoA; TDP, 3,3’-thiodipropionate acid; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; YfdW, formyl-CoA:oxalate CoA-transferase from *E. coli.*
3,3'-thiodipropionate (TDP) is a non-toxic thioether and is widely used as an antioxidant in technical applications (1-4). Furthermore, it is used as a precursor substrate for the microbial production of polythioester (PTE) (5). With TDP in the presence of gluconate or fructose under nitrogen limitation *Ralstonia eutropha* strain H16 accumulates heteropolymers consisting of 3-hydroxybutyrate (3HB) and 3-mercaptopropionate (3MP) (6). PTE-homopolymers are synthesized by applying the artificial BPEC pathway in the recombinant *E. coli* strain JM109 pBPP1 (7). Therefore, 3MP, 3-mercaptopbutyrate (3MB) or 3-mercaptovalerate (3MV) are applied as precursor substrates (7, 8). Only recently, the production of PTE-homopolymers in *Advenella mimigardens* DPN7T applying 3,3'-dithiodipropionate (DTDP) was achieved (9, 10). Unfortunately, PTE-homopolymer production applying TDP is yet not possible. The availability of complete information about enzymes which are involved in TDP-degradation would be beneficial to optimize PTE production.

*Variovorax paradoxus* is a Gram-negative, aerobic β-proteobacterium that belongs to the Comamonadaceae (11, 12). This microorganism could often be isolated from the rhizosphere of cereals (13-16), and growth on carbohydrates like glucose, mannose or galactose is frequently observed (12). Additionally, strains of *V. paradoxus* are able to utilize widespread, xenobiotic compounds, like 2,4-dichlorophenoxyacetic acid (17) or 2,4-dinitrotoluene (18). *V. paradoxus* strain TBEA6 was isolated due to its ability to degrade TDP and to use it as sole source of carbon and energy (19).

In a previous study, a putative degradation pathway for TDP was postulated based on Tn5::mob-mutagenesis with *V. paradoxus* strain TBEA6 and analysis of the obtained mutants (19) (Fig. 1). Accumulation of the supposed degradation intermediate 3-sulfinopropionate (3SP) was observed during cultivation of one of the resulting Tn5::mob-induced mutants.
(mutant 1/1) in MSM containing TDP. In contrast to the wild type, mutant 1/1 was unable to utilize 3SP as sole source of carbon and energy for growth (19). The insertion of Tn5::mob in this mutant was detected in a gene putatively coding for an acyl-CoA-transferase (Act \text{TEBA}_6). RT-PCR analyses of RNA from the wild type revealed constitutive transcription of this gene, irrespectively of whether TDP or succinate was present as sole source of carbon and energy (19). CoA-transferases catalyze the reversible transfer reaction of CoA from a donor to a free acid by formation of a CoA-thioester (20, 21). Therefore, it was expected that the translational product catalyzes the activation of 3SP to its corresponding CoA-ester.

CoA-transferases are classified by sequence similarities and reaction mechanisms into three families (21). (i) In the first family, both substrates (CoA-donor and CoA-acceptor) are not bound to the enzyme simultaneously, but two consecutive enzyme-substrate complexes are formed. Hence, this mechanism is also known as ping-pong mechanism (21, 22). The formation of a covalent CoA-thioester intermediate with an active site glutamate residue is characteristic for members of this family.

(ii) CoA-transferases of the second family are part of a citrate lyase (EC 2.8.3.10) or citramalate lyase (EC 2.8.3.11) complex which consists of three subunits (23). The CoA-transferase catalyzes the exchange of free citrate or citramalate against the acetyl-thioester group of an acyl carrier protein (ACP). During this reaction both substrates (citrate/citramalate and the acetyl-thioester) are not covalently attached to the transferase, and a ternary complex is built (21, 23-25).

(iii) Members of family III differ significantly in sequence and reaction mechanism. They are often involved in unusual biochemical pathways in anaerobic bacteria and activate organic acids for further reactions such as decarboxylation, \(\beta\)-oxidation or elimination of \(\alpha\)/\(\beta\)-hydroxyl groups (21). Their primary structures showed only few conserved amino acids, which makes it difficult to predict the structural conservation within this family (26). Nonetheless, crystal structures of several representatives have been elucidated (20, 26-30).
They indicate that family III CoA-transferases appear as intertwined dimers in which each monomer forms a ring with a hole in the center through which the other monomer is threaded (29). The mechanism proceeds via the formation of anhydrides between a highly conserved aspartate residue (Asp169 with respect to CaiB from *E. coli*) in the active site and the substrates. Crystal structure analysis and kinetic experiments indicate that the reaction is completed prior to the release of any product (20). Consequently, two different mechanisms were discovered, which close the active site during catalysis. In the first mechanism, a glycine-rich loop takes different conformations as described for formyl CoA-transferase (20, 26-28, 31). For CaiB from *E. coli*, a representative of the second mechanism, the binding of the CoA triggers a domain shift that leads to the closure of the active site (30).

This study reports on the enzymatic activation of 3SP, an organosulfur compound, to the corresponding CoA-thioester and the biochemical characterization of ActTBEA6 as a novel member of class III CoA-transferases.

**MATERIALS AND METHODS**

**Bacterial strains and cultivation conditions.** All strains used in this study are listed in Table 1. Cells of *V. paradoxus* were cultivated at 30 °C on solid MSM (32) containing 20 mM gluconate or 20 mM TDP or 20 mM 3SP as sole source of carbon and energy to test carbon source utilization. Cells of *E. coli* were cultivated in lysogeny broth (LB) medium at 37 °C under the same conditions (33). Carbon sources were supplied as filter-sterilized stock solutions as indicated in the text. For maintenance of plasmids, antibiotics were prepared according to the method of Sambrook et al. (33) and added to the media at the following concentrations (µg/ml): ampicillin (75) and kanamycin (50), gentamycin (20), tetracycline (12.5). In *E. coli* heterologous expression of genes under the control of a lac-promotor was...
achieved by cultivation in ZYP-5052-medium, an auto-inductive medium according to Studier et al. (34), or by induction with 0.4 mM IPTG in LB medium.

**Chemicals.** TDP of high purity grade was purchased from Sigma Aldrich (Steinheim, Germany). 3-Sulfinopropionate was synthesized according to Jollès-Bergeret (35); the procedure was modified by one repetition of the step for alkaline cleavage of the intermediate bis-(2-carboxyethyl)sulfone (36). Synthesis and purity of the substance was confirmed by GC/MS as described elsewhere (37) and was at least 95.0 %. Acetic anhydride, propionic anhydride, butyric anhydride, valeric anhydride, isobutyric anhydride, isovaleric anhydride, maleic anhydride, crotonic anhydride, succinic anhydride, itaconic anhydride and glutaric anhydride were purchased from Sigma Aldrich (Steinheim, Germany). Thiodiglycolic anhydride for synthesis of 3-thiaglutaryl-CoA was purchased from Alfa Aesar (Karlsruhe, Germany). Mercaptosuccinic acid was purchased from Acros Organics (Geel, Belgium).

Acetyl-CoA was purchased from Sigma-Aldrich (Steinheim, Germany) or synthesized according to the method of Simon and Shemin (38). For synthesis of propionyl-CoA, butyryl-CoA, valeryl-CoA, isobutyryl-CoA, isovaleryl-CoA, crotonyl-CoA, maleyl-CoA, succinyl-CoA, itaconyl-CoA, glutaryl-CoA or 3-thiaglutaryl-CoA according to the method of Simon and Shemin (38) 10 mg of the trilithium salt of coenzyme A (Merck KGaA, Darmstadt, Germany) were dissolved in 0.5 M K₂CO₃ or Tris-HCl (pH 8.0). The solution was stirred on ice, and small portions of the respective anhydride were added to this solution until no free coenzyme A was detectable by the Ellman’s spot test (39). The pH value of the solution was then adjusted to 4.5 by addition of concentrated phosphoric acid or 9 M hydrochloric acid. The resulting acyl-CoA thioesters were immediately applied for enzyme assays or stored at -20 °C.

**Isolation and manipulation of DNA.** Chromosomal DNA of *V. paradoxus* strain TBEA6 was isolated according to the method of Marmur (40). Plasmid DNA was isolated from *E. coli* using the peqGOLD plasmid miniprep kit I from PEQLAB Biotechnologie.
GmbH (Erlangen, Germany) according to the manufacturer’s manual. DNA was digested with restriction endonucleases under conditions described by the manufacturer. PCR were carried out in an Omnigene HBTR3CM DNA thermal cycler (Hybaid, Heidelberg, Germany) using Platinum\textsuperscript{©} Taq DNA polymerase (Invitrogen, Carlsbad, USA). PCR products were isolated from an agarose gel and purified using the NucleoTrap kit (Macherey and Nagel, Düren, Germany) according to the manufacturer’s instructions. T4-DNA-Ligase was purchased from Invitrogen (Carlsbad, USA). Primers were synthesized by MWG-Biotech AG (Ebersberg, Germany) and are listed in Supplemental Table S1.

**Transfer of DNA.** Competent cells of *E. coli* strains were prepared and transformed by the CaCl\textsubscript{2} procedure (33).

**DNA sequencing and sequence data analysis.** DNA sequencing was performed by Seqlab (Göttingen, Germany) or by the Institut für Klinische Chemie und Laboratoriumsmedizin at the Universitätsklinikum Münster (Germany). The latter sequenced the samples according to the method of Sanger et al. (41) by applying the BigDye\textsuperscript{®} Terminator v3.1 cycle sequencing kit according to the manufacturer’s manual (Applied Biosystems, Darmstadt, Germany). Samples were submitted to the Institut für Klinische Chemie und Laboratoriumsmedizin for purification of the extension products and sequencing in an ABI Prism 3700 DNA Analyser (Applied Biosystems, Darmstadt, Germany). Sequences were analyzed using the program BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/) (42). The program BioEdit (43) was used for multiple sequence alignments. Secondary structure predictions were performed using the Jpred3 server (44) with Jnet version 2.2 and UniRef90 release 15.4. Prediction of molecular weight and extinction coefficient of heterologously expressed Act\textsubscript{TBEA6} were performed using Expasy Protparam (45).

**Elucidation of the upstream and downstream region of the act-acd-bug cluster.** A PCR-based two-step genome walking method (46) was used to sequence the upstream and...
downstream region adjacent to the known *act-acd-bug* cluster. Walking and sequencing
primers were constructed as described by Pilhofer et al. (46) and are listed in Supplemental
Table S1. Genomic DNA of the wild type was isolated according to Marmur (40). Starting
from the known sequence of *act*$_{TBEA6}$ (19), the upstream region was amplified with three
walking steps (walking primers 1 to 3). The amplification products were sequenced with
primers ActSeq1, ActSeq2 and ActSeq6 in the forward (upstream) direction. For validation of
the obtained sequence the sequencing primers ActSeq3rev, ActSeq4rev and ActSeq5rev with
a reverse orientation were used. As reported previously (19), the sequence of *bug*, an
extracytoplasmatic solute receptor (*Bordetella* uptake gene) downstream of *act*$_{TBEA6}$, was
incomplete. Hence, another walking step starting from the known sequence of *bug* applied the
primers ActWalk5 and ActSeq7 and revealed the missing sequence information of *bug*.

**Cloning of Act$_{TBEA6}$.** Act$_{TBEA6}$ was amplified from total genomic DNA of
*V. paradoxus* strain TBEA6 by PCR using Platinum® Taq DNA polymerase (Invitrogen,
Karlsruhe, Germany) and the following oligonucleotides: *act*$_{Hind}$III$_{For}$ and *act*$_{Xho}$I$_{Rev}$_oS (Table 1). PCR products were isolated from agarose gels using the
peqGOLD GelExtraction Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and
ligated with pCR®2.1-TOPO® DNA (Invitrogen, Carlsbad, USA). Ligation products were
used for transformation of CaCl$_2$ competent cells of *E. coli* OneShot Mach1™-T1R, and
transformants were selected on LB agar plates containing IPTG, X-Gal plus ampicillin. For
heterologous expression in the T7 promotorm/polymerase based expression vector pET22b(+)
(Novagen, Madison, USA), *act*$_{TBEA6}$ was obtained by digestion of hybrid plasmid pCR®2.1-
TOPO®::act$_{TBEA6}$ with restriction endonucleases *Hind*III and *Xho*I and purified from an
agarose gel using thepeqGOLD GelExtraction Kit (PEQLAB Biotechnologie GmbH,
Erlangen, Germany). After ligation with the expression vector pET22b(+), which was
linearized with the same restriction endonucleases, the ligation product, pET22b(+)::act$_{TBEA6}$
(Supplemental Fig. S1), was used for transformation of CaCl$_2$-competent cells of *E. coli*
Top10. After selection of transformants using LB-media containing ampicillin, the hybrid plasmids were isolated, analyzed by sequencing and used for transformation of CaCl₂ competent cells of *E. coli* Lemo21 (DE3) (New England Biolabs® Inc., Ipswich, USA).

**Construction of an act precise deletion gene replacement plasmid.** The 526- and 691-bp fragments upstream and downstream of act₁BEA₆ were amplified by using the primers XbaI_upAct/NdeI_upAct or NdeI_downAct/XbaI_downAct, respectively. The oligonucleotides used for PCR are listed in Supplemental Table S1. The resulting fragments were NdeI digested and ligated to yield a 1223-bp fragment. This fragment was amplified using XbaI_upAct/XbaI_downAct, and the resulting PCR product was cloned into the XbaI site of pJQ200mp18Tc (47-49) to yield pJQ200mp18Tc::∆act₁BEA₆.

**Construction of an act gene deletion strain using the sacB system.** Standard protocols were adapted to accomplish gene replacement in strain *V. paradoxus* (47-49). Plasmid pJQ200mp18Tc::∆act₁BEA₆ was used to generate the mutant *V. paradoxus* Δact₁BEA₆. The plasmid was mobilized from *E. coli* donor strain S17-1 to the *V. paradoxus* strain TBEA₆ recipient strain by the spot agar mating technique (50). Positive transconjugants were screened on MSM containing 50 mM gluconate plus tetracycline. After cultivation in liquid nutrient broth for 20 hours, samples were transferred to solid NB medium containing saccharose (10 % wt/vol). Growing strains had lost the suicide plasmid. A successfully generated gene replacement strain was identified and confirmed by PCR analyses and DNA sequencing using oligonucleotides listed in Supplemental Table S1. Oligonucleotides up_act_proof and down_act_proof served to verify that act₁BEA₆ was deleted in the act-acd-bug cluster. Oligonucleotides act_int_fwd and act_int_rev were used to verify that act₁BEA₆ was not incorporated at a different position in the genome.

**Construction of *V. paradoxus* TBEA₆ 1/1 pBBR1MCS-5::acd₂PN7.** The complementation vector pBBR1MCS-5::acd₂PN7 was constructed and described in a previous study (51, 52). In this study, the vector was first transferred to CaCl₂ competent cells of *E.
coli S17-1. Vector harboring clones were screened on LB agar plates containing gentamycin. The vector was then transferred to *V. paradoxus* TBEA6 1/1 by conjugation (48).

**Preparation of crude extracts.** Cells from 50-100 ml cultures were harvested by centrifugation (15-45 min, 4 °C and 3,400 × g), washed twice with sterile saline and resuspended in appropriate buffers. For purification of histidine-tagged fusion proteins, the buffers were prepared as recommended by the manufacturer of the His Spin Trap affinity columns (GE Healthcare, Uppsala, Sweden). Cells were resuspended in 50 mM sodium phosphate binding buffer or 50 mM Tris-HCl buffer, both pH 7.4, containing 500 mM sodium chloride and 20 mM imidazole and afterwards disrupted by a threefold passage through a French press (100 × 10^6 Pa). Soluble protein fractions of crude extracts were obtained in the supernatants after 1 h centrifugation at 100,000 × g and 4 °C and were used for enzyme purifications. Protein concentrations were determined as described by Bradford (59) or applying a NanoDrop 2000 spectrophotometer (Fisher Scientific, Schwerte, Germany) and the calculated extinction coefficient of (51.590 mM⁻¹ cm⁻¹) at 280 nm.

**Immobilized metal-chelate affinity chromatography (IMAC).** To obtain purified histidine-tagged fusion proteins, His Spin Trap affinity columns (GE Healthcare, Uppsala, Sweden) were used according to the manufacturer’s instructions. Ni-NTA columns were equilibrated with 20 mM sodium phosphate buffer, pH 7.4, containing 20 mM imidazole and 500 mM sodium chloride. The same buffer containing 40 mM imidazole was used for the washing step while the elution buffer contained 500 mM imidazole.

**Analytical size-exclusion chromatography.** The molecular weight of ActTBEA6 was determined by analytical size-exclusion chromatography (SEC) using a Superdex 200 HR column. The column was equilibrated with 50 mM sodium phosphate buffer, pH 7.5, containing 150 mM sodium chloride. Calibration was performed applying chymotrypsinogen A (25 kDa), ovalbumin (44 kDa), conalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa) and blue dextran 2000 (GE Healthcare, Uppsala, Sweden).
according to the manufacturer’s instructions. For each determination 300 μg of purified heterologous Act\textsubscript{TBEA6} were applied to the column. The column was operated at a flow rate of 0.750 ml/min.

**Enzyme assays.**

(i) Initial identification of an appropriate CoA donor for Act\textsubscript{TBEA6}. The heterologously expressed Act\textsubscript{TBEA6} was assayed by incubating 20 μg/ml purified enzyme in 50 mM sodium phosphate buffer (pH 7.4) for 1 h at 30 °C in presence of 5 mM 3SP and 5 mM of acetyl-CoA, propionyl-CoA, butyryl-CoA, crotonyl-CoA, or succinyl-CoA, respectively. The reaction was stopped by addition of 50 μl trifluoroacetic acid (TFA, 10 % wt/vol). The samples were analyzed by HPLC-ESI-MS for the formation of 3SP-CoA. Samples with heat inactivated protein (15 min at 95 °C) and soluble protein fractions from cells harboring only the expression vector without \textit{act}\textsubscript{TBEA6} (vector control) served as a control or one of the substrates was omitted at a time.

(ii) Determination of kinetic parameters. 3SP-CoA formation by Act\textsubscript{TBEA6} was measured by applying 3SP-CoA desulfinase Acd\textsubscript{DPN7} as a coupling enzyme in an aerobic continuous spectrophotometric assay (51). Kinetic parameters were determined in cuvettes with a total volume of 1 ml containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.2 mM 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) and purified Acd\textsubscript{DPN7} as an auxiliary enzyme. Different amounts of Acd\textsubscript{DPN7} were tested to ensure that the auxiliary enzyme was not rate limiting. After preincubation for 2.5 min at 30 °C the reaction was started by addition of purified recombinant Act\textsubscript{TBEA6} (0.5 μg). The increase in absorption was measured at 412 nm, \( \varepsilon = 14.150 \text{ mM}^{-1} \text{ cm}^{-1} \) and corrected for the observed increase in absorbance based on the non-enzymatic decomposition of succinyl-CoA at pH 7.6. Activity was measured for ten different concentrations of succinyl-CoA ranging from 0.0 mM to 1.0 mM (with a constant concentration of 0.2 mM succinyl-CoA). All measurements were done in
triplicate at 30 °C. Apparent \( V_{\text{max}} \) and \( K_m \) were determined by fitting the obtained data to the Michaelis-Menten equation.

(iii) Utilization of other CoA-donors than succinyl-CoA. The assay mixture contained 0.2 mM DTNB, 10 mM 3SP and an excess of AcdDPN7 in Tris-HCl 50 mM, pH 7.6, 150 mM NaCl, in a final volume of 1 ml. After preincubation for 1.5 min at 30 °C one of the following CoA esters was added to a final concentration of 0.13 mM: Acetyl-CoA, propionyl-CoA, butyryl-CoA, valeryl-CoA, isobutyryl-CoA, isovaleryl-CoA, crotonyl-CoA, maleyl-CoA, succinyl-CoA, itaconyl-CoA, glutaryl-CoA and 3-thiaglutaryl-CoA. After incubation for another minute, the reaction was started by addition of 42 µg of purified recombinant ActTBEA6. The increase in absorbance was followed at 412 nm.

(iv) Utilization of other CoA acceptors than 3SP. The assay mixture with a final volume of 1 ml in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl contained 0.1 mM succinyl-CoA, 10 µg purified heterologous ActTBEA6 and 5 mM of the following putative CoA-acceptors: sodium acetate, sodium propionate, itaconic acid, sodium fumarate, mercaptosuccinic acid or sodium glutarate. Stock solutions of the corresponding substrates were adjusted to a pH range of 7.0 to 8.0 in advance. After 15 min incubation at 30°C the reaction was stopped by addition of 30 µl trichloroacetic acid (15 % wt/vol). Samples were analyzed for formation of the corresponding CoA esters by HPLC-ESI-MS.

Inactivation experiments. Hydroxylamine and sodium borohydride were applied in two inactivation experiments.

(i) Inactivation by hydroxylamine. 210 µg purified recombinant ActTBEA6 were incubated for 10 min at 30 °C in 490 µl Tris-HCl 50 mM, pH 7.6, with 150 mM NaCl, either containing or lacking succinyl-CoA (2 mM). Subsequently, 5 µl 1 M hydroxylamine solution (in H2O, pH 7.0 adjusted with 5 M NaOH) were added to a final concentration of 10 mM, and the reaction mixture was incubated for additional 10 min at 30 °C. Afterwards, the reaction mixture was diluted 1:10 with Tris-HCl 50 mM, pH 7.6, 150 mM NaCl, and stored on ice.
until enzyme activity was determined with the coupled spectrophotometric assay. Activity was measured in triplicate for the enzyme solutions incubated with or without succinyl-CoA.

(ii) Inactivation by sodium borohydride. 210 µg purified recombinant ActTBEA6 (from the same batch mentioned above) were incubated for 10 min at 30 °C in 490 µl Tris-HCl 500 mM, pH 7.6, either containing or lacking succinyl-CoA (2 mM). Subsequently, 5 µl 1 M sodium borohydride in 1 M NaOH were added, followed by addition of 5 µl 1 M HCl immediately afterwards. The reaction mixture was incubated for additional 10 min at 30 °C. Afterwards, the reaction mixture was diluted 1:10 with Tris-HCl 50 mM, pH 7.6, 150 mM NaCl, and stored on ice until enzyme activity was determined with the coupled spectrophotometric assay. Activity was measured in triplicate for the enzyme solutions incubated with or without succinyl-CoA.

Analysis of CoA-ester formation by HPLC-ESI-MS. Formation of 3SP-CoA during enzyme assays was followed by High Pressure Liquid Chromatography (HPLC) in combination with an electron-spray ionization mass spectrometry (HPLC-ESI-MS) based on a method described earlier (46). Analyses were carried out using an UltiMate® 3000 HPLC apparatus (Dionex GmbH, Idstein, Germany) connected directly to an LXQ™ Finnigan™ (ThermoScientific, Dreieich, Germany) mass spectrometer. An Acclaim 120 C18 Reversed-Phase LC Column (4.6 x 250 mm, 5 µm, 120 Å pores; Dionex GmbH, Idstein, Germany) served to separate the CoA-esters at 30 °C. A gradient system was used, with 50 mM ammonium acetate, pH 5.0 adjusted with acetic acid (A), and 100 % (vol/vol) methanol (B) as eluents. Elution occurred at a flow rate of 0.3 ml/min. Ramping was performed as follows: Equilibration with 90 % A for 2 min before injection and afterwards a change from 90 % to 45 % eluent A in 20 min, followed by holding for 2 min and then returning to 90 % eluent A within 5 min. Detection of CoA-esters occurred at 259 nm by a photodiode array detector. The instrument was tuned by direct infusion of a solution of 0.4 mM CoA at a flow rate of 10 µl/min into the ion source of the mass spectrometer to optimize the ESI-MS system for
maximum generation of protonated molecular ions (parents) of CoA derivatives. The following tuning parameters were retained for optimum detection of CoA-esters: capillary temperature 300 °C; sheat gas flow, 12 l/h; auxiliary gas flow, 6 l/h; sweep gas flow, 1 l/h. The mass range was set to m/z 50 - 1000 Da when run in the scan mode. The collision energy in the MS-MS mode was set to 30 V and delivered fragmentation patterns which are in good accordance with those found in other publications (53).

Data deposition. The DNA sequence and the deduced amino acid sequence of the gene cluster harboring actTBEA6 were deposited in the GenBank database under accession number EU449952.3. The accession number for actTBEA6 is ACC69030.2. DNA sequences for Act from V. paradoxus strain B4 and A. mimigardefordensis strain DPN7T are available under JN675924.1 (actB4) and JN675925.1 (actDPN7), respectively.

RESULTS

Gene organization. In this study, the sequence of these regions was revealed applying the genome-walking method as described in the Materials and Methods section (Fig. 2). The new sequence information was deposited as an update of EU49952 in the Genbank database.

The gene organization in proximity to actTBEA6 resembles the gene organization found in A. mimigardefordensis DPN7T and Burkholderia xenovorans LB400 which harbor act genes encoding for 76 % and 57 % identical amino acids, respectively, in comparison to ActTBEA6. In all strains, lysR, most likely coding for a LysR-type transcriptional regulator, is located upstream of act (Fig. 2), and a gene showing homology to acyl-CoA dehydrogenases (acd) is found in the downstream region. Only recently it was shown that acdDPN7 encodes a 3SP-CoA desulfinase (51). AcdTBEA6 shows high homology to AcdDPN7 (79 % identical and 88 % similar amino acid residues) and to AcdLB400 (64 % / 76 %).
Genes with high similarity to actTBEA6 were searched within the available genome sequences of *V. paradoxus* strains EPS, S110 and B4 to investigate if this gene cluster is generally present in strains of *V. paradoxus*. Homologs (*V. paradoxus* EPS, YP_004152464.1; *V. paradoxus* S110, YP_002942048.1; *V. paradoxus* B4, JN675924.1) showed 49% identical amino acids in comparison to actTBEA6. Although a gene coding for a putative acyl-CoA dehydrogenase was found in the upstream region, comparison of its sequence to Acd<sub>DPN7</sub> indicates that amino acid residues putatively characteristic for 3SP-CoA desulfinases (R84, C122 and Q246 according to Acd<sub>DPN7</sub> numbering, data not shown) (51) are absent. Hence, these acd genes are most probably not coding for 3SP-CoA desulfinases.

**Utilization of TDP or 3SP by different strains of *V. paradoxus***. *V. paradoxus* strains TBEA6, EPS, S110 and B4 were cultivated on MSM agar plates containing 20 mM gluconate or 20 mM TDP or 20 mM 3SP, respectively. While all strains showed growth on gluconate, only *V. paradoxus* strain TBEA6 was able to utilize TDP or 3SP as sole source of carbon and energy.

**Precise deletion mutant *V. paradoxus* Δact and complementation of the transposon-induced disruption of act in mutant *V. paradoxus* 1/1**. The precise deletion mutant *V. paradoxus* Δact was constructed to verify the observed phenotype and to exclude polar effects of the transposon insertion. Surprisingly, *V. paradoxus* Δact showed normal growth when cultivated on solid MSM plates containing 20 mM TDP or 20 mM 3SP. After complementation with pBBR1MCS-5::acd<sub>DPN7</sub>, harboring the 3SP-CoA desulfinase gene from *A. mimigardefordensis* strain DPN7<sup>T</sup> (51), growth of mutant *V. paradoxus* 1/1 was restored on MSM agar plates containing 20 mM 3SP, but not on MSM agar plates containing 20 mM TDP. In liquid MSM containing 50 mM 3SP both, *V. paradoxus* wild type and Δact, showed similar growth behavior (Fig. 3). *V. paradoxus* TBEA6 1/1 showed no growth while slow but significant growth was observed for the complemented strain *V. paradoxus* TBEA6 1/1 pBBR1MCS-5::acd<sub>DPN7</sub> under the same conditions. These results indicated a
polar effect of the transposon on \( acd_{TBEA6} \), located downstream of \( act_{TBEA6} \). This 3SP-CoA desulfinase catalyzes the hydrolysis of 3SP-CoA, the potential reaction product of \( Act_{TBEA6} \).

**Sequence analyses of \( Act_{TBEA6} \).** Sequence analyses showed that the N-terminal part (residues 81-270) of \( Act_{TBEA6} \) affiliates the enzyme to Pfam02515 (CoA-transferase family III, Supplemental Fig. S2). It contains a highly conserved residue (Asp180 in \( V. \ paradoxus \) strain TBEA6, Asp169 with respect to CaiB, indicated by an asterisk in Supplemental Fig. S2) (30) which is located in the active site and binds the organic acid substrate via an anhydride bond (30, 31). Other residues (Arg16, Gly37, Ala38, Val40, Asp90, Leu184, His185, Gly193 and Thr190 referring to CaiB numbering; indicated by ▼ in Supplemental Fig. S2) (30) are considered to be important for folding, and they are conserved throughout CoA-transferase family III (30). Most of them are found in the same position in \( Act_{TBEA6} \) as well. Two minor exceptions are the substitution of a residue corresponding to Arg16\textsuperscript{CaiB} by lysine (Lys13\textsuperscript{TBEA6}) in \( V. \ paradoxus \) strain TBEA6 and an additional glutamine residue (Gln196\textsuperscript{TBEA6}) between Leu195\textsuperscript{TBEA6} (corresponding to Leu184\textsuperscript{CaiB}) and His197\textsuperscript{TBEA6} (corresponding to His185\textsuperscript{CaiB}).

**Secondary structure analyses.** Amino acid sequences of \( Act_{TBEA6} \) and its orthologues were subjected to secondary structure prediction applying the Jpred server (44) (Supplemental Fig. S2). Due to their available solved crystal structures, formyl-CoA:oxalate CoA-transferase from \( E. \ coli \) (YfdW) (27, 28), its orthologue Frc from \( Oxalobacter \ formigenes \) (20, 26) and crotonobetainyl-CoA:carnitine CoA-transferase from \( E. \ coli \) (CaiB) (29, 30) as members of the CoA-transferase III family were included for comparison. As shown in Supplemental Fig. S2, amino acid sequences of \( Act_{TBEA6} \), \( Act_{DPN7} \) and \( Act_{LB400} \) (YP_553419.1) are truncated by about 13 to 15 amino acid residues in comparison to all other included sequences.

**Cloning of the putative acyl-CoA-transferase gene \( act_{TBEA6} \) into the vector pET22b(+), overexpression in \( E. \ coli \) Lemo21 (DE3), purification and characterization of the translational product.** Based on nucleotide sequence data (GenBank accession...
number ACC69030.2) native Act\textsubscript{TBEA6} has a calculated molecular weight of 43.322 kDa (isotopically average), consists of 398 amino acids and has a calculated pI of 5.46. In this study, the putative act gene of \textit{V. paradoxus} strain TBEA6 was heterologously expressed as a His\textsubscript{6}-tagged protein using the T7-promotor/polymerase-based expression vector pET22b(+) and \textit{E. coli} Lemo21 (DE3) as host strain. For this, the protein was equipped with an additional C-terminal His\textsubscript{6}-tag plus two vector encoded amino acids (leucin and glutamate) and an N-terminal pelB signal sequence (22 amino acids plus 17 amino acids between pelB and start of act) for potential periplasmatic localization (see Materials and Methods section; Supplemental Fig. S1). Consequently, the heterologously expressed protein consisted of 445 amino acids, and it exhibited a theoretical molecular weight of 48.372 Da (isotopically average) and a theoretical pI of 5.65. The overproduced enzyme was purified by immobilized metal chelate affinity chromatography to electrophoretic homogeneity (Fig. 4). Afterwards, Act\textsubscript{TBEA6} was applied to analytical size-exclusion chromatography. It revealed an apparent molecular weight of 96 kDa ± 3 kDa. This corresponds to a homodimer of the protein with a theoretical molecular mass of 96.7 kDa including the His\textsubscript{6}-tag and the additional 39 amino acid residues of the N-terminal pelB signal sequence. The UV-visible spectrum (\(\lambda = 200\) nm – 800 nm) of purified Act\textsubscript{TBEA6} showed a single peak at 280 nm which indicates the absence of any chromophoric cofactor.

\textbf{Act enzyme activity assays applying the heterologously expressed and purified protein.}

(i) \textbf{Initial identification of an appropriate CoA-donor for Act\textsubscript{TBEA6}.} In an early test, acetyl-CoA, propionyl-CoA, butyryl-CoA, crotonyl-CoA and succinyl-CoA were applied as potential CoA-donors of Act\textsubscript{TBEA6} as described in the Materials and Methods section. Formation of 3SP-CoA (m/z = 888) was only observed when succinyl-CoA was applied in the assay mixture but not for any of the other CoA esters (data not shown). No 3SP-CoA was detected in negative controls containing heat inactivated enzyme (15 min at 95 °C), applying
soluble protein fractions from cells harboring only the expression vector without actTBEA6 (vector control) or by omitting one of the substrates at a time.

(ii) Determination of kinetic parameters. Only recently, we reported the characterization of Acd_{DPN7}, a 3SP-CoA desulfinase from A. mimigardefordensis strain DPN7\(^7\) (51). The equimolar release of sulfite from 3SP-CoA by Acd_{DPN7} was quantified in a continuous spectrophotometric assay with DTNB, Ellman’s reagent, and served to determine the kinetic parameters of Acd_{DPN7}. In this study, we applied Acd_{DPN7} as an auxiliary enzyme in a coupled enzyme assay and indirectly followed the formation of 3SP-CoA by ActTBEA6 which resulted in an increase in absorption at 412 nm (\(\varepsilon = 14.150 \text{ mM}^{-1} \text{ cm}^{-1}\)). The apparent \(V_{\text{max}}\) for succinyl-CoA was 44.6 \(\mu\text{mol min}^{-1} \text{ mg}^{-1}\) which corresponds to a turnover number of 36.0 \(\text{s}^{-1}\) per subunit of ActTBEA6. The apparent \(V_{\text{max}}\) for 3SP was 46.8 \(\mu\text{mol min}^{-1} \text{ mg}^{-1}\) which corresponds to a turnover number of 37.7 \(\text{s}^{-1}\) per subunit of ActTBEA6. The \(K_m\) values were 0.08 mM for succinyl-CoA and 5.9 mM for 3SP (Table 2).

(iii) Utilization of other CoA-donors than succinyl-CoA. ActTBEA6 utilized only CoA esters of dicarboxylic acid as CoA donors in the following order: succinyl-CoA >> glutaryl-CoA > itaconyl-CoA > 3-thiaglutaryl-CoA (Fig. 5 A and Fig. 6). Interestingly, maleyl-CoA did not serve as a CoA-donor. Furthermore, ActTBEA6 was not active with CoA-esters of monocarboxylic acids like acetyl-CoA, propionyl-CoA, butyryl-CoA, valeryl-CoA, isobutyryl-CoA, isovaleryl-CoA or crotonyl-CoA (Fig. 5 B).

(iv) Equilibrium between succinyl-CoA or glutaryl-CoA and 3SP-CoA. HPLC-ESI-MS analyses indicate that at equilibrium more 3SP-CoA is formed than succinyl-CoA (Fig. 7). In contrast to this, the reaction equilibrium favors the side of the educts when glutaryl-CoA was used as a CoA-donor.

(v) Utilization of other CoA-acceptors than 3SP. ActTBEA6 can catalyze the CoA transfer from succinyl-CoA to itaconate and glutarate as shown by HPLC-ESI-MS analyses
Acetate, propionate, fumarate or mercaptosuccinate were no appropriate CoA acceptors (data not shown).

**Inactivation experiments with hydroxylamine and sodium borohydride.** Members of the CoA transferase family I are inactivated by low concentrations of hydroxylamine or sodium borohydride in presence of an appropriate CoA donor due to their ping-pong mechanism. Enzymes that belong to CoA transferase family II are subunits within a lyase complex. Members of this family catalyze the reaction via a ternary complex which renders them insensitive to hydroxylamine or sodium borohydride (21). Results for inhibition experiments with members of CoA tranferase family III applying hydroxylamine and sodium borohydride are ambiguous (20, 54-58). Hence, both compounds were tested for a potential inactivating effect on ActTBEA6 (see Materials and Methods section). When purified ActTBEA6 was preincubated for 10 min in presence of 2 mM succinyl-CoA and 10 mM hydroxylamine, 75% of the activity was retained. After preincubation with 1 mM NaBH₄ in presence of 2 mM succinyl-CoA nearly 75% of the activity was retained and the activity was reduced to 9% when ActTBEA6 was preincubated in presence of 2 mM succinyl-CoA and 10 mM NaBH₄.

**DISCUSSION**

Bruland et al. (19) observed the accumulation of 3SP as a proposed TDP degradation product during cultivation of a Tn₅::mob-induced *V. paradoxus* TBEA6 mutant in MSM containing TDP. In contrast to the wild type, this mutant was unable to utilize 3SP as sole source of carbon and energy for growth. The insertion of Tn₅::mob in this mutant was mapped in a gene putatively coding for an acyl-CoA-transferase (ActTBEA6) (19). The aim of the present study was to characterize the role of ActTBEA6 during TDP degradation.

**Identification of a gene cluster potentially essential for degradation of 3SP.** The gene region upstream of actTBEA6 was unknown. As revealed in the current study, the same...
gene organization in proximity to act is found in V. paradoxus TBEA6 and in
A. mimigardefordensis DPN7\textsuperscript{T} (Fig. 2). This gene cluster is absent in V. paradoxus strains
S110, EPS and B4 from which the whole genome sequences are available (59-61) (U. Brandt
et al., publication in preparation). Furthermore, the latter three strains were unable to utilize
TDP or 3SP as sole sources of carbon and energy. A. mimigardefordensis strain DPN7\textsuperscript{T} is a β-
proteobacterium that can utilize 3,3′-dithiodipropionate (DTDP), a structural analogue of
TDP, and is able to grow on 3SP (9). The catabolic pathway of DTDP has been completely
eclucidated (37, 51, 62) (Fig. 1). Both strains possess a lysR-act-acd gene cluster with high
similarity regarding the amino acid sequence of the translation products (lysR, 74 % identical
amino acid residues, 88 % similar amino acid residues; act, 76 % / 84 %; acd, 79 % / 88 %)
(Fig. 2). Only recently, the last step of DTDP degradation in A. mimigardefordensis strain
DPN7\textsuperscript{T} has been affiliated to Acd\textsubscript{DPN7} from the aforementioned gene cluster (51).
Interestingly, Acd\textsubscript{TBEA6} shows high homology to Acd\textsubscript{DPN7} from A. mimigardefordensis strain
DPN7\textsuperscript{T} (79% identical and 88 % similar amino acid residues). Hence, it was likely that the
degradation of TDP and DTDP occurs, at least in part, via a similar pathway. It might be
interesting to investigate, if B. xenovorans LB400 can also utilize 3SP as sole source of
carbon and energy.

**Activation of 3SP to 3SP-CoA prior to the final desulfination step.** Activation of
3SP to 3SP-CoA is necessary prior to sulfur abstraction by Acd as shown in a previous study
(51). In the study of Bruland et al. (19), the gene act\textsubscript{TBEA6} was found in close proximity to
acd\textsubscript{TBEA6} and annotated as an acyl-CoA transferase. Hence, we assumed that Act\textsubscript{TBEA6} might
catalyze the activation of 3SP to 3SP-CoA in V. paradoxus strain TBEA6 and we investigated
the biochemical characteristics of the purified enzyme.

**Biochemical characterization and physiological role of Act\textsubscript{TBEA6}.** First attempts to
express act\textsubscript{TBEA6} in E. coli using hybrid plasmids of pET23a(+) and pET19b (Novagen,
Madison, USA) resulted in the formation of insoluble protein. Finally, act\textsubscript{TBEA6} was
heterologously expressed in *E. coli* strain Lemo21 (DE3) harboring pET22b(+):actTBEA6 (Fig. 4), and the protein was purified to electrophoretic homogeneity. It was not investigated in detail whether the pelB leader sequence enabled (partial) secretion into the periplasm or if it helped to enhance the solubility of the heterologously expressed ActTBEA6. However, the apparent molecular weight of 96 kDa ± 3 kDa for ActTBEA6, as revealed by size-exclusion chromatography, corresponds to a homodimer of the protein. Up to now, all solved protein structures indicate that family III CoA-transferases appear as intertwined dimers (29). Therein, each monomer forms a ring with a hole in the center through which the other monomer is threaded (29). Without crystal structure information it is not clear, if this applies to ActTBEA6 as well.

It was an initial task to identify appropriate CoA donors and to verify the formation of 3SP-CoA by ActTBEA6. After identification of succinyl-CoA as an active CoA-donor and verification of 3SP-CoA formation using HPLC-ESI-MS (Fig. 7), kinetic parameters were determined for ActTBEA6 in a continuous spectrophotometric enzyme assay with AcddPN7 as an auxiliary enzyme (Table 2). Although both enzymes belong to different enzyme classes, ActTBEA6 was compared with SucCDPN7 which catalyzes the activation of 3SP in *A. mimigardefordensis* DPN7T (Table 2). SucCDPN7 is a Mg^{2+}-dependent succinate:CoA ligase that can activate dicarboxylic acids to the corresponding CoA-thioesters under consumption of ATP (or GTP) (37). In contrast to this, ActTBEA6 as a representative of the acyl-CoA transferases, conserves the energy of the thioester bond of a CoA donor during transfer of the CoA moiety to another carboxylic acid. In terms of *k*<sub>cat</sub>, ActTBEA6 showed an about 370-fold higher catalytic activity in comparison SucCDPN7 with regard to 3SP. In contrast to this, ActTBEA6 shows less affinity towards 3SP than SucCDPN7 as indicated by the about 7-fold higher *K*<sub>m</sub> value for the sulfur-containing substrate. Nonetheless, the catalytic efficiency of ActTBEA6 towards 3SP is higher as indicated by *k*<sub>cat</sub>*K*<sub>m</sub>. Thus, it might depend on the physiological concentration of 3SP or the other substrates in the cells at a given point of
time whether Act\textsubscript{TBEA6} or SucCD\textsubscript{DPN7} is better suited for the activation of 3SP. If SucCD can compensate for the disruption (mutant 1/1) or the deletion (mutant \(\Delta\text{act}\)) of Act is discussed further below.

Additional tests showed that Act\textsubscript{TBEA6} is not completely specific for just one CoA donor. Instead, Act\textsubscript{TBEA6} accepts succinyl-CoA, itaconyl-CoA, glutaryl-CoA and 3-thiaglutaryl-CoA, respectively (Fig. 5 A, Fig. 6). In contrast to this, CoA-thioesters of monocarboxylic acids such as acetyl-CoA or propionyl-CoA are not accepted as CoA-donors (Fig. 5 B). This indicated that a second, terminal carboxy group in the acyl-moiety is mandatory. The same seems to apply for CoA-acceptor molecules as Act\textsubscript{TBEA6} could activate itaconate and glutarate, respectively, but not acetate or propionate. Interestingly, Act\textsubscript{TBEA6} was unable to utilize maleyl-CoA as a CoA-donor, and fumarate as a potential CoA-acceptor was not activated to the corresponding CoA-thioester. Hence, both a \textit{cis}- or a \textit{trans}-double bond appear to prevent catalysis. The impaired rotation of the carboxy group probably results in sterical hindrance or improper binding of the carboxy group in the catalytical center. With regard to side groups in CoA-acceptor molecules, the methylene group in itaconate appears to be less impeding than the sulphydryl group in mercaptosuccinate. This might be due to the fact that thiols are rather acidic and thus are negatively charged which might interfere with a proper reaction.

Concerning a potential physiological function, Act\textsubscript{TBEA6} showed highest activity with succinyl-CoA (Fig. 6) which is thus expected to be the physiological CoA-donor. The ability to activate glutarate to glutaryl-CoA might indicate that Act\textsubscript{TBEA6} can act as an succinyl-CoA:glutarate CoA-transferase. The enzyme assay that was utilized was based on the formation of 3SP-CoA which was then cleaved to sulfite and propionyl-CoA by Acd\textsubscript{DPN7} as an auxiliary enzyme. Hence, the exchange of 3SP and determination of \(K_m\) values for other potential CoA acceptors was not possible. Consequently, we could not identify the physiological CoA-acceptor of Act\textsubscript{TBEA6}. The ability of Act\textsubscript{TBEA6} to activate 3SP to 3SP-CoA...
is most likely due to the structural similarities of succinyl-CoA and 3SP-CoA or succinate and 3SP, respectively. In the latter, a carboxyl group is exchanged by a sulfino group, which is essentially an exchange of a carbon atom by a sulfur atom. Thus, all four of them are recognized by ActTBEA6. RT-PCR analyses in the previous study (19) revealed the constitutive transcription of the gene in the wild type, irrespectively of whether V. paradoxus strain TBEA6 was grown in presence of TDP or succinate. Nonetheless, the inactivation of ActTBEA6 in mutant 1/1 did not affect growth on other carbon sources (19). This indicates that ActTBEA6 is not essential for growth, or that other enzymes can compensate for inactivated ActTBEA6. Thus, the physiological role of ActTBEA6 in absence of TDP or 3SP remains to be elucidated.

Multiple sequence alignments and comparison with orthologues of ActTBEA6

A BLAST search affiliated the N-terminal part (residues 80-270) of the actTBEA6 translation product to Pfam02515 (CoA-transferase family III). Additionally, the presence of amino acid residues considered to be involved in folding and therefore expected to be highly conserved throughout CoA-transferase family III, allocated ActTBEA6 to this class of CoA transferases (Supplemental Fig. S1).

The first characterized member of family III is a formyl-CoA:oxalate CoA-transferase (Frc) from O. formigenes, which catalyzes the transfer of a CoA moiety between formyl-CoA and oxalate (20, 21, 26, 63, 64). Other enzymes, such as a crotonobetainyl-CoA:L-carnitine CoA-transferase (CaiB) from E. coli (29, 30) or succinyl-CoA:(R)-benzylsuccinate CoA-transferase from Thauera aromatica (56), have been discovered and have been assigned to family III as well. An acyl-CoA:carboxylate CoA-transferase from Aspergillus nidulans was characterized as the first eukaryotic member of this enzyme family (65).

Nonetheless, it has been suggested to best describe the structure of its members in terms of α-helices and β-sheets due to the low number of conserved amino acid residues in CoA-transferase family III (26). Frc and CaiB show an N-terminal βαβ-motif, which
resembles a Rossmann fold and is involved in CoA binding (26). This motif can be found in
ActTBEA6 and all other compared sequences (Supplemental Fig. S2).

Hitherto, all investigated CoA-transferases displayed a C-terminal motif of two
consecutive α-helices (26-30). The prediction of secondary structures for ActTBEA6 and
comparison with several orthologues revealed a truncated amino acid sequence resulting in
the absence of one of the C-terminal α-helices (Supplemental Fig. S2). This absence is also
observed in closely related Acts, e.g. from A. mimigardens strain DPN7T and B.
xenovorans strain LB400. If this truncation has any effect on catalysis or substrate spectrum
remains to be investigated.

Formation of a ternary complex during catalysis has been proposed for members of the
CoA-transferase family III (56). Only recently, the formation of an acid anhydride between an
aspartate residue and CoA-activated acid has been verified (20). Consequently, this anhydride
intermediate should react with sodium borohydrde and hydroxylamine which inactivates the
CoA-transferase permanently. Nonetheless, ambiguous results were obtained regarding
sensitivity towards these inhibitors (20, 54-58). ActTBEA6 was only partially inactivated by
hydroxylamine and sodium borohydrde. However, sodium borohydrde had a stronger effect
(9 % remaining activity) than hydroxylamine (75 % remaining activity). Two different
mechanisms, which close the active site during catalysis, were discovered in members of the
family III CoA-transferases. It has been proposed earlier that closure of the active site
prevents entering of inhibitor molecules (66). A glycine-rich loop, found in formyl-CoA
transferases, is capping the active site if the ligand is bound (20, 26, 28). The glycine-rich
loop is not conserved among family III CoA-transferases but a second mechanism was
identified in CaiB from E. coli. Therein, an induced domain movement could be observed
upon binding of CoA, which results in closure of the active site and thereby in a protection of
the intermediate (30). In Supplemental Fig. S3 the glycine-rich loop is highlighted for formyl-
CoA:oxalate CoA-transferase from E. coli K-12 strain MG1655 (AAC75433.1) and from
O. formigenes (AAC45298.1). Act\textsubscript{TBEA6} and all other aligned sequences show no such motif and about 20 to 30 amino acid residues are missing upstream of the glycine-rich loop (Supplemental Fig. S3). Since such a glycine-rich loop is missing, the second mechanism appears to be more likely for Act\textsubscript{TBEA6}. The ability to properly close the active site might be responsible for the diverse sensitivity towards NaBH\textsubscript{4} and hydroxylamine of different members of the CoA-transferase family III.

**Compensation of Act activity in V. paradoxus TBEA6 Δact.** After biochemical characterization of Act\textsubscript{TBEA6}, deletion of act\textsubscript{TBEA6} in the defined deletion mutant V. paradoxus Δact did not verify the phenotype of the transposon mutant V. paradoxus 1/1 from the previous study. Interestingly, growth of V. paradoxus mutant 1/1 with 3SP was partially restored by complementation with pBBR1MCS-5::\textit{acd}\textsubscript{DPN7} (Fig. 3). This indicated a polar effect of the Tn5::\textit{mob} transposon insertion on \textit{acd}\textsubscript{TBEA6}. The translation product of \textit{acd}\textsubscript{TBEA6}, located downstream of act\textsubscript{TBEA6}, shows homology to a 3SP-CoA desulfinase in A. mimigardefordensis strain DPN7\textsuperscript{T}, which we identified and characterized only recently (51). This enzyme is responsible for the final step during degradation of DTDP. The desulfinase catalyzes the hydrolysis of 3SP-CoA to sulfite and propionyl-CoA which enters the central metabolism via the methylcitric acid cycle (51). In this study, pBBR1MCS-5::\textit{acd}\textsubscript{DPN7} was applied for complementation of an A. mimigardefordensis Δ\textit{acd} mutant. Similarly to the present study, growth could be partially restored with 3SP, but not with the precursor DTDP. It was proposed that this is due to low transcription of Acd\textsubscript{DPN7} and concomitant accumulation of toxic 3MP after cleavage of DTDP which inhibits growth of the cells (51). 3SP was shown to be nontoxic to cells of A. mimigardefordensis DPN7\textsuperscript{T} when supplied as sole carbon source in liquid MSM in concentrations of up to 100 mM (C. Meinert, personal communication). Hence, cells of A. mimigardefordensis DPN7\textsuperscript{T} were expected to have enough time to form a sufficient amount of Acd\textsubscript{DPN7} for growth in presence of 3SP (51). Further explanations for the lack to fully restore growth in comparison to the wild type might
be that a heterologous gene was used or that the ribosomal binding site was not properly recognized.

Moreover, we could confirm desulfination of 3SP-CoA by Acd_{TBEA6} in enzyme assays applying heterologously expressed and purified enzyme (M. Schürmann et al., unpublished results, publication in preparation). Hence, a polar effect of the transposon on Acd_{TBEA6} would impair the final step during TDP degradation (Fig. 1 and Fig. 2).

However, act_{TBEA6} was disrupted or precisely deleted, respectively, in V. paradoxus mutant 1/1 and V. paradoxus Δact. Consequently, the necessary activation of 3SP to the corresponding CoA-thioester prior to sulfur abstraction by Acd_{TBEA6} has to be compensated by other enzymes. In A. mimigardefordensis a succinate-CoA ligase (SucCD) from the citric acid cycle catalyzes this reaction (37) (Fig. 1). Furthermore, only recently SucCDs from E. coli BL21 (accession number for a-subunit YP_002998521.1 and β-subunit YP_002998520.1) and Alcanivorax borkumensis (a-subunit YP_693212.1 and β-subunit YP_693213.1) were investigated with regard to their substrate range in our laboratory (Johannes Nolte et al., manuscript in preparation). Both enzymes accepted 3SP as a substrate with activities comparable to SucCD_{DPN7} reported earlier (67). Hence, we expect this to be a common feature of SucCDs due to the high structural similarity between 3SP and succinate, a physiological substrate of SucCDs in the citric acid cycle. Other strains of V. paradoxus like EPS (59), GenBank accession number of complete genome CP002417.1) and S110 (61), Genbank accession numbers CP001635.1 and CP001636.1) possess two SucCD homologues. Therefore, it is likely that also V. paradoxus strain TBEA6 possesses two SucCD homologues, and we expect them to catalyze the activation of 3SP to 3SP-CoA. Unfortunately, the whole genome sequence of V. paradoxus TBEA6 is unknown, and therefore predictions about structure-substrate specificity relations as well as precise deletion of both SucCDs are not possible at the moment.
Conclusions

In summary, the activation of 3SP to the corresponding CoA-thioester by ActTBEA6 was clearly shown in this study. Therefore, the systematic name of this novel member of the CoA-transferase family III is “succinyl-CoA:3-sulfinopropionate CoA-transferase”. Succinyl-CoA and glutaryl-CoA were identified as potential physiological CoA donors for ActTBEA6. Further studies, which will unravel why deletion of actTBEA6 can be compensated in V. paradoxus TBEA6, are currently in progress. Furthermore, it might be interesting to investigate if the lysR-act-acd gene cluster can transfer the capability of 3SP degradation to other bacteria and how the cluster is regulated during 3SP degradation in more detail.

ACKNOWLEDGMENTS

The LC/MS device used in this study was provided by funds of the DFG (Deutsche Forschungsgemeinschaft, Grant No.: INST 211/415-1 FUGG) which we gratefully acknowledge. Furthermore, we thank Jong-In Han and Paul Orwin for kindly providing V. paradoxus strain S110 and V. paradoxus strain EPS, respectively. Moreover, we sincerely thank Christina Doberstein for assistance in literature research.
REFERENCES


betaproteobacterium capable of utilizing the organic disulfide 3,3'-dithiodipropionic acid. 


37. Schürmann M, Wübbeler JH, Grote J, Steinbüchel A. 2011. Novel reaction of succinyl coenzyme A (Succinyl-CoA) synthetase: activation of 3-sulfinopropionate to 3-


TABLE 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain or plasmid or oligonucleotide</th>
<th>Description or sequence (F-3’)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. paradoxus strain TBEA6</td>
<td>Wild type, TDP and 3SP utilizing</td>
<td>(19)</td>
</tr>
<tr>
<td>V. paradoxus strain TBEA6 mutant 1/1</td>
<td>Tn5-induced mutant, retarded growth on TDP, 3SP-negative, KmR</td>
<td>(19)</td>
</tr>
<tr>
<td>V. paradoxus strain TBEA6 mutant 1/1 pBBR1MCS-5</td>
<td>TDP-negative, partially restored growth on 3SP</td>
<td>This study</td>
</tr>
<tr>
<td>V. paradoxus strain EPS</td>
<td>Wild type, whole genome sequence available, TDP and 3SP-negative</td>
<td>(59)</td>
</tr>
<tr>
<td>V. paradoxus strain B4</td>
<td>Wild type, first genome sequenced from the genus of Fariorovum, TDP and 3SP-negative</td>
<td>(61)</td>
</tr>
<tr>
<td>V. paradoxus TBEA6 ∆act</td>
<td>Precise deletion mutant of V. paradoxus TBEA6, lacking act</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli One Shot® Mach1TM-T1R</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, Φ80 lacZ ΔM15, ΔlacX74, ΔdeoR, ΔaraD139, λDE3, Δ[nicE1, λEmE1, λEmE2, Cam&lt;sup&gt;R&lt;/sup&gt;], i DE3</td>
<td>Invitrogen, Carlsbad, USA</td>
</tr>
<tr>
<td>E. coli Lemo21 (DE3)</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, rpsL, nupG, Δ[mrr-](hsdRMS- mcrBC), Δ(lacI-λDE3), Δ(ara-leu7697), gal&lt;sup&gt;U&lt;/sup&gt;, gal&lt;sup&gt;K&lt;/sup&gt;, endA1, fhuA2, [lon•], ompT, gal, (λDE3), λsBamHIo, ∆EcoRI-B, int::(lacI::PlacUV5::T7 gene1), i21, Δnin5, pLemo = pACYC184-PrhaBAD-lysY</td>
<td>New England Biolabs&lt;sup&gt;®&lt;/sup&gt; Inc.</td>
</tr>
<tr>
<td>pCR®2.1-TOPO®</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;, lacZ&lt;sup&gt;α&lt;/sup&gt;, pCR®2.1-TOPO®</td>
<td>Invitrogen, Carlsbad, USA</td>
</tr>
<tr>
<td>pCR®2.1-TOPO®::actTBEA6</td>
<td>Km&lt;sup&gt;R&lt;/sup&gt;, Ap&lt;sup&gt;R&lt;/sup&gt;, lacZ&lt;sup&gt;α&lt;/sup&gt;, actTBEA6</td>
<td>This study</td>
</tr>
<tr>
<td>pET22b(+):::actTBEA6</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;, N-terminal pelB-leader sequence for potential excretion to cytoplasm, C-terminal His&lt;sub&gt;6&lt;/sub&gt;-tag</td>
<td>Novagen, Madison, USA</td>
</tr>
<tr>
<td>pQX003mp18Ec</td>
<td>sacB, oriV, oriT, masD, T&lt;sup&gt;E&lt;/sup&gt;&lt;sup&gt;R&lt;/sup&gt;, sacB, oriV, oriT, masD, T&lt;sup&gt;E&lt;/sup&gt;&lt;sup&gt;R&lt;/sup&gt;, 526-bp upstream and 691-bp downstream flank of act&lt;sup&gt;TBEA6&lt;/sup&gt; ligated with NdeI (1223 bp)</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR1MCS-5</td>
<td>Broad-host-range cloning vector, Gm&lt;sup&gt;R&lt;/sup&gt;, lacZ&lt;sup&gt;α&lt;/sup&gt;</td>
<td>(52)</td>
</tr>
<tr>
<td>pBBR1MCS-5::actTBEA6</td>
<td>Broad-host-range cloning vector containing the act gene of Advenella mimigardefordensis DPN&lt;sup&gt;T&lt;/sup&gt; and a 14 bp upstream region as a 1220 bp ApaI-PstI fragment, Gm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(51)</td>
</tr>
</tbody>
</table>

<sup>a</sup> for abbreviations used in genotypes of E. coli, see reference (67). Ap<sup>R</sup>, ampicillin resistance; Km<sup>R</sup>, kanamycin resistance. Tc<sup>R</sup>, tetracycline resistance. Gm<sup>R</sup>, gentamycin resistance.
<table>
<thead>
<tr>
<th>Subunit composition</th>
<th>Molecular mass (subunit) kDa</th>
<th>Subunit</th>
<th>Substrate</th>
<th>( V_{\text{max}} ) (( \mu \text{mol min}^{-1} \text{mg}^{-1} ))</th>
<th>( K_m ) (mM)</th>
<th>( k_{\text{cat}} ) (s(^{-1} ))</th>
<th>( k_{\text{cat}}/K_m ) (s(^{-1} \text{mM}^{-1} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActTBEA6</td>
<td>48.4</td>
<td>( \alpha_2 )</td>
<td>Succinyl-CoA</td>
<td>44.6</td>
<td>0.08</td>
<td>36.0</td>
<td>448.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3SP</td>
<td>46.8</td>
<td>5.9</td>
<td>37.7</td>
<td>6.4</td>
</tr>
<tr>
<td>SucCD\text{DPN7} ( a )</td>
<td>72.2( b )</td>
<td>( (\alpha\beta)^2 )</td>
<td>3SP</td>
<td>0.12</td>
<td>0.818</td>
<td>0.1( c )</td>
<td>0.18( c )</td>
</tr>
</tbody>
</table>

\( a \) \( V_{\text{max}} \) and \( K_m \) for succinyl-CoA synthetase (SucCD) from *A. mimigardefordensis* DPN7 have been reported previously (37).

\( b \) Calculation is based on available amino acid sequences of SucCD\text{DPN7} subunits (ACB59226.1 and ACB59227.1).

\( c \) Kinetic parameter has been calculated based on values available from the literature.
Legends to figures

FIG. 1. Putative degradation pathways of 3,3'-thiodipropionate (TDP) in *V. paradoxus* strain TBEA6 and of 3,3'-dithiodipropionate (DTDP) in *A. mimigardefordensis* strain DPN7\(^T\). Bruland et al. (19) postulated that in *V. paradoxus* strain TBEA6 the organosulfur compound TDP is initially cleaved to 3-hydroxypropionate (3HP) and 3-mercaptopropionate (3MP), putatively by an FAD-dependent oxidoreductase (step I a). In *A. mimigardefordensis* strain DPN7\(^T\) a dihydrolipoamide dehydrogenase (LpdA) catalyzes the initial cleavage of DTDP (step I b) yielding two molecules of 3MP (62). In both bacteria, 3MP is further oxygenated to 3-sulfinopropionate (3SP) by a 3MP-dioxygenase (step II) (19). The acyl-CoA-transferase (Act\(_{TBEA6}\)), investigated in this study, can catalyze the transformation of 3SP to the corresponding CoA-thioester, 3SP-CoA (step III a). In *A. mimigardefordensis* DPN7\(^T\), 3SP is activated by SucCD\(_{DPN7}\), a succinate-CoA ligase, to 3SP-CoA (step III b) (37). Subsequent abstraction of the sulfur moiety is catalyzed by a desulfinase, Acd, yielding sulfite and propionyl-CoA (step IV) (51). The latter enters the central metabolism via the methylcitric acid cycle.

FIG. 2. Gene organization in proximity of act orthologues in *V. paradoxus* strain TBEA6 and other bacteria. lysR, transcription factor; act, acyl-CoA-transferase; acd, acyl-CoA dehydrogenase; ech, enoyl-CoA hydratase/isomerase; mdo, 3-mercaptopropionate dioxygenase; ahpd, alkylhydroperoxidase; bug, *Bordetella* uptake gene.

FIG. 3. Growth on 3-sulfinopropionate (3SP). Cells of the wild type *V. paradoxus* TBEA6, the *V. paradoxus* TBEA6 Δact mutant, the transposon-induced mutant *V. paradoxus* TBEA6 1/1, and the *V. paradoxus* mutant 1/1 harboring pBBR1MCS-5::acd\(_{DPN7}\) were precultivated in liquid MSM containing 50 mM sodium gluconate, supplied with gentamycin if necessary. Prior to inoculation of the main culture, cells were harvested and washed twice with sterile...
saline. Cultivation was done in liquid MSM containing 50 mM 3SP in Klett flasks with baffles at 30 °C and with agitation at 120 rpm. ■, *V. paradoxus* TBEA6 wild type; ▲, *V. paradoxus* TBEA6 Δact mutant; ♦, *V. paradoxus* TBEA6 mutant 1/1; ●, *V. paradoxus* mutant 1/1 harboring pBBR1MCS-5::*acd*<sub>DPN7</sub>. Bars indicate standard deviation (n = 3).

FIG. 4. Purification of Act<sub>TBEA6</sub> by affinity chromatography as revealed by SDS-PAGE. Lane 1, crude extract of cells; lane M, molecular weight marker; lane 2, soluble fraction after centrifugation; lane 3, elution fraction after Ni-NTA affinity chromatography column; lane 4, pooled fractions recovered after Superdex 200 HR size-exclusion chromatography. 40 µg protein were applied in lane 1 and 2. Lane 3 and 4 were loaded with 5 µg protein. The SDS-gel was stained with Coomassie® Brilliant Blue R.

FIG. 5. Structures of acyl-CoA-thioesters used in this study. A) CoA-thioesters that were identified as CoA-donors of Act<sub>TBEA6</sub>. B) CoA-thioesters that were not accepted as CoA-donors by Act<sub>TBEA6</sub>.

FIG. 6. Identification of putative CoA-donors of Act<sub>TBEA6</sub>. The assay mixture contained 0.2 mM DTNB, 10 mM 3SP and an excess of Acd<sub>DPN7</sub> in Tris-HCl 50 mM, pH 7.6, 150 mM NaCl, in a final volume of 1 ml. CoA esters were added to a final concentration of 0.13 mM. Addition of assay components are indicated: 1) 50 µl 3SP solution, 2) 50 µl solution containing Acd<sub>DPN7</sub> as an auxiliary enzyme, 3) 10 µl of the respective CoA-thioester, 4) 10 µl containing 42 µg of purified Act<sub>TBEA6</sub>. Rise in absorption at time points of addition is due to opening of the spectrophotometer.
FIG. 7. Formation of 3SP-CoA by Act\textsubscript{TBEA} as revealed by HPLC-ESI-MS analyses. 

**A)** CoA-transfer from succinyl-CoA to 3SP. 
1) Assay solution containing 0.1 mM succinyl-CoA and 5 mM in 50 mM Tris-HCl, pH 7.4. 
2) Subsequently, 25 µg of purified Act\textsubscript{TBEA} were added and incubated for 10 min at 30 °C. 
3) ESI-MS in the positive mode revealed formation of 3SP-CoA (888 m/z) and presence of remaining succinyl-CoA (868 m/z).

**B)** CoA-transfer from glutaryl-CoA to 3SP. 
1) Assay solution containing 0.1 mM glutaryl-CoA and 5 mM in 50 mM Tris-HCl, pH 7.4. 
2) Subsequently, of 25 µg of purified Act\textsubscript{TBEA} were added and incubated for 10 min at 30 °C. 
3) ESI-MS in the positive mode revealed formation of 3SP-CoA (888 m/z) and presence of remaining glutaryl-CoA (882 m/z). CoA-thioesters were detected at 259 nm.

**C)** Mass spectra of the respective CoA-thioesters. 
1) Succinyl-CoA, RT: 18.2 in A1, NL: 5.65E3; 
2) 3SP-CoA, RT: 16.3 min in A2, NL: 5.67E3; and 
3) glutaryl-CoA, RT: 20.1 min in B2, NL: 1.08E4. RT, retention time; NL, normalization level.
FIG. 2

Burkholderia xenovorans LB400 [YP_593419.1]
A. minigordii f. sp. DIPHT [JN870925.1]
V. paradoxus TBVEA6 [ACC69030.2]

lysR  act  acf  ecB

lysR  act  acf  mdp  ahgD

lysR  act  acf  lbg

1 kbp
Fig. 5

A)

\[ \text{Succinyl-CoA} \]
\[ \text{Itaconyl-CoA} \]
\[ \text{Glutaryl-CoA} \]
\[ \text{3-Thiaglutaryl-CoA} \]

B)

\[ \text{Acetyl-CoA} \]
\[ \text{Propionyl-CoA} \]
\[ \text{Butyryl-CoA} \]
\[ \text{Valeryl-CoA} \]
\[ \text{Isobutyryl-CoA} \]
\[ \text{Isovaleryl-CoA} \]
\[ \text{Crotonyl-CoA} \]
\[ \text{Maleyl-CoA} \]
FIG. 6

[Graph showing various time points and peaks labeled with different chemicals: Succinyl-CoA, Glutaryl-CoA, Iaeryl-CoA, 3-Thiaplialyl-CoA]
V. paradoxus strain TBEA6

3,3'-Thiodipropionate (TDP)

H₂O

1a

3-Hydroxypropionate

3-Mercaptopropanoate

O₂

II

A. mimigardelfordensis strain DPW7

3,3'-Dithiodipropionate (DTDP)

1/2 NaOH + H⁺

1/2 NAD⁺

III a

3-Sulfonpropionate

Succinyl-CoA

Succinate

III b

3-Sulfonpropionyl-CoA

ATP

Mg⁺

ADP + P_i

IV

3-Sulfonpropionyl-CoA

2H⁺ + SO₄²⁻
Burkholderia xenovorans LB400 [YP_553419.1]

A. mimigardefordensis DPN7 [JN675925.1]

V. paradoxus TBEA6 [ACC69030.2]
A)

\[
\begin{align*}
\text{Succinyl-CoA} & \quad \text{Itaconyl-CoA} \\
\text{Glutaryl-CoA} & \quad \text{3-Thiaglutaryl-CoA}
\end{align*}
\]

B)

\[
\begin{align*}
\text{Acetyl-CoA} & \quad \text{Propionyl-CoA} \\
\text{Butyryl-CoA} & \quad \text{Valeryl-CoA} \\
\text{Isobutyryl-CoA} & \quad \text{Isovaleryl-CoA} \\
\text{Crotonyl-CoA} & \quad \text{Maleyl-CoA}
\end{align*}
\]