Novel Reaction of Succinyl Coenzyme A (Succinyl-CoA) Synthetase: Activation of 3-Sulfinopropionyl to 3-Sulfinopropionyl-CoA in Advenella mimigardefordensis Strain DPN7T during Degradation of 3,3′-Dithiodipropionic Acid\textsuperscript{7,1}

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The \textit{suuCD} gene of \textit{Advenella mimigardefordensis} strain DPN7\textsuperscript{T} encodes a succinyl coenzyme A (succinyl-CoA) synthetase homologue (EC 6.2.1.4 or EC 6.2.1.5) that recognizes, in addition to succinate, the structural analogues 3-sulfinopropionate (3SP) and itaconate as substrates. Accumulation of 3SP during 3,3′-dithiodipropionionic acid (DTDP) degradation was observed in \textit{Tn}\textsubscript{5}::\textit{mob}-induced mutants of \textit{A. mimigardefordensis} strain DPN7\textsuperscript{T} disrupted in \textit{suuCD} and in the defined deletion mutant \textit{A. mimigardefordensis ΔsucCD}. These mutants were impaired in growth with DTDP and 3SP as the sole carbon source. Hence, it was proposed that the succinyl-CoA synthetase homologue in \textit{A. mimigardefordensis} strain DPN7\textsuperscript{T} activates 3SP to the corresponding CoA-thioester (3SP-CoA). The putative genes coding for \textit{A. mimigardefordensis} succinyl-CoA synthetase (\textit{SuuCD\textsubscript{D}}) were cloned and heterologously expressed in \textit{Escherichia coli} BL21(DE3)/pLysS. Purification and characterization of the enzyme confirmed its involvement during degradation of DTDP. 3SP, the cleavage product of DTDP, was converted into 3SP-CoA by the purified enzyme, as demonstrated by \textit{in vitro} enzyme assays. The structure of 3SP-CoA was verified by using liquid chromatography-electrospray ionization-mass spectrometry. \textit{SuuCD\textsubscript{D}} is Mg\textsuperscript{2+} or Mn\textsuperscript{2+} dependent and unspecific regarding ATP or GTP. In kinetic studies the enzyme showed highest enzyme activity and substrate affinity with succinate (\(V_{\text{max}} = 9.85 \pm 0.14 \mu\text{mol min}^{-1} \text{mg}^{-1}, K_m = 0.143 \pm 0.001 \text{mM}\)). In comparison to succinate, activity with 3SP was only ca. 1.2% (\(V_{\text{max}} = 0.12 \pm 0.01 \mu\text{mol min}^{-1} \text{mg}^{-1}\) and the affinity was 6-fold lower (\(K_m = 0.818 \pm 0.046 \text{mM}\)). Based on the present results, we conclude that \textit{SuuCD\textsubscript{D}} is physiologically associated with the citric acid cycle but is mandatory for the catabolic pathway of DTDP and its degradation intermediate 3SP.

3,3′-Dithiodipropionic acid (DTDP) is an organic disulfide and a precursor for the production of polyesters (PTEs) by bacteria (25). Further applications for DTDP are thermodynamic studies (40), development of secondary batteries (52), amino acid analysis (53), and the construction of self-assembling monolayers (10). Microbial production of PTEs from simple carbon sources and inorganic sulfur is currently not possible. Knowledge of the catabolism of organic sulfur compounds in bacteria could provide a reasonable strategy to engineer strains suitable for PTE production. A first step in this direction was the isolation of bacteria able to utilize DTDP as the sole source of carbon and energy. \textit{Advenella mimigardefordensis} strain DPN7\textsuperscript{T}, a betaproteobacterium, found in mature compost in a waste management facility was one of the isolates (15, 56).

To elucidate the degradation pathway of DTDP and to identify the genes involved, transposon mutagenesis was applied to this bacterium (57). Two of the obtained \textit{Tn}\textsubscript{5}::\textit{mob}-induced mutants affected in growth on DTDP accumulated 3-sulfinopropionic acid (3SP). 3SP is a structural analogue of succinate, in which one carbon atom is substituted by a sulfur atom (see Fig. 2c and d). The \textit{Tn}\textsubscript{5}::\textit{mob} insertion in one mutant was mapped in a region 298 bp upstream of \textit{sucC}, coding for a homologue of the \(\beta\)-chain of succinyl coenzyme A (succinyl-CoA) synthetases, and resulted in partially impaired growth on DTDP (see Fig. 4). Insertion of \textit{Tn}\textsubscript{5}::\textit{mob} into \textit{sucC} completely impaired growth on DTDP in the other mutant. Thus, it was predicted that the succinyl-CoA synthetase homologue from \textit{A. mimigardefordensis} strain DPN7\textsuperscript{T} (\textit{SuuCD\textsubscript{D}}) is involved in the catabolic pathway of DTDP (see Fig. 9) (57).

Succinyl-CoA synthetases (\textit{SuccCD}; EC 6.2.1.4 or EC 6.2.1.5) occur in prokaryotes and eukaryotes and are widely known for catalyzing the only substrate-level phosphorylation in the citric acid cycle (7, 31). Therein, the conversion of succinyl-CoA to succinate yields nucleoside triphosphates during aerobic metabolism. The reaction is completely reversible and supplies also succinyl-CoA for heme biosynthesis and ketone body activation, in particular during anaerobic growth (32). Several studies elucidating a variety of regulation systems, indicated the importance of \textit{SuccCD} as a control point of the citric acid cycle (5). Succinyl-CoA synthetases consist of \(\alpha\) (\textit{SuccD}) and \(\beta\) (\textit{SuccC}) subunits, with mass ranges of 29 to 34 kDa and 41 to 45 kDa, respectively (49). In higher organisms and Gram-positive bacteria \(\alpha\)-\(\beta\)-heterodimers are found, whereas in Gram-negative bacteria an \(\alpha\)-homodimeric structure usually occurs (6, 55).

The \(\alpha\)-subunit comprises the active reaction site with a conserved histidine residue, which is phosphorylated during enzy-
matic catalysis. The phosphate moiety is subsequently transferred to a nucleoside diphosphate to yield the corresponding nucleoside triphosphate. Substitution of the conserved histidine residue by other amino acids upon mutagenesis yields an inactive enzyme (6, 26). The \( \beta \)-subunit confers the nucleotide binding site and determines the nucleotide specificity (18, 20).

SucCDs in Gram-negative bacteria such as *Escherichia coli* tend to be nonspecific with regard to the cofactor, and they use both coenzymes ATP and GTP. In *Pseudomonas aeruginosa* SucCD has a very broad nucleotide specificity and is able to use ADP, GDP, UDP, and CDP in combination with inorganic phosphate and succinyl-CoA to synthesize the corresponding nucleoside triphosphates (21). In eukaryotes, SucCDs with higher specificity are found. In mammals, the ATP-specific form predominates in the testes and brain (23). The enzyme in the yeast *Saccharomyces cerevisiae* is specific for ATP (36).

However, although many investigations concerning structure, regulation, and nucleotide specificity have been conducted, only a few studies have reported on the specificity of the organic acid or its CoA thioester as the other substrate of SucCDs. The conversion of succinyl-CoA to succinate and CoA, yielding ATP by SucCD, was first reported by Kaufman (22) (Fig. 1a). Adler et al. (1) reported also on the activation of itaconic acid (Fig. 1c), a structural analogue of succinate, to itaconyl-CoA by the succinyl-CoA synthetase of liver mitochondria as an initial step of itaconic acid dissimilation. The same reaction was described in *Pseudomonas fluorescens* and *Pseudomonas* sp. strain BSaba, respectively (12, 29, 30). Only recently, a SucCD from the hyperthermophilic archaean *Thermococcus kodakarensis*, a succinyl-CoA synthetase with a non-classical domain distribution that resembles the acetyl-CoA synthetases from *Pyrococcus furiosus*, was reported (42). This enzyme could use, in addition to succinate, isovalerate, 3-methyl thiopropionate, glutarate, adipate, and butyrate as substrates. Unfortunately, there are no reports about substrate utilization except for succinate and itaconate in SucCDs with classical domain distributions. In summary, the two substrates succinate and itaconate are activated to the corresponding CoA thioester by succinyl-CoA synthetases with a classical domain distribution that can be compared to the SucCD investigated here.

Only little is known about 3SP (Fig. 2c) and its metabolism. 3SP is a structural analogue of succinate and was first described as a degradation product of homohypotaurin, which is an inhibitor of nervous conduction (4). In the past, it was also considered as a promising antiradiation drug (46, 51). In A. vacciniana...
mimigardefordensis strain DPN7<sup>T</sup>, as well as in Variovorax para-
dactus strain TBEA6, 3SP was found to be an intermediate of 3-
mercaptopyruvopropionic acid (3MP) degradation (8). There are no pre-
vious reports of succinyl-CoA synthetases catalyzing the
activation of 3SP to 3SP-CoA (Fig. 1b). In the present study,
we demonstrate this reaction and verify its essential involve-
ment in the catabolism of DTDP in A. mimigardefordensis
strain DPN7<sup>T</sup>.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. All of the bacterial strains used in
the present study are listed in Table 1. A. mimigardefordensis strain DPN7<sup>T</sup>
and mutants were cultivated in nutrient broth (NB) medium (38) or mineral salt
medium (MSM) (41) under aerobic conditions on a rotary shaker at an agitation
of 130 rpm and at 30 or 37°C. Carbon sources were supplied as filter
sterilized stock solutions as indicated in the text. For the maintenance of plas-
mids, antibiotics were prepared according to the method of Sambrook et al. (38).
DNA isolation and recombinant DNA techniques. Chromosomal DNA of A.
mimigardefordensis strain DPN7<sup>T</sup> was isolated according to the method of Mar-
mur (27). Plasmid DNA was isolated by using the GeneJET plasmid mini prep kit
from Fermentas (St. Leon-Rot, Germany) according to the manufacturer’s man-
ual. DNA was digested with restriction endonucleases under conditions de-
scribed by the manufacturer or according to the method of Sambrook et al. (38).
PCR products were isolated from an agarose gel and purified by using a Nucleo-
Bind Trap kit (Macherey & Nagel, Düren, Germany) according to the manufacturer's manual (Applied Biosystems, Darmstadt, Germany) or an LI-COR
ster with a BigDye Terminator v3.1 cycle sequencing kit according to the man-
ufacturer’s procedure. DNA sequencing were used as primers after construction as
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PCR products were isolated from an agarose gel and purified by using a Nucleo-
Trap kit (Macherey & Nagel, Düren, Germany) according to the manufacturer’s instructions. T4 DNA ligase was purchased from Invitrogen (Karlsruhe,
Germany). Primers were synthesized by MWG-Biotech AG (Ebersberg, Germany).

Transfer of DNA. Competent cells of E. coli strains were prepared and trans-
formed by the CaCl<sub>2</sub> procedure (38).

DNA sequencing and sequence data analysis. DNA sequences were deter-
mined according to the method of Sanger et al. (39). Sequencing was done by using an ABI Prism 3730 capillary sequencer at the Universitätsklinikum Mün-
ster with a BigDye Terminator v3.1 cycle sequencing kit according to the man-
ufacturer’s manual (Applied Biosystems, Darmstadt, Germany) or an LI-COR
4000L automatic sequencing apparatus (LI-COR, Inc., Biotechnology Division,
Lincoln, NE) using a Thermo Long-Read cycle sequencing kit (Epigenet Tech-
nologies, Madison, WI) and ERD 800-labeled oligonucleotides (MWG-Biotech
AG). The program BlastX (National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov]) was used for the determination of nucleotide
identity (2). The program BioEdit (16) was used for multiple sequence align-
ments.

Genome walking. For sequencing of flanking genomic regions of known se-
quences, a PCR-based two-step genome walking method (34) was performed.
ISS0 walking and ISS9 sequencing were used as primers after construction as
described by Pilhofer et al. (34) (the primers are listed in Table 1).

### Table 1. Strains, plasmids, and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid, or oligonucleotide</th>
<th>Description or sequence (5’-3’)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td>A. mimigardefordensis</td>
<td>Wild type, DTDP-degrading bacterium</td>
<td>1 (DSM 17165&lt;sup&gt;T&lt;/sup&gt;, LMG 22922&lt;sup&gt;T&lt;/sup&gt;)</td>
</tr>
<tr>
<td>E. coli Top10</td>
<td>Deletion of sucCD, no growth on DTDP and 3SP</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli BL21(DE3)/pLysS</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; ompT hisS&lt;sub&gt;B&lt;/sub&gt;(r&lt;sub&gt;B&lt;/sub&gt; m&lt;sub&gt;B&lt;/sub&gt;) gal dcm (DE3), pLysS (Cm&lt;sup&gt;T&lt;/sup&gt;)</td>
<td>Invitorgen, Carlsbad, CA</td>
</tr>
<tr>
<td>E. coli BL21(DE3)</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; ompT hisS&lt;sub&gt;B&lt;/sub&gt;(r&lt;sub&gt;B&lt;/sub&gt; m&lt;sub&gt;B&lt;/sub&gt;) gal dcm (DE3)</td>
<td>Novagen, Madison, WI</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
</tr>
<tr>
<td>pSUP5011</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; Km&lt;sup&gt;R&lt;/sup&gt; Tn5::mob</td>
<td>27</td>
</tr>
<tr>
<td>pBluescript SK(−)</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; lacPOZ&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Stratagene, San Diego, CA</td>
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<tr>
<td>pBluescript SK::sucCDBs</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; lacPOZ&lt;sup&gt;−&lt;/sup&gt;</td>
<td>Promega, Madison, WI</td>
</tr>
<tr>
<td>pGEM-T Easy::sucCDBs</td>
<td>Ap&lt;sup&gt;R&lt;/sup&gt; lacPOZ&lt;sup&gt;−&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pJQ2000mp18Tc</td>
<td>Te&lt;sup&gt;R&lt;/sup&gt; sacB oriV oriT trd</td>
<td>35</td>
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<tr>
<td>pJQ2000mp18Tc::ΔsucCD</td>
<td>Te&lt;sup&gt;R&lt;/sup&gt; sacB oriV oriT trd</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Oligonucleotides</strong></td>
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<tr>
<td>M13 forward</td>
<td>GAAAAAGCCGAGCCGAGT</td>
<td>MWG Biotech AG, Ebersberg, Germany</td>
</tr>
<tr>
<td>M13 reverse</td>
<td>CAGGAAAAAGCATGATGAC</td>
<td>MWG Biotech AG, Ebersberg, Germany</td>
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<tr>
<td>IS50 walking</td>
<td>TGGCCCCGAGCTAGAGAC</td>
<td>MWG Biotech AG, Ebersberg, Germany</td>
</tr>
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<td>IS50 sequencing</td>
<td>CGTATTACGATTAGAGGTAGCCGAC</td>
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</tr>
<tr>
<td>sucCDforward_PstI</td>
<td>CTGCAGTGATCTAATCTGTCGGCG</td>
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<td>sucCDreverse_XhoI_stop</td>
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<td>sucCbxal</td>
<td>GAATTCTGATACATTAACTTAACGTAGCTAGAAAAAC</td>
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<td>sucCecoRI</td>
<td>TCTGAGCCTTCCTATCGTCGTGC</td>
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<tr>
<td>sucDxbal</td>
<td>AAAAAGATCTGTACCAGCGGTCTTGTGTC</td>
<td>MWG Biotech AG, Ebersberg, Germany</td>
</tr>
<tr>
<td>sucDecoRI</td>
<td>AAAAAGATCTGTACCAGCGGTCTTGTGTC</td>
<td>MWG Biotech AG, Ebersberg, Germany</td>
</tr>
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</table>

Each strain and plasmid is used in this study is referenced by a superscript number indicating the source or reference. For example, pSUP5011 is referenced as 27, which indicates that the plasmid is from Stratagene, San Diego, CA. The table also includes oligonucleotides used in the study, with each one referenced by a superscript number indicating the source or reference. For example, pJQ2000mp18Tc::ΔsucCD is referenced as 35, which indicates that the oligonucleotide is from this study. The table provides a comprehensive overview of the strains, plasmids, and oligonucleotides used in the study, allowing for a clear understanding of the experimental setup and methodology.
To the A. mimigardefordensis DNA, which was linearized with the organic acids were assayed in the range of 0.1 to 10 mM (succinate) and 0.1 to 100 mM (citrate) and 155 to 210 min, 50 mM NaCl; 65 to 110 min, 75 mM NaCl; 110 to 155 min, 100 mM NaCl; and 155 to 210 min, 150 mM NaCl. SucCD was used to generate the mutant A. mimigardefordensis ΔsucCD. The plasmid was mobilized from E. coli donor strain S17-1 to the A. mimigardefordensis strain DPN7 recipient strain by the spot agar mating technique (14). A successfully generated gene replacement strain was identified and confirmed by PCR analyses and DNA sequencing.

Cloning of sucCD. sucCD was amplified from total genomic DNA of A. mimigardefordensis strain DPN7 by PCR using PsiI-DNA polymerase (Invitrogen) and the primer sucCD办事处_PstI and sucDEcoRI or sucDEcoRI/sucDXbaI, respectively. The resulting fragments were ligated into the EcoR I digested and ligated to yield a 1,192-bp fragment. This fragment was amplified using sucCBlam and sucDXbaI and the resulting PCR product was cloned into the Xba I site of pJQ200mp18Tc (35) to yield pJQ200mp18Tc::ΔsucCD.

Construction of sucCD gene replacement strain using the sucCD system. Gene replacement was accomplished by adaptation of standard protocols (37, 45). Plasmid pJQ200mp18Tc::ΔsucCD was used to generate the mutant A. mimigardefordensis ΔsucCD. The plasmid was mobilized from E. coli donor strain S17-1 to the A. mimigardefordensis strain DPN7 recipient strain by the spot agar mating technique (14). A successfully generated gene replacement strain was identified and confirmed by PCR analyses and DNA sequencing.

Analysis of 3SP by GC and GC/MS. Lysophilized cells and cell-free supernatants were analyzed by gas chromatography (GC). Samples were subjected to methylation in the presence of 1 ml of chloroform, 0.850 ml of methanol, and 0.150 ml of sulfuric acid for 2 to 4 h at 100°C. Upon methylation, 2 ml of H2O was added to the samples, which were vigorously shaken for 30 s. After phase separation, the organic layer containing the resulting methyl esters of the organic acids was analyzed in an HP6890 gas chromatograph equipped with a BP21 capillary column (50 m by 0.22 mm; film thickness, 0.25 μm; GE, Darmstadt, Germany) and a flame ionization detector. Aliquots of synthesized sucCD and supernatants, which showed unknown substances during GC analysis, were analyzed by GC/MS. The samples were subjected to acid-catalyzed esterification in the presence of methanol as described previously. The resulting methyl esters were then characterized in an HP6890 gas chromatograph equipped with a model 5973 EI MSD mass selective detector (Hewlett-Packard, Waldbronn, Germany) and a flame ionization detector. Aliquots of synthesized 3SP and 3SP-CoA during enzyme assays was monitored by HPLC in the scan mode. The collision energy in the MS mode was set to 30 V and yielded fragmentation patterns that were in good accordance with those found in other publications (13).

Preparation of crude extracts. Cells from 50- to 1,000-ml cultures were harvested by centrifugation at 20°C, using a continuous spectrophotometric assay that from the latter was used for enzyme assays.

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accompanied by the formation of CoA thioester was measured as a decrease in NADH absorption at 340 nm. The auxiliary enzymes were tested to ensure that they were not rate limiting.

The utilization of Mn\(^{2+}\) instead of Mg\(^{2+}\) was measured under the same conditions as described above with 5 mM succinate as an organic acid. The concentration of MnCl\(_2\) was 1 mM. The utilization of GTP instead of ATP was also assayed by incubating 30 µg of purified enzyme in 1 ml of 100 mM Tris-HCl (pH 7.4) for 5 min to 2 h at 30°C under the following conditions: 1 mM CoA, 5 mM 3SP, and 10 mM MgCl\(_2\). The concentration of the GTP was 1 mM.

The formation of the expected CoA esters was verified by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). For this analysis, the reactions were stopped by the addition of 30 µl of 15% (wt/vol) trifluoroacetic acid. The samples were subsequently analyzed as described above.

**Data deposition.** The complete nucleotide sequence and the deduced amino acid sequence for SucCD\(_{\text{a},n}\) have been deposited in the GenBank database under accession number EU423870.

**RESULTS**

Analysis of synthesized 3SP by HPLC, GC/MS, and NMR spectroscopy. 3SP applied for screening of Tn\(5::\text{mob}\)-induced mutants and for enzyme assays was chemically synthesized by using a modification of the method of Jollès-Bergeret (19). The reaction was started from 110 g of sodium formaldehyde sulfite (99.5% purity) and yielded 143 g (0.68 mol, 97% yield) of the foxylate (purity, reaction was started from 110 g of sodium formaldehyde sulfite (pH 7.4) for 5 min to 2 h at 30°C under the following conditions: 1 mM CoA, 5 mM 3SP, and 10 mM MgCl\(_2\). The concentration of the GTP was 1 mM.

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**Molecular characterization of Tn\(5::\text{mob}\)-induced mutants and construction of A. mimigardefordensis SucCD\(_n\).** Two methods were used to map the insertions of Tn\(5::\text{mob}\) in these mutants. A two-step genome walking method (34) was applied, and genomic libraries were also constructed. For the latter, genomic fragments of either mutant conferring Km resistance were cloned in E. coli Top10. Sequencing of these DNA fragments using oligonucleotides hybridizing to the terminal region of IS50L and the multiple cloning site of the used cloning vector pBluescript SK(−) revealed a continuous sequence of 3,764 bp. In mutant JG-4, which was fully impaired in growth with 3SP, Tn\(5::\text{mob}\) had been inserted into an intergenic region upstream of sucC. The insertion of Tn\(5::\text{mob}\) in mutant JG-11 disrupted an open reading frame (ORF) coding for the \(\alpha\)-chain (sucD) of SucCD\(_{\text{a},n}\) and also led to a 3SP-negative phenotype. The phenotypes of the mutants and the affected genes were summarized in Table 2, and the gene organization and insertion loci are shown in Fig. 4. An ORF putatively coding for tellurium resistance (terC) is located downstream of SucCD\(_n\) (57). The ORF upstream of SucCD\(_n\) was annotated to a conserved hypothetical protein. To verify the results obtained with the Tn\(5::\text{mob}\)-induced mutants, the defined deletion strain A. mimigardefordensis SucCD\(_n\) was generated in addition.

**Degradation of DTDP and accumulation of 3SP.** To identify the accumulation of putative intermediates, the wild type and mutants JG-4, JG-11, Jhw38, and Jhw121, which were obtained in this and a previous study (57), were precultivated in MSM containing 0.3% (wt/vol) sodium propionate. After 48 h, the cells were washed and transferred to Erlenmeyer flasks without baffles with MSM containing 0.3% (wt/vol) DTDP, which corresponds to about 14 mM as the sole carbon source. Samples were taken every 48 h, and aliquots of cell-free supernatants were analyzed by GC and GC/MS (data not shown). The wild type and any mutant were able to partially degrade DTDP. In the cultures of the wild type, as well as of the mutants Jhw38 and JG-4, a transient accumulation of 3SP was observed. Mutant Jhw121 accumulated 3SP at a comparably high concentration of up to 5 mM, whereas mutant JG-11 showed no
accumulation of 3SP. The *A. mimigardefordensis* Δ*sucCD* strain accumulated even more 3SP in the supernatant if cultivated in MSM supplied with DTDP and another utilizable carbon source. The highest concentrations of 3SP were detected after preincubation of *A. mimigardefordensis* strain DPN7^T^ in MSM containing 20 mM succinate for 48 h. The cells were then washed, transferred into fresh MSM containing 10 mM succinate and 20 mM DTDP, and cultivated at 30°C and 120 rpm for 9 days. Within this incubation time, almost all DTDP was consumed, and 26 mM 3SP was detectable in the supernatant (Fig. 5).

**Analysis of the primary structure of SucCD_{Am}**. *In silico* analyses of the amino acid sequences of SucC and SucD of *A. mimigardefordensis* strain DPN7^T^ showed the highest similarities with the α-chain of succinyl-CoA synthetase from the *Bordetella pertussis* strain Tohama I (93% identity) and with the β-chain of succinyl-CoA synthetase from *Bordetella avium*.

FIG. 3. NMR spectra of 3SP. (A) ^1^H-NMR spectrum at 400 MHz in D_{2}O. Peaks are dedicated to the corresponding CH\textsubscript{2}-groups of 3SP. The inset depicts zoomed peaks. An asterisk indicates a solvent peak (HDO). (B) ^13^C-NMR spectrum at 100 MHz in D_{2}O. Indices positions a, b, and c indicate the corresponding carbon atoms of 3SP.
strain 197N (89% identity), respectively (Table 2). The α-chain consists of 293 amino acids and comprises a Rossmann fold and a CoA-ligase domain. The β-chain consists of 386 amino acids and encloses a d-alanine–d-alanine ligase domain and an ATP binding site. A multiple sequence alignment of SucCD homologues is shown in Fig. S1 in the supplemental material.

**Purification of SucCD<sub>Δmob</sub>**. The first attempt toward the heterologous expression of SucCD<sub>Δmob</sub> using several vectors of the pET system (Novagen) in various expression strains such as E. coli Tuner (DE3), E. coli Rosetta (DE3)/pLysS, or E. coli HMS174 (DE3) in combination with induction by IPTG failed. The α-subunit was expressed but formed inclusion bodies. Measures to prevent the formation of inclusion bodies, such as cultivation at lower temperature or the addition of small amounts of ethanol to the medium as described by Strandberg and Enfors (48), had no effect. Finally, construction of pBluescriptSK::sucCD<sub>Δmob</sub>, transfer into E. coli BL21(DE3)/pLysS, and subsequent induction using ZYP-5052 medium, an autoinduction medium according to Studier et al. (50), solved the problem. The soluble protein fraction, obtained after cell disruption and centrifugation, was applied to a Q-Sepharose Fast-Flow column. The column was equilibrated with 50 mM Tris-HCl (pH 7.4) containing no NaCl. Elution was then carried out by applying a step gradient of increasing sodium chloride concentrations. The purified enzyme was eluted in the 150 mM NaCl step and used for enzyme assays (Fig. 6). After centrifugation, the enzyme was shown to be soluble in the supernatant.

**SucCD<sub>Δmob</sub> enzyme activity assay.** After expression and purification of soluble SucCD from *A. mimigardefordensis* strain DPN7<sup>T</sup>, the enzyme activity was determined by use of a continuous spectrophotometric assay as described in Materials and Methods. To verify the *in vitro* formation of the expected CoA esters, samples were withdrawn after finishing the spectrophotometric measurements and subjected to LC-ESI-MS. When succinate, itaconate, or 3SP was added to the assay, the formation of the respective CoA esters could be observed (Fig. 7). However, kinetic studies showed clear differences between the three substrates (Table 3). We found that, upon comparing the *V<sub>max</sub>* values, the highest activity of the enzyme occurred with succinate (*V<sub>max</sub> = 9.85 ± 0.14 μmol min<sup>-1</sup> mg<sup>-1</sup>*), whereas the activity with itaconate was only ca. 15% and with 3SP only ca. 1.2% of the activity with succinate. With regard to the *K<sub>m</sub>* values, SucCD<sub>Δmob</sub> revealed the highest affinity to succinate (*K<sub>m</sub> = 0.143 ± 0.001 mM*) as a substrate. A 3-fold-higher *K<sub>m</sub>* value of the enzyme was obtained for itaconic acid and an ~6-fold-higher *K<sub>m</sub>* value was obtained for 3SP, both leading to the assumption of a lower catalytic efficiency of SucCD<sub>Δmob</sub> for these substrates.

As expected for a nucleotide triphosphate-dependent enzyme, no enzyme activity was observed in the absence of Mg<sup>2+</sup>. However, the formation of succinyl-CoA was also detected when 1 mM MgCl<sub>2</sub> was exchanged by equimolar amounts of Mg<sup>2+</sup>.

**FIG. 4.** Localization of Tn<sup>5</sup>:mob insertions in the genomes of four independent Tn<sup>5</sup>-induced mutants of *A. mimigardefordensis* strain DPN7<sup>T</sup>. The positions of Tn<sup>5</sup>:mob insertions in the respective mutants are indicated as arrows. A region of 3,764 bp was sequenced. Abbreviations: ORF 1, open reading frame 1; sucC, β-chain of succinyl-CoA synthetase; sucD, α-chain of succinyl-CoA synthetase; terC, fragment of integral membrane protein that is putatively involved in tellurium resistance.

**FIG. 5.** Degradation of DTDP and accumulation of 3MP and 3SP. *A. mimigardefordensis* ΔsucD cells were preincubated for 48 h in mineral salt medium (MSM) containing 20 mM succinate. The cells were then washed, transferred into fresh MSM containing 10 mM succinate and 20 mM DTDP, and cultivated at 30°C and 120 rpm for 9 days. Within this incubation period, almost all of the DTDP was consumed, and 26 mM 3SP was detectable in the supernatant. Symbols: triangles, DTDP; squares, 3MP; diamonds, 3SP.

**TABLE 2.** Phenotypic and genotypic characterization of Tn<sup>5</sup>:mob-induced mutants of *A. mimigardefordensis* strain DPN7<sup>T</sup> relevant for this study

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Phenotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Insertion locus of Tn&lt;sup&gt;5&lt;/sup&gt;:mob (gene product)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Highest homology&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JhwA8/121</td>
<td>– –</td>
<td>sucC (succinyl-CoA synthetase, beta-chain)</td>
<td>93 (Bordetella pertussis Tohama I)</td>
<td>3</td>
</tr>
<tr>
<td>Jhw38</td>
<td>– +/–</td>
<td>289 bp upstream of sucC*</td>
<td>93 (Bordetella pertussis Tohama I)</td>
<td>3</td>
</tr>
<tr>
<td>JG-4</td>
<td>– –</td>
<td>78 bp upstream of sucC*</td>
<td>93 (Bordetella pertussis Tohama I)</td>
<td>This study</td>
</tr>
<tr>
<td>JG-11</td>
<td>– –</td>
<td>sucD (succinyl-CoA synthetase, beta-chain)</td>
<td>89 (Bordetella avium 197N)</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup> –, No growth; +/–, weak growth.
<sup>b</sup> *: Closest specified gene identified adjacent to the Tn<sup>5</sup>:mob insertion locus.
<sup>c</sup> The relevant strain is indicated in parentheses.
MnCl₂ in the standard assay, applying 5 mM succinate as an organic acid.

Substitution of ATP by GTP was tested with a modified enzyme assay as described in Materials and Methods. The formation of 3SP-CoA in the presence of 3SP as an organic acid and GTP instead of ATP verified the recognition of GTP as a cofactor by SucCDₐm.

Confirming the structure of 3SP-CoA. The formation of 3SP-CoA has, to our best knowledge, never been described before either enzymatically or chemically. Thus, 3SP-CoA could not also be purchased and could not be used as a reference for enzyme assays. To verify the formation of 3SP-CoA and its proposed chemical structure, LC-ESI-MS experiments were conducted. In full positive MS mode without collision-induced dissociation a parental ion (m/z: 11005 Da) was observed (Fig. 8). The fragmentation of this ion led to two main fragments (m/z: 428 Da and m/z: 381 Da). Subsequent fragmentation of the daughter ion (m/z: 381 Da) yielded two additional fragments (m/z: 381 Da and m/z: 261 Da).

**DISCUSSION**

We report here that SucCD from *A. mimigadeordensis* strain DPN7ᵀ can catalyze the activation of 3SP, a DTDP degradation intermediate, to the corresponding 3SP-CoA thioester. Therefore, the enzyme is promiscuous, since it is involved in the citric acid cycle and is mandatory for the catalysis of DTDP and, in particular, its degradation intermediate 3SP. Succinyl-CoA synthetases catalyze the conversion of succinyl-CoA to succinate and CoA in the citric acid cycle. The energy of the thioester bond is conserved through the coupled phosphorylation of nucleoside diphosphates such as ADP or GDP. However, the reverse reaction is also impor-

![FIG. 6. Purification of SucCDₐm applying Q-Sepharose FF as revealed by SDS-PAGE (11.5% wt/vol acrylamide). Lane 1, crude extract of cells; lane 2, soluble protein fraction after 90 min of centrifugation at 100,000 x g and 4°C; lane M, low-molecular-weight calibration kit (GE Healthcare, Uppsala, Sweden); lane 3, purified SucCDₐm eluted with 50 mM Tris-HCl (pH 7.4)–150 mM NaCl. The SDS-gel was stained with Coomassie brilliant blue R.](http://jb.asm.org/)

![FIG. 7. Typical HPLC chromatograms of enzyme assays. Enzyme assays were performed as described in Materials and Methods. Detection of compounds was carried out at 259 nm using a photo diode array detector. Identification of peaks was done using ESI-MS. Shown are the results obtained for a control omitting an organic acid as a substrate but containing SucCDₐm (graph 1) and from assays performed in the presence of 5 mM succinate (graph 2), 5 mM itaconate (graph 3), or 5 mM 3SP (graph 4). * CoA and succinyl-CoA were not separated by the applied HPLC methods. ESI-MS analysis revealed the complete transformation of CoA to succinyl-CoA.](http://jb.asm.org/)
tant for the anabolism. In this case, SucCD activates succinate to the corresponding CoA thioester (24).

Two independent Tn5::mob transposon mutagenesis experiments from this and a previous study (57) yielded a total of 40,000 transconjugants; these were screened for the lack of growth on DTDP or 3SP. Four different Tn5::mob-induced mutants completely or almost completely impaired in DTDP and/or 3SP degradation or in growth on these compounds with Tn5::mob insertions directly in or in the proximity of SucCD (Fig. 4 and Table 2) indicated the involvement of SucCD in DTDP degradation. Due to the sequence similarities of the sucC- and sucD-encoded proteins to the two subunits of succinyl-CoA synthetases and a certain structural similarity of 3SP to succinate, it was predicted that 3SP is activated to the corresponding 3SP-CoA thioester by SucCD<sub>Am</sub>. This assumption was strengthened, since 3SP was accumulated in the supernatant (Fig. 5) during cultivation of the DTDP- and 3SP-negative mutant <i>A. mimigardefordensis</i>/H9004 sucCD<sub>Am</sub> in MSM containing DTDP and succinate as a carbon source for growth. A concentration of up to ~26 mM 3SP in the supernatant indicates

FIG. 8. (Top) Structural formula of 3SP-CoA; (bottom) mass spectrometric data. The first is an ESI spectrum of 3SP-CoA in positive mode. In the middle is an MS spectrum of the parent ion (m/z = 888 Da). Two main fragments (m/z = 428 Da and m/z = 381 Da) were obtained. At the bottom, further fragmentation of the parent m/z 381 Da yielded daughter ions (m/z = 315 Da and m/z = 261 Da).
FIG. 9. Proposed degradation pathway of DTDP. Initial cleavage by a dihydrolipoamide dehydrogenase (step I) yields two molecules of 3MP, which are further oxygenated by a dioxygenase (step II), yielding 3SP. The latter is activated to the corresponding CoA thioester by SucCD (step III), as shown in the present study. The next step is most probably the dehydrogenation of 3-sulfino-2,3-dehydropropionyl-CoA by an acyl-CoA-dehydrogenase homologue (step IV) in an FAD-dependent step. The sulfur moiety of the dehydrogenation product, 3-sulfino-2,3-dehydropropionyl-CoA, is putatively removed by an enzymatic or an autocatalytic reaction. Propionyl-CoA subsequently enters the central metabolism via the methyl citric acid cycle.

that this compound is neither toxic to the cells nor is it an inhibitor of any other enzyme critical for growth. Furthermore, 3SP seems not to be a substrate for any other enzyme in \textit{A. mimigardefordensis}, except for SucCD. 3SP is formed in the cytoplasm of the cells from 3MP by a dioxygenase (Fig. 9) but was found in the supernatant. Since the compound carries two negative charges and would not diffuse out of the cells, it seems reasonable that a transporter is involved. 3SP exhibits structural similarities to succinate except for the exchange of a carbon atom in one of the carboxyl groups by a sulfur atom. For succinate, different transporters are known (17). Since succinate was utilized as a carbon source during accumulation experiments, \textit{A. mimigardefordensis} must possess at least one succinate transporter. Other steps of DTDP catabolism are catalyzed by enzymes unspecific enough to accept intermediates of DTDP degradation. Therefore, this might also apply to the transport of 3SP. Consequently, it is not very likely that a special 3SP transporter has evolved, but it seems rather probable that the succinate transporter is also active with 3SP.

To confirm the proposed activation of 3SP to the corresponding 3SP-CoA thioester, sucCD was heterologously expressed in a recombinant \textit{E. coli} strain BL21(DE3)/pLysS harboring pSK::\textit{succd}rbs, and the purified enzyme was subsequently applied in an enzyme assay. Analyses by LC-ESI-MS showed the formation of another compound with a similar absorption spectrum as CoA and succinyl-CoA, but with a significantly different retention time. In the concomitant total ion chromatogram a main peak of \(m/z = 888\) Da was observed. This corresponded well to the value estimated for 3SP-CoA. To verify the formation of 3SP-CoA and to confirm its expected chemical structure, the main peak was further fragmented. The obtained daughter ion \(m/z = 428\) Da is the typical ion for CoA derivatives (13) and can be assigned to the adenosine 3',5'-diphosphate moiety. It results from a cleavage in the phosphate backbone region of the proposed 3SP-CoA ester. Since this fragment was derived from the nonvariable region of 3SP-CoA, the second observed daughter ion \(m/z = 381\) Da was more interesting with respect to the structural analysis. It was obtained by the loss of a fragment \(m/z = 507\) Da from the parent ion. Park et al. (33) used the constant decrease of \(m/z = 507\) Da from parent ions of different CoA esters to ascertain the identity of the expected CoA derivative in their study. Therefore, these researchers calculated the expected mass of the second daughter by subtracting \(m/z = 507\) Da from the respective parent ion. The ion at \(m/z = 381\) Da (Fig. 8) corresponded to the expected mass of 3SP attached to the pantetheinyl moiety of CoA. Up to that point it was uncertain how 3SP was bound to CoA. Theoretically, 3SP could be bound as a thioester or as a thiosulfinate [(2-carboxyethyl)sulfinic acid CoA ester] (Fig. 2f and g). Further fragmentation of \(m/z = 381\) Da yielded \(m/z = 315\) Da as a major fragment. This was assigned to the loss of \(\text{H}_2\text{SO}_2\) from the \(m/z = 381\) Da fragment, which is only possible if 3SP is attached to CoA as a thioester. The second fragment received during this fragmentation resulted from the total loss of the 3SP moiety and could be assigned to the dehydroxylated pantetheine residue (Fig. 8, \(m/z = 261\) Da [structure not shown]).

Activation of itaconate by SucCD as an initial step of itaconate degradation in liver mitochondria and in \textit{P. fluorescens} and \textit{Pseudomonas} sp. strain Baba, respectively, was reported earlier (1, 29, 30, 46). This transformation of itaconate to itaconyl-CoA was also verified by LC/MS for SucCD from \textit{A. mimigardefordensis} strain DPNN77.

In all, succinate, itaconate, and the novel substrate 3SP are activated to the corresponding CoA thioesters (Fig. 1a to c). According to the obtained kinetic parameters, SucCD_{Aba} shows a different activity with regard to the three substrates. This might be due to the structural differences of succinate, 3SP, and itaconate. An additional methylene group in itaconate represents a rather minor structural difference in comparison to the substitution of a carbon atom by a sulfur atom in 3SP compared to the well-known substrate succinate (Fig. 2e to e). However, along with succinate and itaconate activation, the transformation of 3SP to the corresponding 3SP-CoA thioester, which is reported for the first time here, is the third reaction catalyzed by SucCDs with a classical domain distribution (Fig. 1a to c). In addition, proof of this reaction confirms the suggestion made by Jollès-Bergeret in 1974 that 3SP, as a close
structural analogue of succinate, would undergo, at least in part, the same metabolic fate (19). Due to the possibility of substituting the cofactor Mg$^{2+}$ by Mn$^{2+}$ and the coenzyme ATP by GTP, SucCD$_{Am}$ shows behavior similar to that of other SucCDs from Gram-negative bacteria (21).

Another point to be discussed is whether the physiological function of SucCD$_{Am}$ is dedicated to the citric acid cycle (TCC) or to the catabolism of the DTDP degradation intermediate 3SP or to both. Tn$^{5}$:mob insertions directly in or in the vicinity of the same sucCD resulted in a phenotype completely or partially impaired in growth on 3SP as well as on DTDP. For the A. mimigardefordensis $\Delta$sucCD strain no growth on DTDP as the sole carbon source and accumulation of 3SP when succinate was applied as a carbon source in addition to DTDP were observed. Growth on any other tested carbon sources was not affected. The latter finding is in accordance with results reported recently for E. coli mutants carrying deletions of sucCD. E. coli MG1655 $\Delta$sucCD mutants were generated in two independent studies (9, 54). In both studies, no growth retardation of the deletion mutants in LB medium or in minimal medium containing glucose as the sole carbon source was observed. Byung et al. (9) showed that in these mutants succinyl-CoA was supplied by $\alpha$-ketoglutarate dehydrogenase encoded by sucAB, whereas a simultaneous deletion of both sucCD and sucAB was lethal. Thus, despite the lack of an active SucCD and hence a disrupted TCC in A. mimigardefordensis strain DPN7$^T$ mutants, growth on MSM agar plates containing 0.2% (wt/vol) gluconate, propionate, taurine, or succinate as the sole sources of carbon and energy was not detectably affected in all mutants compared to the wild type. The amino acid sequence of SucCD$_{Am}$ shows high similarity to other SucCDs, and the formation of 3SP-CoA was also observed in crude extracts from E. coli BL21(DE3)/pLysS not harboring the gene for SucCD from A. mimigardefordensis strain no 3.

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