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Novel Pathway for Catabolism of the Organic Sulfur Compound 3,3′-Dithiodipropionic Acid via 3-Mercaptopropionic Acid and 3-Sulfinopropionic Acid to Propionyl-Coenzyme A by the Aerobic Bacterium *Tetrathiobacter mimigardefordensis* Strain DPN7[▽]

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The hitherto unstudied microbial degradation of the organic disulfide 3,3′-dithiodipropionic acid (DTDP) was investigated with the recently described bacterium *Tetrathiobacter mimigardefordensis* strain DPN7^T (DSM 17166^T; LMG 22922^T), which is able to use DTDP as the sole carbon source for growth. 3-Mercaptopropionic acid (3MP) and 3-sulfinopropionic acid (3SP) were detected in the growth medium and occurred as intermediates during DTDP degradation. To identify genes coding for enzymes of DTDP catabolism, Tn*5*::*mob*-induced mutants of *T. mimigardefordensis* were generated. Screening of transposon mutant libraries yielded many mutants fully or partially impaired in utilizing DTDP as a carbon source. Mapping of the insertion loci in some mutants identified four disrupted open reading frames (ORFs) with putative metabolic functions. The ORFs were assigned function on the basis of homologies with *lpdA* (EC 1.8.1.4), *cdo* (EC 1.13.11.20), *sucCD* (EC 6.2.1.5), and *acnB* (EC 4.2.1.3). Tn*5*::*mob* insertions occurred additionally in the vicinity of heat shock protein-encoding genes. The predicted function of the LpdA homologue in *T. mimigardefordensis* is cleavage of the disulfide bond of DTDP to form two molecules of 3MP. Cdo catalyzes the conversion of the sulfhydryl group of 3MP, yielding the corresponding sulfinic acid, 3SP. SucCD exhibits thiokinase activity, ligating coenzyme A (CoA) with 3SP to form 3SP-CoA. Afterwards, an elimination of sulfite via a putative desulfinase is expected. *acnB* encodes a putative 2-methylisocitrate dehydratase. Therefore, a new pathway is proposed for the catabolism of DTDP via 3MP, 3SP, and 3SP-CoA toward propionyl-CoA, which is then further catabolized via the 2-methylcitric acid cycle in *T. mimigardefordensis*.

The biotechnological relevance of 3,3′-dithiodipropionic acid (DTDP) is its application as a precursor substrate for microbially synthesized polythioesters (PTEs) (36). Furthermore, this organic sulfur compound (OSC) is employed in electrochemical and thermodynamic studies (49), for development of secondary batteries (58), in amino acid analysis (59), and for construction of self-assembly monolayers (15). The chemical structure of DTDP is very similar to that of the disulfide amino acid cystine. The absence of amino groups in DTDP is the only difference, yielding a higher melting point of cystine (247 to 249°C) than of DTDP (152 to 157°C). The occurrence of DTDP in natural habitats has not been described, to our knowledge, although this OSC may well be formed, because it is the disulfide of two molecules of 3-mercaptopropionic acid (3MP) and an oxidative disulfide formation is not unlikely. 3MP, along with cysteine and glutathione, belongs to the most frequently detected thiols in natural aquatic environments (1, 69). However, it occurs only in nanomolar concentrations (24). 3MP was found as a central intermediate during catabolism of the marine alga osmolyte dimethylsulfoniopropionate (11, 29, 57, 60, 61, 64) and also in freshwater habitats, as an anaerobic degradation product of sulfur-containing organic compounds, such as homocysteine and methionine (31, 43). Furthermore, it is generated by abiotic reactions of dissolved sulfide with dissolved organic matter in hypolimnetic waters (24). Therefore, 3MP is not a xenobiotic, though it is chemically produced at the scale of several thousand tons for applications as a bisphenol A cocatalyst. Moreover, it is used as a convulsant for studies of experimental epilepsy (16, 53) and for gold nanoparticle arrays to form three-dimensional monolayers (68). During application as a precursor substrate in PTE production, the microbial utilization of 3MP has been demonstrated clearly (35, 37, 38, 55). However, nothing is known about the pathway and the enzymes involved in the catabolism of 3MP or of its dimer, DTDP. Obviously, none of the PTE-producing microorganisms and only a few characterized strains utilize these two compounds as sole sources of carbon and energy (66). This is presumably due to the toxicity of these OSCs or of their intermediates during degradation (47).

Most information regarding the catabolism of naturally occurring OSCs is available for cysteine and methionine and also for dimethyl sulfoxide, dimethylsulfoniopropionate, and dimethylsulfide as intermediates of the sulfur cycle (28, 30, 33, 67). In addition, biodesulfurization of benzothiophenes (41) and of the fluorinated OSC *bis*-(3-pentafluorophenylpropyl)-sulfide (62) has been studied in detail. However, these OSCs are not related structurally to DTDP or 3MP.

It is presently not possible to synthesize PTEs from simple carbon sources and inorganic sulfur sources. To establish mi-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
Escherichia coli		
S17-1	*thi-1 proA hsdR17* (r_K^- m_K^+) *recA1*; *tra* genes of plasmid RP4 integrated into the genome	52
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1* λ⁻ *lac* [F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)]	10
Tetrathiobacter mimigardefordensis (DSM 17166^T; LMG 22922^T)	Wild type, DTDP-degrading organism	66
Plasmids		
pSUP5011	Ap^r Cm^r Km^r Tn*5*::*mob*	51
pBluescript SK (−)	Ap^r *lacPOZ′*	Stratagene, San Diego, CA

[a] For abbreviations used in genotypes of *E. coli*, see reference 6.

crobial synthesis independently of toxic and very expensive OSCs, the metabolism of a suitable microorganism has to be engineered. A first step in this direction was to obtain bacteria able to utilize one or more of the precursors as a carbon source. Because symmetric cleavage of DTDP via a putative disulfide reductase will yield two molecules of 3MP (36) and because DTDP is far less toxic to bacteria than 3MP is, we investigated the catabolism of DTDP. A proposal for the degradation pathway of this OSC in *Tetrathiobacter mimigardefordensis* strain DPN7^T is presented in this report.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were cultivated aerobically in Luria-Bertani (LB) medium (48) at 37°C with the addition of applicable antibiotics, if necessary. *E. coli* XL1-Blue and the vector pBluescript SK (−) were used for DNA cloning and construction of genetic libraries. *E. coli* S17-1 harboring the suicide plasmid pSUP5011 was used for Tn*5*::*mob* mutagenesis of *Tetrathiobacter mimigardefordensis* strain DPN7. This betaproteobacterium was isolated from a matured compost heap and was found to grow on DTDP as the sole carbon source. Strain DPN7 was later characterized in more detail and allocated as the type strain in the new taxon *T. mimigardefordensis* (66). Deposition was accomplished at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 17166^T) and at the Laboratorium Microbiologie Universiteit Gent (LMG 22922^T). Cells of *T. mimigardefordensis* were grown aerobically at 30°C in 0.8% (wt/vol) nutrient broth or in mineral salts medium (MSM) (50) containing the carbon source indicated in the text. Carbon sources were prepared as filter-sterilized 20% (wt/vol) stock solutions and adjusted to pH 7.0. Solid media contained 1.8% (wt/vol) purified agar-agar. Antibiotics were added to growth media at the following concentrations: ampicillin (Ap), 75 μg/ml; and kanamycin (Km), 50 μg/ml.

Chemicals and synthesis of 3-sulfinopropionic acid. Sulfur-containing substrates were purchased from Acros Organics (Geel, Belgium). Since 3-sulfinopropionate (3SP) could not be purchased, it was synthesized as a disodium salt according to the methods described by Jollés-Bergeret (26); the described procedure was slightly modified by one repetition of the alkaline cleavage of the intermediate *bis*-(2-carboxyethyl)sulfone. Starting from 111 g sodium formaldehyde sulfoxylate (purity, >98%) plus 108 ml acrylic acid (99.5%), 119 g of the intermediate *bis*-(2-carboxyethyl)sulfone was chemically synthesized. After alkaline scission, precipitation, and washing procedures, 99 g of the disodium salt of 3SP, with a purity of about 90%, was finally obtained. The success of synthesis and purity of the synthesized compound were confirmed by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) analyses.

Tn*5* mutagenesis. For transposon mutagenesis of *T. mimigardefordensis* strain DPN7, the suicide plasmid technique described previously (52) was employed by transferring the vector pSUP5011 from *E. coli* S17-1 to the Km-susceptible strain DPN7 by conjugation, using the spot agar mating technique (21). Tn*5*-induced mutants were selected on MSM agar plates containing 50 μg Km ml^{-1} (MSM^{Km}) and 0.5% (wt/vol) sodium gluconate, 0.2% (wt/vol) sodium propionate, or 0.2% (wt/vol) sulfinopropionate (master plates). Putative mutants were transferred in a coordinated pattern to MSM^{Km} agar plates containing 0.4% (wt/vol) DTDP (selection plates) and to corresponding master plates for further analysis.

Isolation and manipulation of DNA. Chromosomal DNAs of the Tn*5*-induced mutants of *T. mimigardefordensis* strain DPN7 and of the wild type were isolated as described by Marmur (39). Plasmid DNA was isolated by the method of Birnboim and Doly (7). Restriction enzymes and ligases were used according to the instructions of the manufacturers.

Transfer of DNA. Competent cells of *E. coli* were prepared by the $CaCl_2$ procedure and transformed with genomic DNA of Tn*5*-induced mutants of *T. mimigardefordensis* strain DPN7 ligated into plasmid pBluescript SK (−) (23).

Genotypic characterization of Tn*5*-induced mutants of *T. mimigardefordensis* strain DPN7. Genomic DNAs of Tn*5*-induced mutants were digested with BamHI or SalI, and the resulting fragments were ligated into plasmid pBluescript SK (−). Recombinant *E. coli* clones were selected due to their Km resistance conferred by Tn*5*. The hybrid plasmids of the resulting clones harbored a BamHI or a SalI restriction fragment, respectively, which included the region of Tn*5* located between the *mob* site and the IS*50*L element (including the Km resistance gene) plus genomic DNA adjacent to the Tn*5* insertion locus. These recombinant plasmids were sequenced using an oligonucleotide (5′-GTTAGGAGGTCACATGG-3′) which hybridized specifically to the IS*50*L element of Tn*5*::*mob* and the oligonucleotide universal primers M13-forward (5′-GTAAAACGACGGCCAGT-3′) and M13-reverse (5′-CAGGAAACAGCTATGAC-3′).

PCR. Amplifications of plasmid or genomic DNA were done as described previously (25). Inverse PCR (44) and direct genome walking using PCR (42) were done as specified to receive more information about the downstream and upstream regions of Tn*5* insertion loci.

DNA-DNA hybridization. Southern hybridizations to confirm Tn*5* insertion were done by the method of Oelmüller et al. (45).

DNA sequencing and sequence analysis. DNA sequencing was performed by applying a SequiTherm long-read cycle sequencing kit (Epicenter Technologies, WI) and IRD800-labeled oligonucleotides (MWG-Biotech, Ebersberg, Germany). Sequence reactions were accomplished by using a GeneReadIR 4200 DNA analyzer (LI-COR Inc., Biotechnology Division). Sequences were analyzed using the program BLAST (National Centre for Biotechnology Information [http://www.ncbi.nlm.nih.gov/BLAST/]) by searching the protein database using the translated nucleotide query (BlastX) (2, 3).

GC-MS analyses. The compositions of the cell-free supernatants of cultures and of the obligate standards of the important OSCs were determined upon methanolysis after lyophilization in the presence of 15% (vol/vol) sulfuric acid (H_2SO_4) by GC analysis of the resulting methyl esters as described previously (8, 56).

HPLC analyses. HPLC analysis was carried out with a LaChrom Elite HPLC apparatus (VWR-Hitachi International GmbH, Darmstadt, Germany) consisting of a Metacarb 67H advanced C column (Varian, Palo Alto, CA; Bio-Rad Aminex equivalent) and a 22350 VWR-Hitachi column oven. The column (300 mm by 6.5 mm) consisted of sulfonated polystyrene resin in the protonated form. The primary separation mechanism included ligand exchange, ion exclusion, and adsorption. A VWR-Hitachi refractive index detector (type 2490) with an active flow cell temperature control and automated reference flushing eliminating temperature effects on the refractive index baseline was used for detection. Aliquots of 20 μl were injected and eluted with 0.005 N sulfuric acid in double-distilled water at a flow rate of 0.8 ml/min. Online integration and analysis were done with EZ Chrome Elite software (VWR International GmbH, Darmstadt, Germany).

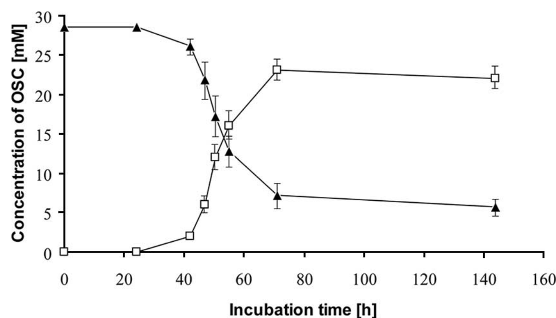

FIG. 1. Degradation of DTDP by *T. mimigardefordensis* strain DPN7^T and occurrence of 3MP. Cells were cultivated in a 250-ml Erlenmeyer flask without baffles on a rotary shaker at 30°C and 120 rpm in MSM containing 0.6% (wt/vol) or 28.6 mM DTDP as the sole carbon source. Analyses of DTDP and 3MP in the cell-free supernatants were done by GC. Data points presented are mean values for four replicates; error bars indicate the standard deviations. Symbols: ▲, DTDP; □, 3MP.

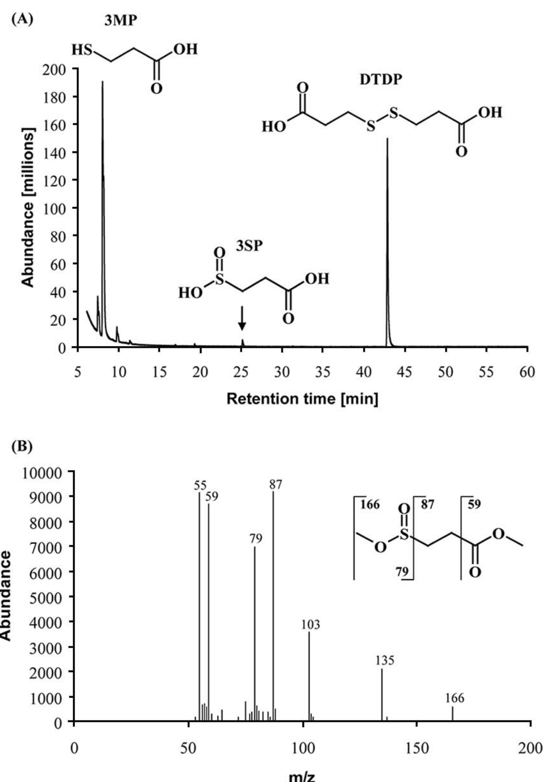

FIG. 2. GC-MS analysis of DTDP and of intermediates occurring during degradation. Cultivation was accomplished under the conditions described in the legend to Fig. 1. (A) Besides DTDP, the intermediates 3MP and 3SP were separated in the chromatogram and identified. (B) Electron ionization mass spectrum of methylated 3SP as an example. Main fragmentations are indicated.

Nucleotide sequence accession numbers. The complete DNA sequences and deduced amino acid sequences for *lpdA* (accession number EU423868), *ddiox* (accession number EU423869), and *sucCD* (accession number EU423870) and the partial DNA sequence for *acnB* (accession number EU423871) of *T. mimigardefordensis* strain DPN7^T have been deposited in the GenBank database.

RESULTS

Biodegradation of DTDP by *T. mimigardefordensis* strain DPN7. DTDP is utilized by *T. mimigardefordensis* strain DPN7 as the sole source of carbon and energy. Growth was initially observed on solid agar plates and then confirmed in liquid MSM, where an increase of turbidity was accompanied by a concomitant decrease of DTDP (Fig. 1). Controls (cultures without inoculum or without a carbon source) did not show any decrease of the DTDP concentration or increase of the optical density, respectively. A concentration of 60 μmol DTDP per mg protein was metabolized by cells of *T. mimigardefordensis* strain DPN7^T. GC-MS analysis of the lyophilized cell-free supernatants identified, in addition to DTDP, two most likely intermediates of DTDP degradation (Fig. 2; see below), as they were absent in the sterile control.

Identification of DTDP degradation metabolites in cell-free supernatants of cultures from *T. mimigardefordensis* strain DPN7^T. Degradation products of DTDP could be identified as 3MP and 3SP by GC-MS analysis when the data were compared with information provided by the National Institute of Standards and Technology library (Fig. 2). During cultivation of the cells in MSM at 30°C and at 120 rpm, with an incubation time of more than 20 hours, cleavage of DTDP resulted in a decrease of the DTDP concentration and an increase of the 3MP concentration in the supernatant, as revealed by GC analysis (Fig. 1). This was observed although 3MP was also spontaneously converted to DTDP, as revealed in control experiments using 3MP as the sole OSC in MSM. 3SP, in contrast, was detectable in the exponential growth phase; however, it occurred only at low concentrations in cultures of the wild type (Fig. 2).

Isolation and phenotypic characterization of auspicious Tn*5*::*mob*-induced mutants. Transposon mutagenesis of *T. mimigardefordensis* strain DPN7 employing Tn*5*::*mob* was performed to generate mutants for investigation of DTDP catabolism and to identify genes coding for the involved enzymes in this bacterium. Insertion of Tn*5*::*mob* into the genomes of these mutants was confirmed by Southern hybridization using ApaI-digested genomic DNAs isolated from the mutants and the digoxigenin-labeled Km resistance gene of Tn*5* as a probe. Extensive physiological characterizations preceded the mapping of the Tn*5* insertion loci into the genome of each mutant relevant for this study. The genotypic characteristics of these mutants are summarized in Table 2. Eleven mutants (Jhw51c, Jhw90, Jhw101, KK14, KK15, Jhw17, Jhw103, JhwA8/121, Jhw13b, JhwI, and JhwV) exhibited fully impaired growth on MSM containing DTDP as the sole carbon source and were referred to as DTDP-negative mutants. Five mutants (Jhw38, JhwAA14, KK13, JhwIX, and JhwX) exhibited slower growth in the same medium and were therefore referred to as DTDP-leaky mutants. Most mutants grew like the wild type in MSM containing the following carbon sources at appropriate concentrations: acetate, gluconate, glucose, propionate, succinate, 3SP, sulfinoalanine, or taurine. Two exceptions were mutants JhwA8/121 and Jhw38, which exhibited diminished growth with 3SP (3SP-negative and -leaky, respectively). Mutant JhwA8/121 also showed partially impaired growth with sulfinoalanine as the sole carbon and energy source.

Genotypic characterization of Tn*5*::*mob*-induced mutants. Total genomic DNA was isolated from all 16 transposon-in-

TABLE 2. Genotypic characterization of Tn*5*::*mob*-induced mutants of *T. mimigardefordensis* defective in the utilization of DTDP

Mutant	Phenotype regarding DTDP	Insertion locus of Tn*5*::*mob* (gene product)[a]	% Amino acid identity (strain)
Jhw51c	Negative	*lpdA* (dihydrolipoamide dehydrogenase)	79 (*Bordetella pertussis* TohamaI)
Jhw90	Negative	*lpdA* (dihydrolipoamide dehydrogenase)	79 (*Bordetella pertussis* TohamaI)
Jhw101	Negative	*lpdA* (dihydrolipoamide dehydrogenase)	79 (*Bordetella pertussis* TohamaI)
KK14	Negative	*cdo* (cysteine dioxygenase type I)	65 (*Verminephrobacter eiseniae* EF01)
KK15	Negative	*cdo* (cysteine dioxygenase type I)	65 (*Verminephrobacter eiseniae* EF01)
Jhw17	Negative	*cdo* (cysteine dioxygenase type I)	65 (*Verminephrobacter eiseniae* EF01)
Jhw103	Negative	*cdo* (cysteine dioxygenase type I)	65 (*Verminephrobacter eiseniae* EF01)
JhwA8/121	Negative	*sucC* (succinyl-CoA synthetase, beta chain)	93 (*Bordetella pertussis* TohamaI)
Jhw38	Leaky	*sucC** (succinyl-CoA synthetase, beta chain)	93 (*Bordetella pertussis* TohamaI)
JhwAA14	Leaky	*acnB* (putative bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase)	86 (*Burkholderia* sp. strain 383)
Jhw13b	Negative	*acnB* (putative bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase)	86 (*Burkholderia* sp. strain 383)
KK13	Leaky	*lonA* (ATP-dependent Lon protease)	39 (*Bacillus clausii* KSM-K16)
JhwI	Negative	MOSC domain* (sulfur carrier)	44 (*Bordetella bronchiseptica* RB50)
JhwV	Negative	*dnaK* (chaperone protein)	88 (*Bordetella bronchiseptica* RB50)
JhwIX	Leaky	*dnaJ* (molecular chaperone)	80 (*Bordetella avium* 197N)
JhwX	Leaky	*dnaJ* (molecular chaperone)	80 (*Bordetella avium* 197N)

[a] *, closest specified gene identified adjacent to the respective Tn*5*::*mob* insertion locus.

duced DTDP-negative and -leaky mutants. The genomic regions comprising the Tn*5*::*mob* insertions were cloned into *E. coli* by selecting clones conferring Km resistance mediated by the transposon. Subsequently, the sequences directly adjacent to the Tn*5*::*mob* insertions were obtained by employing oligonucleotides hybridizing to Tn*5*. These sequences were then extended toward the upstream and downstream regions of the transposon insertions as described in Materials and Methods. By this method, in several cases identical, overlapping sequences were obtained, thus indicating that some transposon insertions had occurred in closely adjacent regions. The results of these analyses are summarized and presented graphically in Fig. 3, which provides the array of open reading frames (ORFs), including the positions of 13 transposon insertions in four different genomic regions relevant for DTDP catabolism in *T. mimigardefordensis* strain DPN7.

Mapping of the Tn*5*::*mob* insertion in three DTDP-negative mutants (Jhw51c, Jhw90, and Jhw101) revealed disruption of a dihydrolipoamide dehydrogenase gene (sequence similarity of 79% identical amino acids to the E3 component of the pyru-

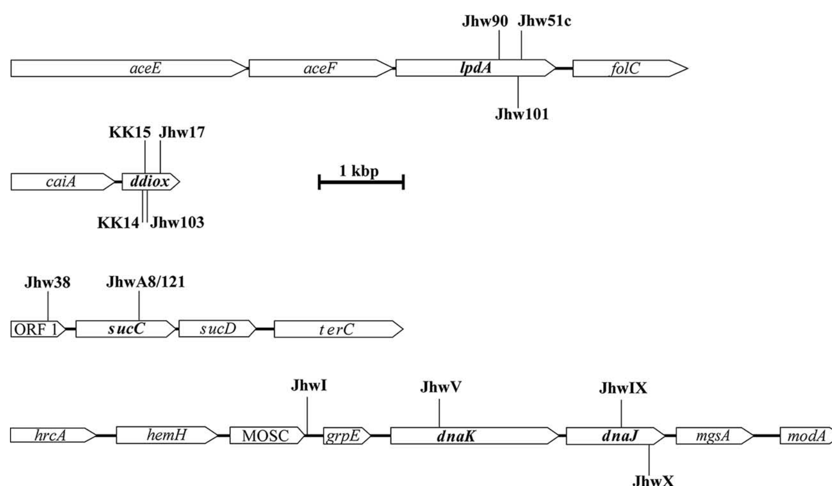

FIG. 3. Tn*5*::*mob* insertions in the genome of *T. mimigardefordensis* strain DPN7 and identification of genes adjacent to the insertion loci. The diagrams show the localization of Tn*5*::*mob* insertions in four regions of the *T. mimigardefordensis* strain DPN7 genome in the 13 independent mutants showing fully or partially impaired growth on DTDP. The positions of Tn*5*::*mob* insertions in the respective mutants (mutant codes are given in Table 2) are indicated. Lengths and directions of arrows showing the genes indicate the proportional lengths and directions of transcription, respectively, of the corresponding genes. Putative identities of the gene products suggested from the amino acid sequence identities to proteins in the GenBank database are as follows: *aceE*, pyruvate dehydrogenase; *aceF*, dihydrolipoamide acetyltransferase; *lpdA*, dihydrolipoamide dehydrogenase; *folC*, folylpolyglutamate synthase/dihydrofolate synthase; *ddiox*, thiol dioxygenase; *caiA*, acyl-CoA dehydrogenase; ORF1, no sequence similarities; *sucC*, succinyl-CoA synthetase, beta chain; *sucD*, succinyl-CoA synthetase, alpha chain; *terC*, tellurium resistance protein; *hrcA*, heat-inducible transcription repressor; *hemH*, ferrochelatase; MOSC, molybdenum cofactor sulfurase, C-terminal end; *grpE*, nucleotide exchange factor; *dnaK*, Hsp70-like chaperone; *dnaJ*, molecular chaperone; *mgsA*, methylglyoxal synthase; and *modA*, molybdate binding protein. Bar, 1,000 base pairs.

vate dehydrogenase complex of *Bordetella pertussis* TohamaI [EC 1.8.1.4]). Dihydrolipoamide dehydrogenases are flavoproteins belonging to the family of disulfide-oxidoreductases (40). These enzymes consist of three domains, including a biotin/lipoyl attachment domain in the amino-terminal region; a pyridine nucleotide-disulfide oxidoreductase domain, which is actually a small NADH binding domain within a larger FAD binding domain (Rossmann fold superfamily); and a dimerization domain of the pyridine nucleotide-disulfide oxidoreductases, found in the carboxy-terminal region of the protein. The Tn*5*::*mob* insertions in the three mutants occurred distal to the NADH binding domain (mutant Jhw90) or between this domain and the dimerization domain (mutants Jhw101 and Jhw51c) (Fig. 3). Furthermore, these mutants grew normally on 3SP as the sole carbon and energy source.

In the genomes of four DTDP-negative mutants (KK14, KK15, Jhw17, and Jhw103), the transposon was localized in a 0.6-kbp ORF (Fig. 3) putatively coding for a type I cysteine dioxygenase (Cdo; EC 1.13.11.20). The translational product of this ORF showed 65% sequence similarity to the Cdo of *Verminephrobacter eiseniae* EF02. Those mutants were unable to utilize DTDP, but they grew on 3SP as the sole source of carbon and energy. Although they did not utilize DTDP, cleavage of this OSC was detected in cultivation experiments in MSM containing DTDP as a cosubstrate and gluconate or succinate as an alternative carbon source. In this experiment, accumulation of 3MP was detected, but at only slightly higher concentrations than those in the wild type. This is most likely due to the fact that under the cultivation conditions applied in this study, two molecules of 3MP are spontaneously oxidized to the dimer, thus yielding one molecule of DTDP. Therefore, accumulation of 3MP to high concentrations could not be detected in the supernatants. A negative effect on the utilization of cysteine or 3MP could not be demonstrated for any of these mutants because the wild type did not utilize either compound as the sole source of carbon and energy.

Amino acid sequences of the putative translational products deduced from the adjacent genomic DNA of the Tn*5* insertion loci in the genomes of two mutants (JhwA8/121 and Jhw38) (Fig. 3) disclosed strong similarities to a gene coding for the β-chain of a succinyl-coenzyme A (succinyl-CoA) synthetase (EC 6.2.1.5). The direct vicinity of the Tn*5*::*mob* insertion in mutant Jhw38 revealed no sequence similarities; instead, the transposon insertion was mapped 298 base pairs upstream of *sucC*. To the corresponding protein of *B. pertussis* TohamaI, 93% amino acid identity was obtained. The mutants exhibited fully (JhwA8/121) or partially (Jhw38) impaired growth with DTDP as the sole carbon source and were also defective in the utilization of 3SP. If cultivated in MSM containing succinate or gluconate as a carbon source in addition to DTDP, mutant JhwA8/121 accumulated >5 mM 3SP in the supernatant, which is a significant amount in comparison to the wild type, which produces barely detectable amounts of this OSC (up to approximately 2 mM).

In one DTDP-negative (Jhw13b) and one DTDP-leaky (JhwAA14) mutant, Tn*5*::*mob* was mapped in a gene encoding a putative bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase (AcnB; EC 4.2.1.3). AcnB dehydrates 2-methylcitric acid to 2-methyl-*cis*-aconitic acid and subsequently hydrates it to 2-methylisocitric acid (5). Cultivation of mutant JhwAA14 in MSM containing 0.6% (wt/vol) succinate and 0.3% (wt/vol) DTDP revealed accumulation of 2-methylcitrate in the supernatant and therefore provided further evidence for an involvement of AcnB in DTDP catabolism.

Furthermore, for five mutants, Tn*5*::*mob* insertions were located in or adjacent to ORFs coding for various proteins involved in the heat shock response (32). In four of these mutants, the transposon was mapped in the chromosomal region between genes encoding the carboxy-terminal region of a molybdenum cofactor sulfurase (MOSC domain) (4) and a methylglyoxal synthase (*mgsA*) (Fig. 3). Two of those mutants exhibited a DTDP-negative phenotype (JhwI and JhwV). The Tn*5* insertion in mutant JhwI was mapped in a noncoding region localized downstream of the MOSC-encoding gene and upstream of *grpE*, which encodes the nucleotide exchange factor GrpE (70). In mutant JhwV, Tn*5* insertion occurred in the Hsp70-like chaperone-encoding gene *dnaK*. In two DTDP-leaky mutants (JhwIX and JhwX), insertions were located downstream of *dnaK*, inside *dnaJ*. Furthermore, in mutant KK13, the transposon disrupted an ORF coding for a putative LonA protein. The latter is an ATP-dependent protease (14) and is involved in intracellular protein degradation as a chaperone (22, 46).

DISCUSSION

Here we report on the first studies to unravel the biodegradation of DTDP by microorganisms. The gram-negative bacterium *T. mimigardefordensis* strain DPN7^T was enriched for this purpose in a previous study (66) and was chosen for this investigation. Based on identified intermediates of degradation, in silico analyses of the Tn*5* insertion loci, and the phenotypes of the mutants, we propose the pathway shown in Fig. 4.

The initial step in the biodegradation of DTDP was predicted to yield two molecules of 3MP (36). Further evidence for this was obtained in this study. First, 3MP formation always accompanied degradation of DTDP (Fig. 1). Second, disruption of an *lpdA* homologue in three independent mutants (Table 2; Fig. 3) resulted in a DTDP-negative phenotype, whereas growth of these mutants on 3SP was not affected. Flavoprotein disulfide reductases exhibited high sequence and structural similarities. These enzymes catalyze the reduction of compounds which are linked by disulfide bonds (65). Therefore, the predicted function of the LpdA homologue in *T. mimigardefordensis* is the symmetric cleavage of DTDP into two molecules of 3MP (Fig. 4).

In four other independent mutants (Table 2), Tn*5*::*mob* was mapped in a *cdo*-homologous putative thiol dioxygenase-encoding gene (*ddiox*) (Fig. 3). Cdos from eukaryotes are well characterized (34), whereas Cdos were detected only recently in prokaryotes (19). The gene product of *ddiox* and homologous enzymes belong to the nonheme Fe^{2+}-dioxygenases and the cupin superfamily. An occurrence of these enzymes was shown for mammalian cells, some yeast species, and a few bacteria (27). Already described thiol dioxygenases catalyze the irreversible oxidation of the OSC sulfhydryl group to a sulfinic acid, i.e., cysteine is oxidized to sulfinoalanine (19) (Fig. 5). Oxidation of cysteamine to hypotaurine by a Cdo homologue was also shown (12, 18), but only in eukaryotic cells until now. 3MP is a hitherto atypical substrate for a Cdo and

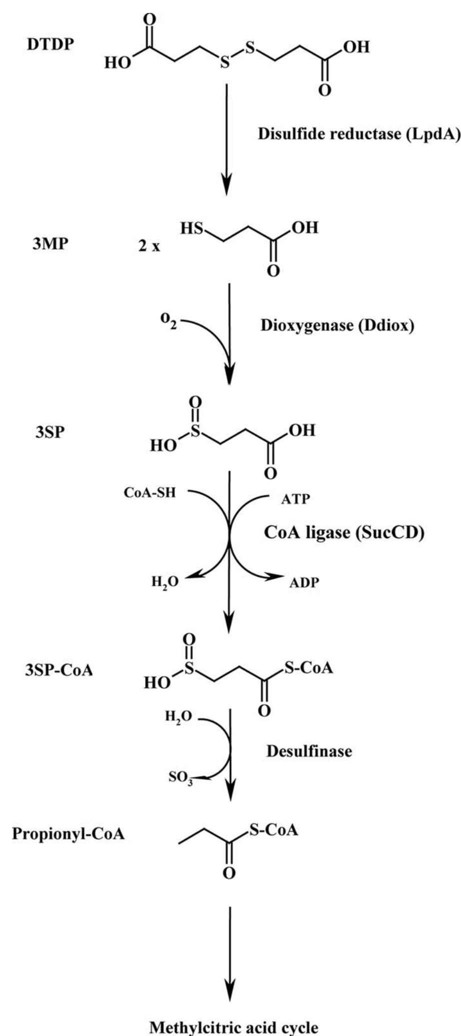

FIG. 4. Putative pathway for degradation of DTDP in *T. mimigardefordensis*. Initially, DTDP is cleaved into two molecules of 3MP, which are then oxygenated by a dioxygenase, thereby yielding 3SP. After linkage to CoA by a thiokinase, the sulfur moiety is putatively removed by a desulfinase, resulting in propionyl-CoA, which is then metabolized via the methylcitric acid cycle toward intermediates of the central metabolism. Whereas most enzyme reactions are based on experimental data and on general predictions of the respective enzymes, the desulfination reaction is hypothetical and is based on theoretical considerations and indirect experimental evidence.

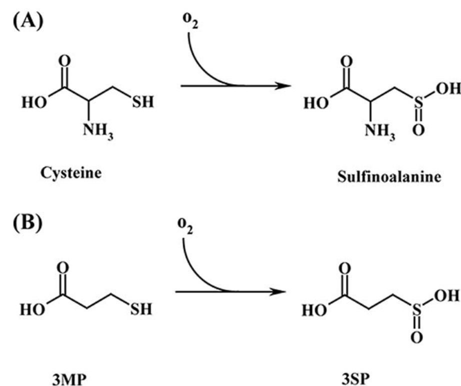

FIG. 5. Reaction schemes of the enzymatic conversions of cysteine into sulfinoalanine by cysteine dioxygenases (EC 1.13.20.11) (A) and of 3MP into 3SP by the 3MP dioxygenase encoded by *ddiox* from *T. mimigardefordensis* (B).

was described only as an inhibitor of Cdo activity without the detection of any oxidation product of 3MP (13, 19). Furthermore, recent studies on the biodegradation of 3,3′-thiodipropionic acid in a different bacterium provide strong evidence for the involvement of a Cdo in the catabolism of 3MP (N. Bruland, J. H. Wübbeler, and A. Steinbüchel, unpublished data). Therefore, conversion of 3MP into 3SP as shown in Fig. 5 is most likely catalyzed by the Cdo-homologous enzyme in strain DPN7^T.

The third proposed step in the catabolism of DTDP is the activation of the resulting 3SP and its ligation to CoA, yielding 3SP-CoA (Fig. 4). In gram-negative bacteria, the holoenzyme of SucCD is a tetramer consisting of two $\alpha\beta$ dimers, which are encoded by *sucC* (β-chain) and *sucD* (α-chain) (9). The bacterial enzyme catalyzes the phosphorylation of ADP to ATP during aerobic metabolism in the citric acid cycle, coupled to cleavage of succinyl-CoA to succinate plus CoA. However, the thiokinase activity in the reverse direction is also relevant, e.g., in anabolism. Therefore, during catabolism of DTDP, the SucCD homologue of *T. mimigardefordensis* strain DPN7^T should exhibit thiokinase activity. This is not unlikely, because 3SP is structurally analogous to succinate; the only difference in the structures between succinate and 3SP is the exchange of the carboxyl group with a sulfinic group.

Further degradation of the resulting 3SP-CoA and the fourth step of DTDP catabolism must be catalyzed by a desulfinase-like enzyme. This step should yield propionyl-CoA (Fig. 4) but is most unknown for *T. mimigardefordensis* because none of the DTDP-negative or -leaky mutants pointed to a gene encoding such an enzyme. The reasons for this could by manifold, and further investigations are necessary to identify the desulfinating enzyme. It is most certain that propionyl-CoA is actually being formed, because in two of the Tn*5*::*mob*-induced mutants the transposon was mapped in a gene coding for AcnB (Table 2). This aconitase is one of the typical enzymes of the 2-methylcitric acid cycle, which catalyzes in many bacteria the conversion of propionyl-CoA to pyruvate (54). Accumulation of significant amounts of 2-methylcitrate in the medium during cultivation of these mutants in MSM containing DTDP plus succinate confirms this conclusion. This also means that the fifth step of DTDP degradation is catalyzed by the 2-methylcitric acid cycle in *T. mimigardefordensis*.

It can be concluded that the organic sulfur compound DTDP is biodegradable and is used as the sole carbon and energy source for growth by a few soil microorganisms. It has to be investigated whether the utilization of this OSC depends on rare functions and low substrate specificities of the described well-known enzymes or whether these enzymes are specifically synthesized for degradation of DTDP. Detailed biochemical studies on the enzymes are currently being done. Unusual sulfur-containing metabolites occurred as intermediates during degradation. The potential inhibitory effects exerted by some of these compounds cause stress to the cells and probably require the presence of various heat shock proteins. Hints for involvement of these chaperones were obtained not only in this study (Table 2; Fig. 3) but also during identification

of putative dimethylsulfoniopropionate degradation genes (11). Metabolites of 3MP were identified as potent inhibitors of β-oxidation (17, 47), but activation is important and necessary for the development of toxicity. In contrast to 3MP, 3SP is not known to occur naturally in the environment, and biochemical or microbial studies including this sulfinic acid are very rare. It was used as an analogous substance to succinate and sulfinoalanine in kinetic and substrate specificity studies of enzymes (20) and in studies about the radioprotective activity of various sulfinic acids, which revealed a certain radioprotective activity property but also toxicity of 3SP (63). Therefore, it is not surprising that chaperones are essentially required for the functionality of some enzymes catalyzing DTDP degradation.

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