Biodegradation of the xenobiotic organic disulphide 4,4'-dithiodibutyric acid by *Rhodococcus erythropolis* strain MI2 and comparison with the microbial utilization of 3,3'-dithiodipropionic acid and 3,3'-thiodipropionic acid

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Application of the non-toxic 3,3'-thiodipropionic acid (TDP) and 3,3'-dithiodipropionic acid (DTDP) as precursors for the microbial production of polythioesters (PTEs), a class of biologically persistent biopolymers containing sulphur in the backbone, was successfully established previously. However, synthesis of PTEs containing 4-mercaptobutyrate (4MB) as building blocks could not be achieved. The very harmful 4MB is not used as a PTE precursor or as the carbon source for growth by any known strain. As a promising alternative, the harmless oxidized disulfide of two molecules of 4MB, 4,4'-dithiodibutyric acid (DTDB), was employed for enrichments of bacterial strains capable of biodegradation. Investigation of novel precursor substrates for PTEs and comparison of respective strains growing on TDP, DTDP and DTDB as sole carbon source was accomplished. A broad variety of bacteria capable of using one of these organic sulphur compounds were isolated and compared. TDP and DTDP were degraded by several strains belonging to different genera, whereas all DTDB-utilizing strains were affiliated to the species *Rhodococcus erythropolis*. Transposon mutagenesis of *R. erythropolis* strain MI2 and screening of 7500 resulting mutants yielded three mutants exhibiting impaired growth on DTDB.

Physiological studies revealed production of volatile hydrogen sulphide and accumulation of significant amounts of 4MB, 4-oxo-4-sulphanylbutanoic acid and succinic acid in the culture supernatants. Based on this knowledge, a putative pathway for degradation of DTDB was proposed: DTDB could be cleaved into two molecules of 4MB, followed by an oxidation yielding 4-oxo-4-sulphanylbutanoic acid. A putative desulphydrase probably catalyses the abstraction of sulphur, thereby generating succinic acid and hydrogen sulphide.

Received 6 November 2009
Revised 29 November 2009
Accepted 2 December 2009

INTRODUCTION

4,4'-Dithiodibutyric acid (DTDB), 3,3'-dithiodipropionic acid (DTDP) and 3,3'-thiodipropionic acid (TDP) are deployed in many different areas of research (Appel *et al.*, 1995; Codognato *et al.*, 2007; Jang & Keng, 2006; Kanayama & Kitano, 2000; Saxena & Gupta, 1984; Scott, 1968; Tsutsumi *et al.*, 1998; Tuan & Phillips, 1997). However, the biotechnological significance of these organic sulphur compounds (OSCs) is their application in studies for the improvement of polythioester (PTE) production (Lütke-Eversloh & Steinbüchel, 2003). PTEs are micro-

bially synthesized, non-biodegradable polymers containing sulphur in the thioester linkages of the backbone (Kim *et al.*, 2005; Lütke-Eversloh *et al.*, 2002a). They accumulate as hydrophobic inclusions in the cytoplasm of bacterial cells in a manner similar to polyhydroxyalkanoic acids (PHAs) (Lütke-Eversloh & Steinbüchel, 2004). PHAs and PTEs are industrially and scientifically interesting materials, not only because they possess some unique characteristics regarding their biodegradability, microstructure, thermal behaviour and diverse mechanical properties, but also because many of them can be produced from non-petrochemical resources (Steinbüchel, 2001).

Initially, PTE heteropolymers were detected in *Ralstonia eutropha* (Lütke-Eversloh *et al.*, 2001a). This Gram-negative bacterium naturally accumulates PHAs at levels of up to more than 90 % of the cell dry matter (Pedrós-Alio

Abbreviations: DTDB, 4,4'-dithiodibutyric acid; DTDP, 3,3'-dithiodipropionic acid; 4MB, 4-mercaptobutyric acid; 3MP, 3-mercaptopropionic acid; OSC, organic sulphur compound; PHA, polyhydroxyalkanoic acid; PTE, polythioester; 3SP, 3-sulphinopropionic acid; TBL, γ -thiobutyrolactone; TDP, 3,3'-thiodipropionic acid.

et al., 1985; Schlegel *et al.*, 1961a), and it synthesizes various PTE heteropolymers, employing its unspecific PHA synthase if 3-mercaptopropionic acid (3MP), 3-mercaptoputyric acid (3MB), 3-mercaptopaleric acid (3MV), TDP or DTDP is provided as a precursor in addition to a second compound that serves as a carbon and energy source (Lütke-Eversloh & Steinbüchel, 2003; Lütke-Eversloh *et al.*, 2001a, b, 2002a). Later, PTE homopolymers consisting of 3MP, 3MB or 3MV were synthesized in a recombinant strain of *Escherichia coli* expressing a non-natural pathway (Lütke-Eversloh *et al.*, 2002b; Thakor *et al.*, 2005), if the respective OSC was provided as precursor. The use of DTDB or 4-mercaptoputyric acid (4MB) should yield PTE with 4MB building blocks possessing hitherto unknown and probably interesting properties. However, even though various strategies for biosynthesis have been applied, production of 4MB-containing PTE has not yet been successful (Lütke-Eversloh & Steinbüchel, 2003).

Although most of these precursor substrates are structural analogues of common cell metabolites (for a comparison see Fig. 1), it became obvious from previous studies (Bruland *et al.*, 2009a, b; Wübbeler *et al.*, 2006) that degradation was restricted to only few bacteria. Also, both established PTE-producing strains were not able to use any of the OSCs mentioned above as the sole carbon and energy source for growth. Furthermore, at present it is not possible to synthesize PTEs from simple carbon and inorganic sulphur sources. Thus, the establishment of microbial synthesis processes, independently of toxic and often expensive OSCs, by metabolic engineering of suitable micro-organisms is desirable. Prior to such a strain optimization, it was essential to obtain bacteria with the capacity to utilize precursors of PTE as the sole carbon source, to understand the catabolism of the respective OSC.

Extensive analyses have recently been accomplished to elucidate the catabolic pathways of TDP and DTDP in *Variovorax paradoxus* strain TBEA6 and *Advenella mimi-gardefordensis* strain DPN7^T, respectively. Transposon mutagenesis employing the suicide plasmid technique described by Simon *et al.* (1983), as an established method

to elucidate unknown metabolic pathways, can successfully be applied to both strains (Bruland *et al.* 2009a; Wübbeler *et al.* 2008). The putative pathway of microbial utilization of TDP with hitherto unknown and interesting enzyme reactions has recently been proposed (Bruland *et al.*, 2009a). In the first step, the thioether is cleaved by a yet unidentified enzyme into 3-hydroxypropionate and 3MP. The latter is then sulfoxxygenated by a novel cysteine dioxygenase homologue referred to as 3MP dioxygenase, yielding 3-sulphinopropionate (3SP). After linkage to coenzyme A (CoA) by a family III acyl-CoA transferase, the sulphur moiety is probably removed by a desulphinase, yielding propionyl-CoA, which is then further metabolized, most likely via the methylmalonyl-CoA pathway.

Catabolism of DTDP is initiated by a symmetrical cleavage of DTDP into two molecules of 3MP by a disulphide reductase (J. H. Wübbeler and others, unpublished results). As in TDP degradation (see above), a 3MP dioxygenase converts the sulphhydryl group of 3MP into a sulphinic group, thereby yielding 3SP. A thiokinase then activates 3SP by ligating CoA with 3SP to give 3SP-CoA (M. Schürmann and others, unpublished results). Subsequently, the necessary elimination of sulphite from 3SP-CoA probably requires three enzymes catalysing yet unconfirmed conversions to yield propionyl-CoA, which is then further metabolized via the 2-methylcitric acid pathway (Wübbeler *et al.*, 2008).

DTDB is evidently the oxidized form of two 4MB molecules (Fig. 1e). It is far less toxic and, in direct comparison with its symmetrical cleavage compound 4MB, odourless, and also much cheaper. 4MB is hardly available as commercial product on the market. Therefore, it was of particular interest for the present study to gain preliminary insights into the metabolism of this OSC; especially important to investigate was the overall biodegradability of DTDB by cultivable micro-organisms and the occurrence of 4MB during degradation. Since it was predicted that 4MB is a valuable precursor substrate for the biotechnological production of novel PTEs, the elucidation of the hitherto unknown DTDB, and consequently 4MB, catabolic pathways in bacteria would be helpful to improve

Fig. 1. Comparison of the chemical structures of the OSCs relevant to this study with those of the respective analogous cell intermediates. (a) TDP, (b) cystathionine, (c) DTDP, (d) cystine, (e) DTDB, (f) homocystine.

and establish PTE synthesis to produce polymers containing 4MB building blocks. The use of DTDB as the precursor substrate could be advantageous to establish PTE production in bacterial strains showing a certain susceptibility to the toxic thiol 4MB. Furthermore, a higher 4MB content in such a PTE might be achieved by heterologous expression of DTDB-cleaving enzymes in engineered recombinant strains.

METHODS

Sulphur-containing chemicals. DTDP and TDP were purchased from Acros Organics. DTDB was purchased from Sigma–Aldrich Chemie.

Growth conditions and storage of bacterial strains. All available bacterial strains investigated for their ability to utilize the PTE precursors as the sole source of carbon and energy are shown in Table 1, whereas the characterized natural isolates are listed in Table 2. Strains were maintained either on 0.8 % (w/v) nutrient broth (NB) and Luria–Bertani (LB) medium (Sambrook *et al.*, 1989), or on mineral salts medium (MSM) (Schlegel *et al.*, 1961b) at 30 °C containing the appropriate carbon source indicated in the text. Carbon sources were prepared as filter-sterilized 20 % (w/v) stock solutions and adjusted to pH 7.0. Solid media contained 1.8 % (w/v) purified agar-agar. Long-term storage was accomplished in screw-capped vials at –70 °C as glycerol (20 %, v/v) stocks or as lyophilizates.

Enrichment and isolation of mesophilic natural isolates capable of utilizing the respective OSC as the sole source of carbon and energy. Natural isolates catabolizing the OSCs

investigated and compared in this study were obtained from 250 ml flasks filled with 50 ml sterile MSM (with an initial pH of 6.9) and 1 % (w/v or v/v) of inoculum, which were taken from many different polluted locations. The OSCs were added as carbon source at concentrations between 0.2 and 1.0 % (v/v). The flasks were shaken for several days at 30 °C before aliquots were plated on solid MSM agar plates containing the respective OSC as the sole carbon source. After up to 7 days of incubation, colony material from these plates was repeatedly transferred to fresh plates until axenic cultures were achieved.

The verified available strains and isolates obtained, in association with their geographical origins and relevant phenotypes, are listed in Tables 1 and 2, respectively.

Biochemical characterization of natural isolates. Gram staining, the L-alanyl-aminopeptidase test, the potassium hydroxide test, as well as catalase and oxidase tests were performed according to Gerhardt *et al.* (1994). The susceptibility of the strains towards antibiotics was determined on complex media for the following antibiotics and concentrations: 150 µg ml^{–1} ampicillin (Ap), 50 µg ml^{–1} chloramphenicol (Cm), 10 µg ml^{–1} gentamicin (Gm), 100 µg ml^{–1} kanamycin (Km), 200 µg ml^{–1} penicillin (Pn), 100 µg ml^{–1} streptomycin (Sm), 12.5 µg ml^{–1} tetracycline (Tc) and 25 µg ml^{–1} thiostrepton (Ts). API 20NE and 20E tests (bioMérieux) were performed to determine the assimilation of a variety of carbon sources and the presence of enzymes for taxonomic affiliation. The capacity to utilize different carbon sources, which is not included in the API tests, was investigated on MSM agar plates containing 0.1–1.0 % (w/v) of the respective carbon source.

Detection of inorganic sulphur compounds. Sulphate and sulphite contents in culture supernatants were determined with test strips (Merck), based on either a thorin–barium complex (sulphate detection) or a mixture of potassium hexacyanoferrate(II), zinc

Table 1. Bacterial strains from culture collections with proven inability to utilize DTDB, DTDP and TDP as the sole source of carbon and energy

Abbreviations: ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; ^T, type strain

Strain	Uncommon feature or relevant phenotype	Source
<i>Acinetobacter calcoaceticus</i>	Produces isoprene	DSM 30006 ^T
<i>Bacillus cereus</i>	Produces restriction endonuclease Bce 14579	DSM 31 ^T
<i>Bacillus megaterium</i>	Hydroxylates <i>N,N</i> -dimethylaniline	DSM 32 ^T
<i>Bacillus subtilis</i>	Produces bacilysin and fengymycin	DSM 10 ^T
<i>Corynebacterium glutamicum</i>	Produces L-glutamic acid	DSM 20300 ^T
<i>Gordonia amicalis</i> IEGM	Dibenzothiophene-desulphurizing organism	DSM 44461 ^T
<i>Gordonia desulfuricans</i> 213E	Benzothiophene-desulphurizing organism	DSM 44462 ^T
<i>Gordonia nitida</i> LE31	Degrades 3-ethylpyridine and 3-methylpyridine	DSM 44499 ^T
<i>Gordonia rubripertincta</i>	Produces mycobactin	DSM 43197 ^T
<i>Gordonia westfalica</i> Kb1	Poly(<i>cis</i> -1,4-isoprene)-degrading organism	DSM 44215 ^T
<i>Mycobacterium smegmatis</i> mc ² 155	Transformation efficient mutant	ATCC 700084
<i>Pseudomonas putida</i>	Degrades aromatic compounds	DSM 291 ^T
<i>Ralstonia eutropha</i> H16	Produces polyhydroxybutyrate; extensively used in studies of chemolithotrophic growth and hydrogenase activity	DSM 428
<i>Ralstonia metallidurans</i> CH34	Multiple resistance to heavy metals	DSM 2839
<i>Rhodococcus erythropolis</i>	Produces cholesterol oxidase and 3-keto Delta ^{1,4} steroids. Converts phenol and naphthalene	DSM 43066 ^T
<i>Rhodococcus erythropolis</i>	Produces aromatic steroids	DSM 43297
<i>Rhodococcus opacus</i> PD630	Accumulates triacylglycerol; degrades hydrocarbons	DSM 44193
<i>Streptomyces coelicolor</i> A3(2)	Grows on <i>p</i> -hydroxybenzoate	DSM 40783

Table 2. Bacterial isolates from this and previous studies capable of using the indicated OSC as sole source of carbon and energy

Abbreviations: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Pn, penicillin; Sm, streptomycin; Tc, tetracycline; Ts, thiostrepton.

Strain designation	OSC utilized	Source of sample	Susceptible to the following antibiotics	Source or reference
<i>R. erythropolis</i> DBR3	DTDB	Matured compost	Sm, Ts	This study
<i>R. erythropolis</i> DBR4	DTDB	Matured compost	Gm, Sm, Ts	This study
<i>R. erythropolis</i> DBRW	DTDB	Matured compost	Gm, Sm, Ts	This study
<i>R. erythropolis</i> DBRQ	DTDB	Matured compost	Sm, Ts	This study
<i>R. erythropolis</i> MI2	DTDB	Oil-contaminated material from an absorber in a garage	Cm, Ts	This study
<i>R. erythropolis</i> E6	DTDB	Soil sample of an inner courtyard	Cm, Ts	This study
<i>R. erythropolis</i> BEA1	DTDP	Soil sample in vicinity of a storage site for waste thiochemicals	Ap, Cm, Gm, Km, Pn, Sm, Tc, Ts	This study
<i>R. erythropolis</i> BEA2	DTDP	Soil sample in vicinity of a storage site for waste thiochemicals	Ap, Cm, Gm, Km, Pn, Sm, Tc, Ts	This study
<i>R. erythropolis</i> BEA3	DTDP	Soil sample in vicinity of a storage site for waste thiochemicals	Ap, Cm, Gm, Pn, Sm, Tc, Ts	This study
<i>R. erythropolis</i> BEA4	DTDP	Soil sample in vicinity of a storage site for waste thiochemicals	Ap, Cm, Gm, Km, Pn, Sm, Tc, Ts	This study
<i>Cupriavidus taiwanensis</i> BEA5c	DTDP	Soil sample in vicinity of a storage site for waste thiochemicals	Ap, Cm, Pn, Sm, Tc	This study
<i>Pseudomonas</i> sp. strain MT-I1	DTDP	Sewage sludge	Gm, Km, Sm	Toups <i>et al.</i> (2009)
<i>Bordetella</i> sp. strain MT-I2	DTDP	Sewage sludge	Ap, Cm, Km	Toups <i>et al.</i> (2009)
<i>Pseudomonas</i> sp. strain MT-I3	DTDP	Sewage sludge	Cm, Sm	Toups <i>et al.</i> (2009)
<i>Tetrathobacter (Advenella) mimigardefordensis</i>	DTDP	Matured compost after the sieving process	Ap, Gm, Km, Pn, Sm, Tc	Wübbeler <i>et al.</i> (2006)
<i>Variovorax paradoxus</i> SFWT	TDP	Sediments of a fresh water tank	Cm	Bruland <i>et al.</i> (2009a)
<i>Variovorax paradoxus</i> TBEA6	TDP	Soil sample in vicinity of a storage site for waste thiochemicals	Cm, Km, Tc, Ts	Bruland <i>et al.</i> (2009a)
<i>P. soli</i> TBEA3	TDP	Soil sample in vicinity of a storage site for waste thiochemicals	Cm, Km, Tc, Ts	Bruland <i>et al.</i> (2009a, b)
<i>Schlegelella thermodepolymerans</i>	TDP	Activated sludge	Ap, Cm, Km, Sm, Tc	Elbanna <i>et al.</i> (2003)

sulphate and sodium nitroprusside (sulphite detection). Detection of volatile hydrogen sulphide production by micro-organisms was accomplished with lead acetate test strips (Fluka). Solubilized hydrogen sulphide was determined with the Visicolor Eco sulphide test kit (Macherey–Nagel) based on *N,N'*-dimethyl-1,4-phenylene diamine, which is converted in two steps into methylene blue, if hydrogen sulphide is present. Application of the analytical test strips was performed according to the manufacturers' instructions.

Isolation, manipulation and transfer of DNA. Chromosomal DNA was isolated as described by Marmur (1961). Plasmid DNA was isolated by the method of Birnboim & Doly (1979). Restriction enzymes and ligases were used according to the instructions of the manufacturers. Competent cells of *E. coli* were prepared by the CaCl_2 procedure and transformed with 16S rDNA ligated into plasmid pGEM-T Easy (Promega) (Hanahan, 1983).

Electroporation of *Rhodococcus erythropolis* strain MI2 was accomplished based on the methods described by Desomer *et al.* (1990) and Kalscheuer *et al.* (1999).

PCR. Amplifications of genomic DNA were accomplished according to the procedures described by Innis *et al.* (1990).

Sequencing of 16S rRNA genes. PCR amplifications of the 16S rRNA genes were carried out using the oligonucleotides 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1525r (5'-AGAAAGGAGGT-GATCCAGCC-3') as primers, which are complementary to conserved regions of the 16S rRNA gene of *E. coli*. The PCR products were purified using the NucleotrapCR extraction kit (Macherey–Nagel) and, if necessary, ligated into the cloning vector pGEM-T Easy (Promega) prior to sequencing.

The sequences of the 16S rRNA genes were determined by the chain-termination method (Sanger *et al.*, 1977), applying a SequiTherm long-read cycle sequencing kit (Epicentre Biotechnologies) and IRD800-labelled oligonucleotides (MWG Biotech). Sequence reactions were accomplished by using a GeneReadIR 4200 DNA analyser (LI-COR). The following oligonucleotides were used as primers: 27f, 343r (5'-CTGCTGCCTCCCGTA-3'), 357f (5'-TACGGGAGGC-AGCAG-3'), 519r [5'-G(T/A)-ATTACCGCGGC(T/G)GCTG-3'], 536f [5'-CAGC(C/A)GCCGCGGTAAT(T/A)C-3'], 803f (5'-ATT-AGATACCCTGGTAG-3'), 907r (5'-CCGTCATTCATTTGAGTTT-3'), 1114f (5'-GCAACGAGCGCAACCC-3'), 1385r [5'-CGGTGT-GT(A/G)CAAGGCC-3'] and 1525r, as well as the M13 universal primer (5'-GTAAACGACGGCCAGT-3') and the M13 reverse primer (5'-CAGGAAACAGCTATGAC-3'), which hybridize to

pGEM-T Easy DNA. A consensus sequence was created using the software BioEdit Sequence Alignment Editor (version 7.0.5.3). 16S rRNA gene sequences were analysed using BLAST (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov>) by running the BLASTN program (Altschul *et al.*, 1997).

Identification of degradation intermediates via GC/MS analyses. The compositions of the cell-free supernatants of cultures and of the standards of important OSCs were determined upon methylation after lyophilization in the presence of 15% (v/v) sulphuric acid (H₂SO₄) by gas chromatographic analysis of the resulting methyl esters, as described previously (Brandl *et al.*, 1988; Timm *et al.*, 1990).

Transposon mutagenesis of *R. erythropolis* MI2. For transposon mutagenesis, the suicide vectors pFAJ2571 and pFAJ2527 (Nagy *et al.*, 1997) were transferred into the chloramphenicol-susceptible isolate *R. erythropolis* MI2 by electroporation. Transposon-induced mutants were selected on MSM agar plates containing 50 µg chloramphenicol ml⁻¹ (MSM^{Cm}) and 0.2% (w/v) sodium gluconate (master plates). Putative mutants were transferred in a coordinated pattern to MSM^{Cm} agar plates containing 0.2% (w/v) DTDB (selection plates) and corresponding master plates for further analysis.

RESULTS

Utilization of DTDB, DTDP or TDP by available bacteria

To examine different bacterial strains (Table 1) for their ability to utilize DTDB, DTDP or TDP as the sole source of carbon and energy, growth experiments were conducted with solid MSM containing 0.2% (w/v) of the respective OSCs. However, none of the strains listed in Table 1 was able to grow on any of the three OSCs as the sole carbon and energy source.

Isolation and characterization of bacterial strains utilizing DTDB, DTDP or TDP as sole source of carbon and energy

Isolation and characterization of some DTDP- and TDP-utilizing bacteria have been previously reported (Bruland *et al.* 2009a, b; Toups *et al.*, 2009; Wübbeler *et al.* 2006); the same enrichment procedure was employed during this study to isolate novel bacterial strains capable of using DTDB or DTDP as the sole source of carbon and energy. Enrichment cultures were done under mesophilic and aerobic conditions in MSM (Schlegel *et al.*, 1961b), and were inoculated with diverse samples from polluted or sulphur-containing soils and which contained one of the OSCs mentioned above. These enrichment cultures yielded many bacterial isolates exhibiting morphologically different colonies on OSC-containing MSM agar plates. The evaluation of API test strips and of 16S rRNA gene sequences of initially interesting strains revealed high similarities of the isolates to strains of species belonging to the genera *Alcaligenes*, *Bordetella*, *Cupriavidus*, *Klebsiella* and *Rhodococcus* (Table 2). We focused on the most promising isolates regarding growth

rate, susceptibility to antibiotics (Table 2) and predicted non-pathogenicity to humans. Isolates affiliated into genera with human pathogen species, such as *Alcaligenes*, *Bordetella* and *Klebsiella*, were not considered further. The other isolates were later characterized in more detail and are presented in Table 2. Isolates from very recent studies also comprising members of new taxa, such as *A. mimigardefordensis* strain DPN7^T (Gibello *et al.*, 2009; Wübbeler *et al.*, 2006) and *Pseudorhodoferrax soli* strain TBEA3 (Bruland *et al.*, 2009b), were also investigated (Table 2).

All isolates listed in Table 2 used acetate, propionate and gluconate as the sole carbon and energy source for growth. They all, except strain BEA3, also grew with butyrate. Fructose was not utilized by strain DPN7^T; strains SFWT, TBEA3^T, TBEA6 and DBR3 showed no growth on glucose. None of the strains listed in Table 2 grew with dibutylsulphide, ethanol, 2-mercaptopropionate, 3MP, 4-mercapto-1-butanol or γ -thiobutyrolactone (TBL) as the sole carbon and energy source for growth.

Interestingly, none of these OSC-utilizing isolates was able to use either of the two other OSCs investigated in this study as a carbon source for growth. For example, no DTDB-utilizing isolate was able to grow on the close structural analogue DTDP (Fig. 1c) or on TDP (Fig. 1a).

Isolates able to utilize TDP or DTDP as the sole carbon and energy source

V. paradoxus strain TBEA6, *V. paradoxus* strain SFWT and the recently classified *P. soli* (Bruland *et al.*, 2009a, b) are, besides *Schlegelella thermodepolymerans* (Elbanna *et al.*, 2003), the only known characterized strains using TDP as the sole source of carbon and energy (Table 2).

The degradation and utilization of DTDP are on the one hand accomplished by four closely related Gram-positive bacteria, which were all affiliated to the species *R. erythropolis* (strains BEA1, BEA2, BEA3 and BEA4). On the other hand, five Gram-negative strains with the capability to grow with DTDP as a carbon source have been characterized: two strains of the genus *Pseudomonas*, one *Bordetella* species (Toups *et al.*, 2009), and two further Gram-negative bacteria affiliated as *Cupriavidus taiwanensis* strain BEA5c or *A. mimigardefordensis* strain DPN7^T (Wübbeler *et al.*, 2006) (Table 2).

Biodegradation of DTDB

This study reports for the first time, to our knowledge, the microbial utilization of DTDB. Apparently, DTDB is used as the sole source of carbon and energy exclusively by some novel isolated strains of *R. erythropolis* (Table 2). No representatives of other genera were obtained under the enrichment conditions employed in this study. Growth of these Gram-positive natural isolates was initially observed on solidified MSM agar plates and was then confirmed in

liquid MSM, where an increase in turbidity was accompanied by a concomitant decrease in DTDB, as shown with *R. erythropolis* strain MI2 as an example in Fig. 2(a).

Controls (cultures without inoculum or without a carbon source) did not show any decrease in the DTDB concentration or increase of the optical density, respectively

Fig. 2. Degradation of DTDB by *R. erythropolis* strain MI2 and by the DTDB-negative mutant 1.7/17. Cells were cultivated in 1 l flasks without baffles on a rotary shaker at 30 °C and 120 r.p.m. in MSM containing either 24 mM DTDB as the sole carbon source or 24 mM DTDB in addition to 0.5 % (w/v) sodium gluconate. Analyses of the cell-free supernatants were done by GC and GC/MS. Because 4-oxo-4-sulphanylbutanoic acid and 4MB are not commercially available, they could not be applied as controls, standards or in calibrations. Therefore, detection of these compounds is shown as 'peak area', depending on the respective signal intensity. (a) Utilization of DTDB and increase of turbidity. Symbols: ◇, consumption of DTDB as the sole carbon source by *R. erythropolis* strain MI2; □, consumption of DTDB by *R. erythropolis* strain MI2 during cultivation with sodium gluconate as a second carbon source; △, consumption of DTDB by mutant 1.7/17 during cultivation with sodium gluconate as a second carbon source; ◆, increase of turbidity in cultures of *R. erythropolis* strain MI2 during growth with DTDB as the sole carbon source; ■, increase of turbidity in cultures of *R. erythropolis* strain MI2 during growth with DTDB and sodium gluconate as carbon sources; ▲, increase of turbidity in cultures of mutant 1.7/17 during growth with DTDB and sodium gluconate as carbon sources. (b) Detection of the degradation intermediates 4MB and succinic acid. Symbols: ◇, concentration of 4MB during degradation of DTDB as the sole carbon source by cells of *R. erythropolis* strain MI2; □, concentration of 4MB during degradation of DTDB in addition to sodium gluconate as a second carbon source by cells of *R. erythropolis* strain MI2; △, concentration of 4MB during degradation of DTDB in addition to sodium gluconate as a second carbon source by cells of mutant 1.7/17; ◆, concentration of succinic acid during degradation of DTDB as sole carbon source by cells of *R. erythropolis* strain MI2; ■, concentration of succinic acid during degradation of DTDB and sodium gluconate as carbon sources by cells of *R. erythropolis* strain MI2; ▲, concentration of succinic acid during degradation of DTDB and sodium gluconate as carbon sources by cells of mutant 1.7/17. (c) Subsequent analyses of cell-free supernatants of *R. erythropolis* strain MI2 and mutant 1.7/17 in MSM with 24 mM DTDB as the sole carbon source after preincubation for 48 h in MSM with 0.5 % (w/v) sodium gluconate. Symbols: ◇, consumption of DTDB by *R. erythropolis* strain MI2; △, consumption of DTDB by mutant 1.7/17; ◆, 4MB, *R. erythropolis* strain MI2; ▲, concentration of 4MB, mutant 1.7/17; ○, concentration of succinic acid, mutant 1.7/17; ●, concentration of 4-oxo-4-sulphanylbutanoic acid, mutant 1.7/17.

(data not shown). The following experiments were accomplished to unravel the catabolism of DTDB in *R. erythropolis* strain MI2.

Isolation and phenotypic characterization of transposon-induced mutants of *R. erythropolis* MI2 impaired in DTDB degradation

All further studies were done with *R. erythropolis* strain MI2, because of the strain's stable susceptibility to chloramphenicol. Therefore, this isolate was subjected to transposon mutagenesis employing the suicide vector plasmids pFAJ2571 and pFAJ2572 (Nagy *et al.*, 1997), conferring

chloramphenicol resistance to the recipient. This mutagenesis aimed at the generation of mutants with defective growth on DTDB to investigate the DTDB catabolism. Insertions of Tn5561X1 and Tn5561X2 into the genomes of these mutants were confirmed by PCR using primers hybridizing to *istB* (forward primer) and *cmr* (reverse primer). Genomic DNA of the wild-type was used as negative control.

Among 7500 analysed mutants, three were identified with a defect in the utilization of DTDB. Two mutants (designated 1.7/17 and 2.3/36) exhibited fully impaired growth on MSM agar plates containing DTDB as the sole carbon source, and were referred to as DTDB-negative mutants. One mutant (designated 1.5/45) exhibited slower growth on the same plates,

Fig. 3. Utilization of DTDB and sodium gluconate by the wild-type and transposon-induced mutants of *R. erythropolis* strain MI2 affected in growth on DTDB. Cells were cultivated in 50 ml Klett flasks with baffles on a rotary shaker at 30 °C and 120 r.p.m. in liquid MSM containing 0.2 % (w/v) DTDB or 0.2 % (w/v) sodium gluconate, respectively, as the sole carbon sources. Symbols: ■, wild-type, gluconate; □, wild-type, DTDB; ●, mutant 2.3/36, gluconate; ○, mutant 2.3/36, DTDB; ▲, mutant 1.7/17, gluconate; △, mutant 1.7/17, DTDB; *, mutant 1.5/45, gluconate; ×, mutant 1.5/45, DTDB.

and was therefore referred to as a DTDB-leaky mutant. All three mutants grew otherwise like the wild-type. The growth behaviour of these mutants and the wild-type on liquid MSM with sodium gluconate and DTDB is compared in Fig. 3.

Identification of DTDB degradation metabolites in cultures of the wild-type and of the three DTDB-defective mutants of *R. erythropolis* strain MI2

GC/MS analysis of the lyophilized cell-free supernatant obtained from wild-type cultures of *R. erythropolis* strain MI2 identified, in addition to DTDB, the predicted symmetrical cleavage compound 4MB and succinic acid as discharged degradation intermediates of DTDB catabolism (Fig. 2a, b). Furthermore, no increase of sulphite or sulphate was detected in any of the cultures; instead, sulphur in excess of its need as a sulphur source was released as volatile hydrogen sulphide, detected by the occurrence of a black precipitate on the test strips due to the conversion of lead(II) acetate into lead(II) sulphide. In contrast, no hydrogen sulphide could be detected in the media of DTDB cultures of *R. erythropolis* strain MI2 at any

time; therefore, the concentration of solubilized hydrogen sulphide in the medium was always below 3 µM (the lower limit of detection). Volatile or solubilized hydrogen sulphide could not be detected in cultures of this bacterium utilizing gluconate as the sole carbon source.

To identify intermediates that accumulate during catabolism of DTDB, the three mutants and the wild-type of *R. erythropolis* strain MI2 were cultivated in MSM containing 0.5 % (w/v) sodium gluconate as utilizable carbon source; 24 mM DTDB was added to each culture after 24 h of incubation. Samples were withdrawn every day, and the cell-free supernatant obtained from each culture was analysed by GC/MS. Within 4 days of cultivation, the wild-type and the DTDB-leaky mutant 1.5/45 consumed all available DTDB (data not shown). The easily detectable 4MB, which is most likely the product of symmetrical cleavage of DTDB, was identified as an abundant intermediate in the supernatants of the wild-type (Fig. 4b), of the DTDB-negative mutant 1.7/17 (Fig. 4a) and of the DTDB-leaky mutant 1.5/45 (data not shown). Moreover, succinic acid (Fig. 4) and, in very low

Fig. 4. GC/MS analyses of DTDB-degradation intermediates. Chromatograms of the wild-type and of mutant 1.7/17 of *R. erythropolis* strain MI2 are compared. Cells were cultivated in 250 ml flasks without baffles on a rotary shaker at 30 °C and 120 r.p.m. in MSM containing 0.5 % (w/v) sodium gluconate; after 24 h of cultivation, 24 mM DTDB was added. (a) Cell-free supernatant of mutant 1.7/17, and (b) cell-free supernatant of the wild-type; both were incubated for 48 h after inoculation. Besides DTDB, the intermediates 4MB, 4-oxo-4-sulphanylbutanoic acid and succinic acid were separated in the respective chromatograms and identified.

concentrations, also fumaric acid (not highlighted in Fig. 4) were detected. The analysis of the DTDB-negative mutant 2.3/36 revealed neither a detectable decrease in the DTDB concentration in the supernatant nor any degradation intermediates (data not shown). In contrast, the DTDB-negative mutant 1.7/17 depleted the DTDB concentration down to 2 mM within 7 days of incubation, whereas succinic acid was accumulated and could be detected at a high concentration (up to 21.6 mM) in the medium. Furthermore, considerable amounts of an unexpected OSC could be observed in the culture supernatant; it was identified as 4-oxo-4-sulphanylbutanoic acid by GC/MS analyses (Fig. 4a).

Cell pellet analysis of all four strains revealed barely detectable amounts of 4MB as the only identifiable intermediate.

Comparison of *R. erythropolis* strain MI2 and mutant 1.7/17 with respect to growth behaviour and degradation intermediates

Well-established precultures incubated in MSM containing 0.5 % (w/v) sodium gluconate were used to inoculate the main cultures of both strains. Growth behaviour in MSM containing 24 mM DTDB and 0.5 % (w/v) sodium gluconate was compared also with that of the wild-type cultivated in MSM containing 24 mM DTDB as the sole carbon source, and is presented in Fig. 2(a). In less than 60 h, both wild-type cultures degraded all available DTDB, thereby generating and discharging significant amounts of 4MB (Fig. 2b). In the supernatant of the wild-type culture, containing sodium gluconate as a second carbon source, no succinic acid was detectable (Fig. 2b). Despite utilization of 7.4 mM DTDB, growth of mutant 1.7/17 was weak; the turbidity increased up to an OD₆₀₀ of only 0.4 (Fig. 2a), presumably due to the inhibitory effects of the accumulation of DTDB-degradation intermediates. However, 4MB was detectable and the accumulation of succinic acid in the supernatant could be verified (Fig. 2b). Surprisingly, no 4-oxo-4-sulphanylbutanoic acid was detectable under these cultivation conditions, although solubilized hydrogen sulphide at a concentration of up to 12 µM (after 18 h of incubation) was detected in the culture supernatant. Volatile hydrogen sulphide was always produced by *R. erythropolis* strain MI2 and mutants if DTDB was used as a carbon source.

An additional modified cultivation variant was applied to the wild-type and mutant 1.7/17. Cells of both strains were preincubated in MSM with 0.5 % (w/v) sodium gluconate for 48 h and then transferred into fresh media containing 24 mM DTDB as the sole carbon source. The wild-type utilized 24 mM DTDB within approximately 40 h (Fig. 2c). 4MB was detected during degradation, but neither succinic acid nor hydrogen sulphide was detectable in the supernatant. Mutant 1.7/17 degraded 14.4 mM DTDB within 108 h, and produced easily detectable amounts of 4MB and 4-oxo-4-sulphanylbutanoic acid in the supernatant during this period (Fig. 2c). Accumulation of hydrogen

sulphide in the supernatant was detected only during the first 24 h of incubation, with a maximum of 18 µM after 20 h. Subsequently, the concentration of succinic acid increased steadily (Fig. 2c).

Cleavage of DTDP by *R. erythropolis* strain MI2 and conversion of DTDB by *A. mimigardefordensis* strain DPN7^T

The isolates listed in Table 2 were tested for their ability to convert a particular 'non-utilizable' OSC, DTDB, DTDP or TDP, if cultivated in the presence of a second easily degradable carbon source, e.g. sodium gluconate. No strain yielded any evidence for cleavage or other conversions of the added foreign OSC, as verified by GC and GC/MS. On the other hand, the respective 'non-utilizable' OSC did not exert any toxic effect on the cells if applied at concentrations lower than 1 % (w/v).

To elucidate whether the substrate specificity of strains MI2 and DPN7 results from inducing and regulatory effects, the experiments were done in a modified way. In these experiments, the 'non-utilizable' OSC was added to cultures containing the 'utilizable' OSC. Therefore, both strains were cultivated in MSM containing 20 mM DTDP plus 20 mM DTDB. Samples were withdrawn every 48 h and analysed for the occurrence of putative cleavage products by GC/MS (Fig. 5). After 4 days of incubation, up to 4.5 mM 3MP was detected as the cleavage product of DTDP when cells of strain MI2 were grown with DTDP plus DTDB. In contrast, when cells of strain DPN7 were cultivated for 6 days in MSM containing DTDB plus DTDP, TBL, which is the lactone of 4MB, was detected at a concentration of up to 2.6 mM. Interestingly, *A. mimigardefordensis* strain DPN7^T did not release 4MB into the medium.

DISCUSSION

Although a variety of bacterial strains (Table 1) with uncommon degradation capabilities was cultivated on minimal media containing one of the OSCs, no growth of any of these strains was detected. In fact, only a few cultivable strains are able to utilize DTDB, DTDP or TDP as the sole sources of carbon and energy (Table 2). Furthermore, all DTDB-degrading strains could not utilize DTDP or TDP, while none of the DTDP-utilizing strains grew with DTDB or TDP, and no TDP-catabolizing strain utilized DTDB or DTDP. The inability to grow on the respective non-utilizable OSC is probably caused by an inability to catalyse the initial cleavage steps or to transport the compounds into the cells, or the absence of other means of conversion, if the cells are not cultivated in the presence of a utilizable OSC. Consequently, *R. erythropolis* strain MI2 and *A. mimigardefordensis* strain DPN7^T can cleave the respective non-utilizable disulphide, when DTDB (strain MI2) or DTDP (strain DPN7^T) are at the same time available for growth. This indicates that the

Fig. 5. Conversion of DTDP by *R. erythropolis* strain MI2 and of DTDB by *A. mimigardefordensis* strain DPN7^T, and occurrence of the respective cleavage products 3MP and TBL. Cells were cultivated in 250 ml flasks without baffles on a rotary shaker at 30 °C and 120 r.p.m. in MSM containing 20 mM DTDB and 20 mM DTDP. Analyses of the OSCs in the cell-free supernatants were done by GC and GC/MS. Standard deviations based on three measurements are indicated. □, TBL; ◆, 3MP.

enzymes catalysing the first steps in the catabolism of DTDB and DTDP also react with the other substrate in *R. erythropolis* strain MI2 and *A. mimigardefordensis* strain DPN7^T, respectively.

A. mimigardefordensis strain DPN7^T presumably converts 4MB to TBL due to the high toxicity of 4MB to living cells (Held & Biaglow, 1994). 4MB could be converted to the corresponding lactone TBL, assuming either the involvement of a lipase (Gupta *et al.*, 2004) or a reaction analogous to the error-editing process catalysed by certain tRNA synthetases, converting the non-protein amino acid homocysteine (which is a close structural analogue of 4MB) to the thioester homocysteine-thiolactone (Jakubowski & Fersht, 1981; Jakubowski & Goldman, 1992).

It became evident in this study that all three OSCs are biodegradable and are used as the sole carbon and energy sources for growth by a few soil micro-organisms with exclusive degradation abilities. Whether the utilization depends on rare functions and low substrate specificities of well-known enzymes, or whether the degrading enzymes are specifically synthesized for the degradation of one particular OSC has to be further investigated. Whereas different bacterial species could be isolated using TDP or DTDP as the sole carbon source, the degradation of DTDB seems to be restricted to strains of *R. erythropolis* alone. Rhodococci are widespread in natural habitats, and the members of this genus are known for their broad catabolic diversity and unique catabolic capabilities (Bell *et al.*, 1998). *R. erythropolis* cells contain a large set of enzymes, which allow the cells to perform enzyme-catalysed reactions such as oxidations, dehydrogenations, epoxidations, hydrolysis, hydroxylations and dehalogenations (de Carvalho *et al.*, 2005). Members of the genus *Rhodococcus* also have unique abilities to cleave carbon–sulphur bonds without affecting the carbon–carbon bond. *R. erythropolis*

IGTS8 is therefore used as model organism to study the microbial desulphurization of fossil fuels via a biological system (Grossman *et al.*, 2001). Furthermore, the industrial production of acrylamide is an important biotechnological application of rhodococci (Yamada & Kobayahashi, 1996).

Novel pathways for the degradation of DTDP and TDP have been recently presented (Bruland *et al.*, 2009a; Wübbeler *et al.*, 2008). Based on this knowledge and on the experimental data on the phenotypic and physiological characteristics of DTDB-defective mutants of *R. erythropolis* strain MI2 revealed in this study, we propose the putative degradation pathway of DTDB shown in Fig. 6. DTDB degradation is most likely initiated by a symmetrical cleavage performed by putative disulphide oxidoreductases, thereby yielding two molecules of 4MB. This compound is more toxic than the other mercaptoalkanoates used in this study, as documented in the past during synthesis of PTEs (Lütke-Eversloh & Steinbüchel, 2003), and as described for free thiols in many biochemical processes (Held & Biaglow, 1994). 4MB is proposed as an intermediate, since it is easily detectable in supernatants of cultures with DTDB as the carbon source (Figs 2 and 4). For this initial step, the participation of an oxidoreductase is likely. Flavoprotein disulphide reductases catalyse, for example, the reduction of compounds which are structurally related and linked by disulphide bonds (Williams, 1992). In the catabolism of DTDP, the reduction of the disulphide bond is most likely catalysed by a dihydrolipoamide dehydrogenase homologous enzyme with enhanced substrate specificity (Wübbeler *et al.*, 2008; J. H. Wübbeler and others, unpublished results). Considering the available data on the completely sequenced genome of *Rhodococcus* strain RHA1 (McLeod *et al.*, 2006), more than 500 ORFs that encode reductases, including six genes encoding paralogues of dihydrolipoamide dehydrogenases, are present. Moreover, *A. mimigardefordensis* strain DPN7 and *R. erythropolis* strain MI2 were both able to cleave

Fig. 6. Putative pathway for the degradation of DTDB in *R. erythropolis* strain MI2. Initially, DTDB is putatively cleaved into two molecules of 4MB, which is then probably oxidized by an oxygenase, thereby yielding 4-oxo-4-sulphanylbutanoic acid. Afterwards, the sulphur moieties could be removed as volatile hydrogen sulphide by a putative desulphydrase. The resulting succinic acid is then further metabolized.

the disulphides (DTDB and DTDP) symmetrically, if incubated under inducing conditions, as shown in Fig. 5.

The second proposed step in the catabolism of DTDB is the oxidation of 4MB to the corresponding carboxylic acid 4-oxo-4-sulphanylbutanoic acid. This compound was detected in the culture supernatants of the DTDB-negative mutant 1.7/17 (Figs 2c and 4a). The reaction is presumably performed by an oxygenase, and the subsequent step is thought to be the abstraction of the sulphur, catalysed by a putative desulphydrase. Approximately 200 ORFs encoding oxygenases and two ORFs encoding desulphydrases have been identified in *Rhodococcus* strain RHA1 (McLeod *et al.*, 2006). The putative desulphydrase of *R. erythropolis* strain MI2 presumably converts 4-oxo-4-sulphanylbutanoic acid into volatile hydrogen sulphide and succinic acid; the latter can be further metabolized in the central metabolism via the citric acid cycle. All previously proposed products have been proven to be intermediates.

In conclusion, microbial utilization apparently converts DTDB into two molecules of 4MB, which are then further metabolized. After identification and characterization of the genes involved, the construction of metabolically engineered strains suitable for production of 4MB-containing PTEs should become possible. The system of transpo-

son mutagenesis (Nagy *et al.*, 1997) used in this study, which was the only available and applicable method at the time, is not suitable for the genotypic characterization of transposon-induced mutants, as also described elsewhere (Sallam *et al.*, 2006). However, a promising system for the construction of transposon mutant libraries of strains of *R. erythropolis* has been developed just recently (Sallam *et al.*, 2007). This tool should be employed for the complete elucidation of DTDB catabolism. Subsequently, the successful heterologous expression of genes of the butyrate kinase/phosphotransbutyrylase/PHA synthase (BPEC) pathway (Liu & Steinbüchel, 2000) in genetically manipulated *R. erythropolis* could yield PTE with 4MB building blocks. As already mentioned, detailed knowledge of the DTDB- or 4MB-degrading pathways is a prerequisite to engineer the optimized production strains. The data presented and the anticipated findings are therefore especially supportive of the prospective synthesis of such interesting polymers.

ACKNOWLEDGEMENTS

The authors thank Matthias Arenskötter for helpful discussions and instructions in methods.

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Edited by: H. L. Drake