Biodegradation (2010) 21:309–319
DOI 10.1007/s10532-009-9302-y

ORIGINAL PAPER

Microbial utilization of the industrial wastewater pollutants 2-ethylhexylthioglycolic acid and *iso*-octylthioglycolic acid by aerobic Gram-negative bacteria

Mario Toups · Jan Hendrik Wübbeler · Alexander Steinbüchel

Received: 8 April 2009 / Accepted: 15 September 2009 / Published online: 30 September 2009

Abstract Industrial wastewater from the production of sulfur containing esters and the resulting products of this synthesis, 2-ethylhexylthioglycolic acid (EHTG) and *iso*-octylthioglycolic acid (IOTG), were deployed in this study to enrich novel bacterial strains, since no wastewater and EHTG or IOTG degrading microorganisms were hitherto described or available. In addition, nothing is known about the biodegradation of these thiochemicals. The effect of this specific wastewater on the growth behaviour of microorganisms was investigated using three well-known Gram-negative bacteria (*Escherichia coli*, *Pseudomonas putida*, and *Ralstonia eutropha*). Concentrations of 5% (v/v) wastewater in complex media completely inhibited growth of these three bacterial strains. Six bacterial strains were successfully isolated, characterized and identified by sequencing their 16S rRNA genes. Two isolates referred to as *Achromobacter* sp. strain MT-E3 and *Pseudomonas* sp. strain MT-I1 used EHTG or IOTG, respectively, as well as the wastewater as sole source of carbon and energy for weak growth. More notably, both isolates removed these sulfur containing esters in remarkable amounts from the cultures supernatant. One further isolate was referred to as *Klebsiella* sp. strain 58 and exhibited an unusual high tolerance against the wastewater's toxicity without utilizing the contaminative compounds. If cultivated with gluconic acid as additional carbon source, the strain grew even in presence of more than 40% (v/v) wastewater. Three other isolates belonging to the genera *Bordetella* and *Pseudomonas* tolerated these organic sulfur compounds but showed no degradation abilities.

Keywords Biodegradation · Organic sulfur compounds · Thiochemicals · 2-Ethylhexylthioglycolic acid · *Iso*-octylthioglycolic acid · Water treatment

Abbreviations

DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
TG	Thioglycolic acid
EH	2-Ethyl-1-hexanol
MSM	Mineral salt medium
EHTG	2-Ethylhexylthioglycolic acid
IOTG	Iso-octylthioglycolic acid
LB	Luria Bertani broth
OSC	Organic sulfur compounds
Na	Sodium

M. Toups · J. H. Wübbeler · A. Steinbüchel (✉)
Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, 48149 Münster, Germany
e-mail: steinbu@uni-muenster.de

J. H. Wübbeler
e-mail: wjhw@uni-muenster.de

Introduction

Industrial wastewaters may be a serious threat to receiving waters and their flora and fauna. Therefore, the discharge of such waters is strictly regulated, and companies are responsible to ensure a water quality which is not harmful for the environment. Since chemical and physical treatment methods are often complicated and expensive, also microbiological processes for wastewater treatment were established. Olive mill effluents can be efficiently treated with bacteria (Mantzavinos and Kalogerakis 2005), and a variety of microbiological methods is available for treatment of distillery wastewaters (Pant and Adholeya 2007). In other studies combined methods for treatment of wastewater including a microbiological step such in an integrated electrochemical and biological approach were reported (Rüdiger et al. 2007). Furthermore, there have been various approaches to regain either bioenergy or biochemicals from liquid wastes, turning them into profitable resources (Angenent et al. 2004).

In this study the microbial degradability of organic sulfur compounds (OSC) occurring from ester synthesis in wastewater was investigated. These were 2-ethylhexylthioglycolic acid (EHTG) and *iso*-octylthioglycolic acid (IOTG), which are used as stabilizers in polyvinylchloride and as chain length regulators in polymer synthesis. EHTG (Fig. 1a) consists of 2-ethylhexanol (EH) and thioglycolic acid (TG), whereas IOTG is an isomeric mixture of a variety of esters consisting of thioglycolic acid and different C_8-alcohols; two examples of typical chemical structures are shown in Fig. 1b and c. These substances are very harmful for the environment, and animal testing has proven their potential harm for mammals (Hollenbach et al. 1972; Schmidt et al. 1974).

The degradability of thiodiglycol (Fig. 1d), which is a detoxication product of mustard gas and of thiodiglycolic acid (Fig. 1e) by *Alcaligenes xylosoxidans*, was shown in many studies (Kim et al. 1997; Lee et al. 2000; Tikhonova et al. 2002; Garcia-Ruiz et al. 2002). Because EHTG and IOTG are structurally related to thiodiglycol, degradation of these esters by related strains may be auspicious. To our best knowledge, there have been no reports about microorganisms with the capability of degrading EHTG or IOTG. Hence, an important aim of this study was to isolate bacteria from the environment, which have the ability

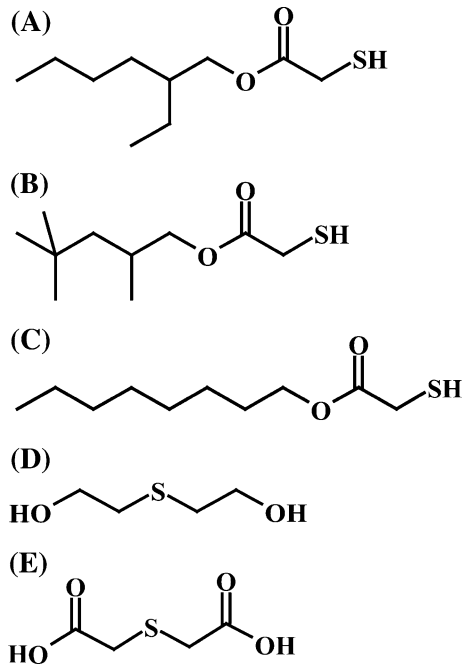

Fig. 1 Chemical structures of OSCs relevant for this study. **a** 2-Ethylhexylthioglycolic acid, **b** 2-methyl-4,4-dimethylpentylthioglycolic acid, **c** 1-octylthioglycolic acid, **d** thiodiglycol, **e** thiodiglycolic acid

to degrade these organic thiochemicals and which use them as sole carbon and energy source for growth. Axenic cultures of these bacteria were characterized, and their degradation performance was verified. Enrichment of such bacteria was accomplished in minimal media containing only EHTG and IOTG as sole carbon and energy source, and also with media containing sodium gluconate in addition to polluted wastewater from an industrial ester synthesis plant. This wastewater contained the two esters together with the precursor substances thioglycolic acid and a variety of alcohols leading to a total concentration of pollutants of about 5% (v/v). A further aim of this study was to determine the toxicity and resulting growth inhibitory effects of this industrial wastewater on three typical Gram-negative bacteria widely occurring in municipal sewage water.

Materials and methods

Bacteria and cultivation conditions

Escherichia coli TOP 10 (Invitrogen, Carlsbad, USA) was cultivated in Luria–Bertani broth medium (LB,

Sambrook et al. 1989) at 30 or 37°C. *Ralstonia eutropha* H16 (DSM 428) was cultivated in nutrient broth (NB) medium (Sambrook et al. 1989) and *Pseudomonas putida* (DSM 291^T) in LB at 30°C. The natural isolates were maintained at 30°C in either LB or mineral salt medium (Schlegel et al. 1961) with appropriate carbon sources. Growth experiments with 1% (v/v) EHTG or IOTG as carbon source were conducted in mineral salt medium (MSM), containing 0.05% (v/v) Cremophor EL (BASF, Ludwigshafen) as emulsifying agent. To examine the ability of the strains to metabolize the investigated compounds as sulfur sources, a sulfur-free MSM was prepared by replacing $MgSO_4$ with $MgCl_2$ and by using the sulfur-free microelement solution SL 7 instead of SL 6 (Pfennig 1974). Due to the property of the emulsified esters to cloud liquid media, growth was recorded by plating appropriate diluted aliquots of samples. Colonies were counted after 24–48 h of incubation. All growth experiments were done under aerobic conditions on a rotary shaker at 120 rpm. Carbon sources were either autoclaved separately or were added from filter-sterilized stock solutions. Solid media contained 1.8% (w/v) agar.

Wastewater and contaminant thiochemicals

Wastewater for the experiments was provided by the company Bruno Bock Chemische Fabrik GmbH & Co KG (Marschacht, Germany). This company is producing TG since several decades and later on also the two esters EHTG and IOTG. During their synthesis, wastewater accumulates, which still contains traces of the educts EH and TG in addition to the products EHTG and IOTG. Physical and chemical properties of these four main substances were compiled in Table 1. According to the company, the wastewater's composition slightly varies from batch to batch, though it contains a total amount of ~5% (v/v) of contaminative substances, including many different alcohols (C_4–C_{18}), but the exact composition can not be determined. The disposal of these compounds is initiated by an energy consuming and therefore cost intensive distillation method.

Enrichment and isolation of natural isolates

Enrichment of natural isolates was conducted in 500 ml flasks filled with 100 ml MSM. Inoculums were a large variety of natural samples like hair, fur, feathers, compost, soil samples, oily cloths from a gas station, and residues from inside a drainage pit. Furthermore, 1 ml of activated sludge, taken from the activated sludge basin of the municipal sewage treatment plant in Münster-Coerde (Germany) was used as inoculum. All collected samples were considered to contain sulfur components and therefore potential degrading bacteria growing in such environments. Only isolates originating from activated sludge showed the ability to utilize or tolerate the esters or the wastewater. Two different enrichment procedures were accomplished. The first procedure was accomplished to enrich strains able to grow in the wastewater, if cultivated additionally with an easy to degrade and common carbon source. Therefore, 12% wastewater plus 1% (w/v) sodium gluconate was added to the enrichment flasks. The other procedure was done to obtain isolates capable to utilize solely EHTG or IOTG for their growth. Accordingly, 0.1–1.0% (v/v) of one of the esters as sole carbon and energy source were deployed. The enrichment flasks were shaken for several days at 30°C before aliquots were plated on solid MSM-agar plates containing the respective OSCs as carbon source. After

Table 1 Physical and chemical properties of respective OSC in wastewater

	EH	TG	EHTG	IOTG
Color	Colorless	Colorless	Colorless	Colorless
Boiling point (°C)	184–185	224	242–259	230–260
Flash point (°C)	79.0	131.5	117.5	116.0
Ignition point (°C)	330	315	n.v.	n.v.
Vapor pressure (hPa)	0.11 (20°C)	0.10 (20°C)	0.97 (25°C)	0.19 (25°C)
Density (g/cm^3)	0.830	1.325	0.973–0.975	0.975–0.977
Water solubility (g/l)	1.1	Completely soluble	0.00473	0.0106

2–5 days incubation, material of colonies that were grown on these plates were consistently transferred to fresh plates until axenic cultures were achieved.

Biochemical characterization of natural isolates

Gram staining, L-alanyl-aminopeptidase (LAAP) test, potassium hydroxide (KOH) test as well as catalase and oxidase tests were performed according to Gerhardt et al. (1994). Susceptibility of the strains towards antibiotics was determined on LB agar plates for the following antibiotics (ml^{-1}): 75 μg ampicillin, 34 μg chloramphenicol, 10 μg gentamycin, 50 μg kanamycin, 100 μg streptomycin, 12.5 μg tetracycline. An Api 20NE test (bioMèrieux, France) was performed to determine the assimilation of a variety of carbon sources and the presence of enzymes for taxonomic affiliation. The ability to utilize different carbon sources, which are not included in the Api 20NE test, was investigated on MSM agar plates containing 0.2% (w/v) of the respective carbon source; only levulinic acid (0.5%, v/v) and 3-mercaptopropionate (3MP, 0.1%, v/v) were employed in different concentrations (Table 2).

Isolation, manipulation and transfer of DNA

Chromosomal DNA was isolated as described by Marmur (1961). Plasmid DNA was isolated by the method of Birnboim and 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 the 16S rDNA ligated into plasmid pGEM®-T Easy (Promega, USA) (Hanahan 1983).

Sequencing of 16S rRNA genes

PCR amplifications of the 16S rRNA genes were carried out according to the procedures described by Innis et al. (1990) using the oligonucleotides 27f (5′-GAGTTTGATCCTGGCTCAG-3′) and 1525r (5′-AGAAAGGAGGTGATCCAGCC-3′) as primers, which were complementary to conserved regions of the 16S rDNA of *E. coli*. The PCR products were purified using the NucleotrapCR extraction kit (Macherey–Nagel, Düren, Germany) and ligated into the cloning vector pGEM®-T Easy (Promega, USA).

The sequences of the 16S rRNA genes were determined by the chain termination method according to Sanger et al. (1977), using an ABI Prism 3730 capillary sequencer at the Universitätsklinikum Münster (UKM) or a LI-COR (USA) thermal cycle sequencer. The following oligonucleotides were used as primers: 27f, 343r (5′-CTGCTGCCTCCCGTA-3′), 357f (5′-TACGGGAGGCAGCAG-3′), 519r (5′-G(T/A)-ATTACCGCGGC(T/G)GCTG-3′), 536f (5′-CAG C(C/A)GCCGCGGTAAT(T/A)C-3′), 803f (5′-ATTA GATACCCTGGTAG-3′), 907r (5′-CCGTCAATTCA TTTGAGTTT-3′), 1114f (5′-GCAACGAGCGCAA CCC-3′), 1385r (5′-CGGTGTGT(A/G)CAAGGCCC-3′) and 1525r as well as the M13 universe primer (5′-GTAAAACGACGGCCAGT-3′) and the M13 reverse primer (5′-CAGGAAACAGCTATGAC-3′), which were both hybridizing to pGEM®-T Easy DNA. A consensus sequence was created using the software Bio Edit Sequence Alignment Editor (version 7.0.5.3.). 16S rDNA sequences were analysed using the programm BLAST (National Center for Biotechnology Information; http://www.ncbi.nml.nih.gov) by running the nucleotide-nucleotide blast (BlastN). The nucleotide sequence of the 16S rDNA of the strains and of related type strains belonging to the same phylogenetic group in addition to well-known representatives of the β-subclass of the *Proteobacteria* were aligned using CLUSTALX (Thompson et al. 1997). 16S rRNA gene sequences were retrieved from the EMBL database and from the Ribosomal Database Project (Maidak et al. 1997). Resulting trees were displayed with TreeView (Page 1996) which were calculated by using the neighbour-joining method (Saitou and Nei 1987).

Analyses of EHTG and IOTG

To analyze the microbial utilization of EHTG and IOTG in media and in cell mass, 10 ml samples were withdrawn from cultivation flasks. For analysis of the supernatant, such samples were centrifuged at 4°C for 1 h in a Megafuge 1.0 R centrifuge (Heraeus Sepatech, Osterode, Germany). The supernatants were transferred to clean tubes and extracted with 2 ml of chloroform by vortexing and inverting for 1 min. Chloroform extracts were then transferred to a GC vial and analyzed. For pellet analysis, the washed cell pellets were frozen at −70°C and then lyophilized for 24 h in a type 1120 freeze-dryer (Christ,

Table 2 Physiological characteristics of bacteria isolated in this study

Characteristic	E1	E3	I1	I2	I3
Gram behaviour	−	−	−	−	−
Oxidase activity	+	+	+	+	+
Catalase activity	+	+	+	+	+
Ampicillin tolerance	−	+	+	−	+
Chloramphenicol tolerance	−	−	+	−	−
Gentamycin tolerance	+	+	−	+	+
Kanamycin tolerance	−	−	−	−	+
Streptomycin tolerance	+	+	−	+	−
Tetracycline tolerance	+	+	+	+	+
Sodium gluconate	−	−	+	−	+
Succinic acid	−	+	+	−	+
Acetic acid	−	+	+	−	+
Levulinic acid	−	−	−	−	−
Propionic acid	−	+	+	−	+
Fructose	−	−	+	−	+
3,3′-Dithiodipropionic acid	−	−	+	+	+
3-Mercaptopropionic acid	−	−	−	−	−
Potassium nitrate	−	+	−	−	+
L-Tryptophane	−	−	−	−	−
D-Glucose (fermentation)	−	−	−	−	−
L-Arginine	−	−	−	−	−
Urea	−	−	−	−	−
Aesculin ferric citric acid	−	−	−	−	−
Gelatine (bovin)	−	−	−	−	−
D-Glucose (assimilation)	−	−	+	−	+
L-Arabinose	−	−	−	−	−
D-Mannose	−	−	−	−	−
D-Mannitol	−	−	−	−	−
N-Acetylglucosamine	−	−	−	−	−
D-Maltose	−	−	−	−	−
Potassium gluconate	−	−	+	−	+
Capric acid	−	−	+	−	+
Adipic acid	+	+	−	+	+
Malic acid	+	+	+	+	−
Trisodium citric acid	+	+	+	+	+
Phenylacetic acid	+	+	+	+	+
4-Nitrophenyl-β-D-Galactopyranosid	−	−	−	−	−

Osterode, Germany). Five to fifteen milligram of dried cell pellets was incubated with 2 ml chloroform and 2 ml of methanol/sulphuric acid (15%, v/v, sulphuric acid in methanol) for 4 h at 100°C (type Ö270 oil bath; Memmert, Schwabach, Germany). Afterwards, the methylated samples were chilled to room temperature, mixed with 2 ml H_2O_{bidest} and shaken vigorously for 20 s. The chloroform phase was then transferred to a GC vial for detection and quantification of the substances in a capillary gas chromatograph (Series 6890 GC System, Hewlett Packard, Waldbronn, Germany).

Nucleotide sequence accession numbers

The complete DNA sequences of the 16S rRNA genes of the isolates presented in the phylogenetic tree (Fig. 2) have been deposited in the GenBank database: *Pseudomonas* sp. strain MT-I3 (accession number EU727192), *Pseudomonas* sp. strain MT-I1 (accession number EU727193), *Bordetella* sp. strain MT-E1 (accession number EU727194), *Bordetella* sp. strain MT-I2 (accession number EU727195) and *Achromobacter* sp. strain MT-E3 (accession number EU727196). The 16S rRNA gene of *Klebsiella* sp. strain 58 was not deposited because sequencing of the 16S rDNA of this isolate was not completed.

Deposition of valuable isolates

Klebsiella sp. strain 58 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig (Germany) and is available under accession number DSM 21696. *Achromobacter* sp. strain MT-E3 and *Pseudomonas* sp. strain MT-I1 were also deposited at the DSMZ and are available under accession numbers DSM 21681 and DSM 21680, respectively.

Results

Toxicity of wastewater from ester production

To estimate the toxicity of the wastewater from industrial ester production on microorganisms, three widely available bacterial strains were cultivated in complex media containing this wastewater at different concentrations. *E. coli* and *P. putida* were cultivated in LB medium containing wastewater from 0 to 5% (v/v), whereas *R. eutropha* was cultivated in NB medium

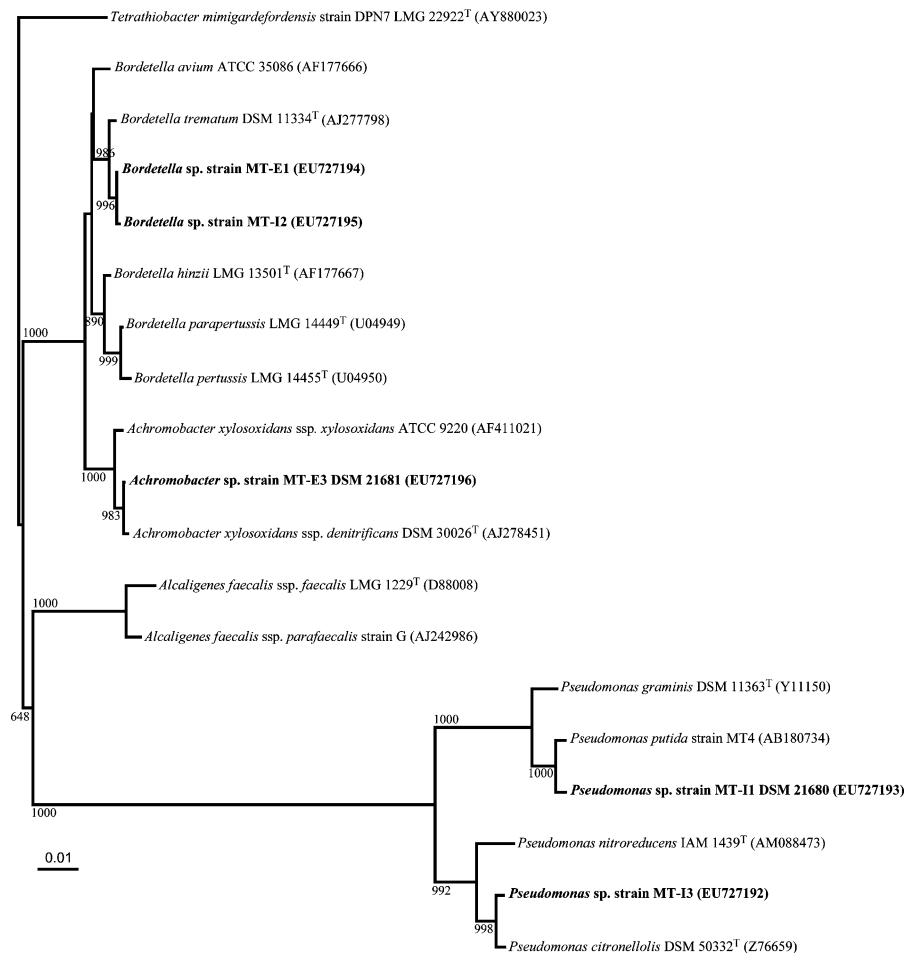

Fig. 2 Phylogenetic tree, based on 16S rRNA gene sequences, showing the positions of strains MT-E1, MT-E3, MT-I1, MT-I2, and MT-I3 within the β-subclass of the *Proteobacteria*. *Tetrathiobacter mimigardefordensis* LMG 22922^T was used as the outgroup. Bootstrap values exceeding 60% are shown at branch points. The *scale bar* represents 0.01 substitutions per nucleotide positions; ^{T}, type strain; accession numbers are given in *parenthesis*

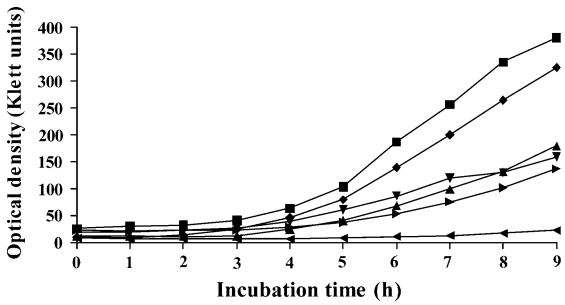

Fig. 3 Toxicity of the investigated wastewater for cells of *Escherichia coli*. Cells were cultivated in 50 ml Klett flasks with baffles on a rotary shaker at 30°C and 120 rpm in liquid LB containing increasing concentrations of wastewater (from 0.0 to 5.0%, v/v). ■, 0.0% wastewater; ◆, 1.0% wastewater; ▼, 2.0% wastewater; ▲, 3.0% wastewater; ▶, 4.0% wastewater; ◀, 5.0% wastewater. Growth was analyzed in a Klett–Summerson photometer

containing wastewater from 0 or 2% (v/v). The experiment was conducted at 30°C, and growth was measured with a Klett Summerson photometer. Figure 3 shows the growth behavior of *E. coli* in presence of varying concentrations of wastewater. Growth slowed down concomitantly with increasing concentrations of wastewater added to the basic complex medium. With 4% (v/v) wastewater a strong inhibition of the growth could be observed, whereas no growth at all occurred with 5% (v/v) or more wastewater. Growth experiments with *P. putida* gave similar results and *R. eutropha* did not even tolerate concentrations of 2% (v/v) wastewater (data not shown). These data clearly indicated a certain toxicity of the wastewater or of its components resulting in growth inhibition of the investigated bacteria and probably also affecting growth of many other bacteria. Therefore, it would be advantageous to identify bacteria, which were capable of degrading the respective esters and which were not inhibited by already low concentrations of the wastewater.

Utilization of EHTG and IOTG by available bacteria

In past, our laboratory has isolated and characterized many different bacterial strains with the capability of utilizing various OSCs (Bruland et al. 2009a, b; Carbajal-Rodríguez et al. 2009; Wübbeler et al. 2006, 2008, 2009). Due to the similarities of the respective substrates regarding the chemical structures, growth experiments were conducted with these bacterial strains using solid MSM containing 0.1% (v/v) EHTG or IOTG as sole carbon and energy source. However, even after incubation of more than 7 days, no growth of any of these strains could be observed.

Isolation and characterization of enriched bacteria

To investigate if the industrial wastewater contained culturable microorganisms, aliquots ranging from 5 to 200 µl were streaked or plated on different solid media. Additionally, liquid media were inoculated with 1% (v/v) wastewater. Despite 72 h of incubation, no growth of any colonies or an increase of turbidity was observed. Therefore, culturable bacteria were obviously absent from the wastewater.

Subsequent enrichments were successfully performed by using activated sludge as inoculum, and six natural isolates were obtained. Isolates MT-E1 and MT-E3 resulted from enrichments with 1% (v/v) EHTG, strains MT-I1, MT-I2, and MT-I3 were isolated with 1% (v/v) IOTG. Strain 58 originated from an enrichment culture in MSM containing 12% (v/v) wastewater plus 1% (w/v) sodium gluconate as additional carbon source.

To unravel the phylogenetic position of the isolated bacteria, Api 20NE tests were performed and the 16S rRNA gene sequences of all six isolates were analyzed. Strains MT-E1 and MT-I2 were found to be species of the genus *Bordetella*. Strains MT-I1 and MT-I3 were affiliated to the genus *Pseudomonas*, but only strain MT-I1 was able to grow in minimal medium with IOTG as sole carbon source. Strain MT-E3 utilized EHTG as sole carbon source for growth and was affiliated as a species of the genus *Achromobacter*, whereas strain 58 belongs to the genus *Klebsiella*.

Figure 2 shows a phylogenetic tree with strains MT-E1, MT-E3, MT-I1, MT-I2, and MT-I3 embedded among their closest relatives. The 16S rRNA gene of strain 58 was only partially sequenced and was therefore not included in the figure. Table 2 summarizes the results of all biochemical tests for the five isolates. It includes Gram staining, LAAP test, KOH test, susceptibility to antibiotics and utilization of a large variety of common carbon sources.

Growth and degradation performances of OSC-tolerating or -utilizing isolates

Since the isolates *Bordetella* sp. strain MT-E1, *Bordetella* sp. strain MT-I2 and *Pseudomonas* sp. strain MT-I3 merely tolerated EHTG or IOTG, but were not able to use these substances or the wastewater for growth, they were no longer of interest and were not further investigated in this study.

Klebsiella sp. strain 58

This new isolate exhibited a remarkable high tolerance towards the toxic wastewater, but could not utilize any of the contaminative compounds as sole source of carbon and energy. Therefore, growth of strain 58 depends on the supply of a utilizable carbon source like sodium gluconate. 10% (v/v) wastewater did not inhibit strain 58 significantly, but growth started to cease at wastewater concentrations of 20% (v/v) or higher (Fig. 4). *Klebsiella* sp. strain 58 was still able to grow in presence of more than 40% (v/v) wastewater.

Achromobacter sp. strain MT-E3 and *Pseudomonas* sp. strain MT-I1

Both strains grew in medium containing the wastewater and EHTG or IOTG, respectively, as sole carbon sources. Various growth experiments were conducted with these isolates: *Achromobacter* sp. strain MT-E3 showed much better growth in baffled Erlenmeyer flasks. In contrast, growth of *Pseudomonas* sp. strain MT-I1 was not altered if cultivated with or without baffles. Furthermore, the dependency of growth on pH (between pH 5.0–9.0) in MSM plus 1% (v/v) EHTG or IOTG, respectively, of both strains was investigated and pH 7 was the optimum. To increase the availability of the water-insoluble esters to the cells, an emulsifying agent (Cremophor EL, Bayer, Germany), which is not utilizable as carbon source by either strain as shown in separate control experiments, was added at low concentrations (0.05%, v/v). Because turbidity increased vigorously due to the emulsified esters, establishment of a growth curve by measuring the optical density was not possible. Instead, a steady increment of their living cell number could be verified; therefore, cells of both strains were utilizing the respective ester as sole carbon and energy source for growth. Volume of inoculation was adjusted to approximately 50×10^6 living cells $\times$ ml^{-1} in the main cultures. *Achromobacter* sp. strain MT-E3 increased its living cell number up to a maximum of 600×10^6 cells $\times$ ml^{-1} if cultivated in a baffled Erlenmeyer flask for 10 days at 30°C and 120 rpm in MSM containing 1% (v/v) EHTG. *Pseudomonas* sp. strain MT-I1 also increased its living cell number, but only up to 200×10^6 cells $\times$ ml^{-1} if cultivated in MSM containing 1% (v/v) IOTG for 7 days at 30°C and 120 rpm.

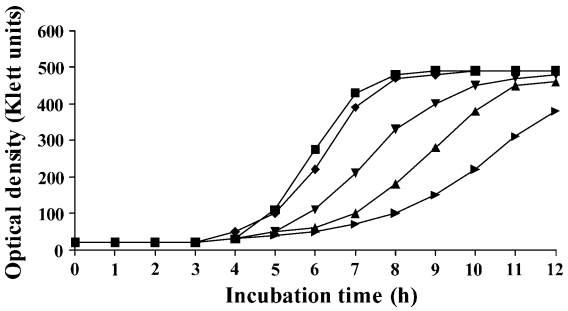

Fig. 4 Tolerance of *Klebsiella* sp. strain 58 towards the wastewater. Cells were cultivated in 50 ml Klett flasks with baffles on a rotary shaker at 30°C and 120 rpm in liquid MSM containing 1% (v/v) sodium gluconate and increasing concentrations of wastewater (from 0.0 to 40.0%, v/v). ■, 0% wastewater; ◆, 10% wastewater; ▼, 20% wastewater; ▲, 30% wastewater; ▶, 40% wastewater. Growth was analyzed in a Klett–Summerson photometer

Growth of both isolates in MSM with other sulfur sources was also investigated. The inorganic sulfur source $MgSO_4$ was replaced by the organic sulfur source EHTG or IOTG, respectively. Whereas strain MT-E3 was able to increase its cell number fourfold with EHTG, strain MT-I1 was unable to grow with IOTG as sole sulfur source.

For the identification of putatively occurring degradation metabolites of the esters during their catabolism, cell-free supernatants and pellets of cultures from *Achromobacter* sp. strain MT-E3 and *Pseudomonas* sp. strain MT-I1 were analyzed with GC and GC/MS. Due to the methylation process, EHTG and IOTG decomposed completely to the corresponding methylated cleavage compounds, methylated thioglycolic acid and methylated 2-ethylhexanol or different *iso*-octanols, respectively, as revealed by GC/MS. However, quantification was a problem, especially with samples containing IOTG, because in the only commercially available reference compound several main peaks belonged to the isomeric mixture of this

ester. Therefore, we focused on the analysis of the catabolism of EHTG by *Achromobacter* sp. strain MT-E3. The main culture of this strain, inoculated from a preculture of well grown cells, and a sterile reference were incubated at 30°C and 120 rpm in baffled flasks with MSM containing 50 mM EHTG as sole source of carbon and energy. Samples were withdrawn every 24 h, starting with the first chloroform extraction of both flasks after inoculation and vigorous shaking of the culture (Fig. 5). Analyses of the supernatant showed a depletion of ~80% of the initial EHTG within 72 h of incubation, which was a very high extent of degradation for the observed weak cell growth. Additional examination of the respective dried cell pellets, and furthermore the analyses of samples of non-centrifuged culture broth were consistent with this presumption: Only some EHTG was utilized for growth, whereas a fraction was associated with the cells, i.e., adhered or stored inside the cells (data not shown). Interestingly, the concentration of EHTG in the pellet was steadily increasing during the time course of incubation, accompanied by the concomitant decrease of this ester in the cell-free supernatant (Fig. 5). Degradation intermediates were not detected.

Further experiments investigated the strains ability to utilize the industrial wastewater or rather the contaminative compounds as sole carbon source for growth. Employed concentrations of the wastewater were 16, 33, and 50% (v/v) in liquid MSM by monitoring the increase or decrease of the living cell numbers. Main cultures of *Achromobacter* sp. strain MT-E3 were inoculated to a living cell number of $\sim 30 \times 10^6$ cells $\times$ ml^{-1} at the beginning. Cultivation conditions with 50% (v/v) wastewater caused the death of all cells within 2 days of incubation. Thirty three percent (v/v) wastewater resulted in an extended lag phase of 6 days, but then the living cell number triples and stayed constant at 90×10^6 cells $\times$ ml^{-1}. With only 16% (v/v) wastewater a steady increase of the living cells to more than $200 \times 10^6 \times$ ml^{-1} was observed within 6 days of incubation.

The growth behavior of *Pseudomonas* sp. strain MT-I1 was different. A significant increase of the living cell numbers occurred only with 16% (v/v) wastewater during the first 3 days of incubation. The cell number at the beginning of the cultivation was about $3 \times 10^6 \times$ ml^{-1} and increased to $60 \times 10^6 \times$ ml^{-1}. Within four additional days of cultivation the living cell number decreased steadily to $25 \times 10^6 \times$ ml^{-1}.

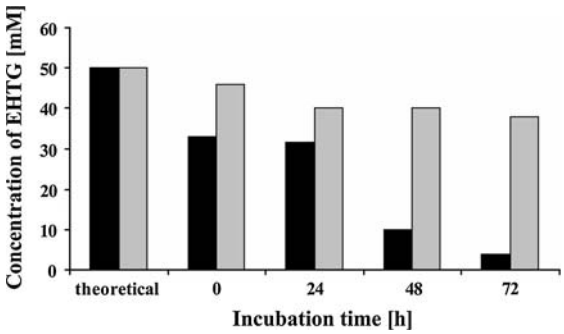

Fig. 5 Depletion of EHTG from the culture's supernantant by *Achromobacter* sp. strain E3. Cells of *Achromobacter* sp. strain E3 and a control flask without inoculum were cultivated each in 500 ml flasks with baffles on a rotary shaker at 30°C and 120 rpm in liquid MSM containing 50 mM EHTG as sole carbon source. Analyses of the cell-free supernatants were done by GC and GC/MS. *Symbols*: *black bar*, concentration of EHTG in the supernatant of *Achromobacter* sp. strain E3; *grey bar*, control without cells of *Achromobacter* sp. strain E3. The theoretical value of maximum extraction (50 mM EHTG) is also presented. First sampling (0 h) was accomplished after vigorous shaking of the inoculated main culture

Discussion

This study presents for the first time results about the microbial utilization of industrial wastewater and its main contaminative compounds, the esters EHTG and IOTG, by novel isolated strains. However, the investigated wastewater was very toxic for microorganisms and total content of contaminative compounds was ~5% (v/v) (Fa. Bruno Bock, unpublished data). Since this wastewater inhibited growth of *E. coli*, *P. putida*, and *R. eutropha* at concentrations of only 2–5% (v/v), a particular toxic OSC has to inhibit growth of these strains at concentrations of 0.1–0.25% (v/v).

Three valuable strains were isolated and characterized during this study. Important characteristics of these novel isolates regarding wastewater treatment are discussed below. Despite *Klebsiella* sp. strain 58 was unable to utilize any of the wastewater components, it instead tolerated comparably high concentrations of the wastewater. This tolerance is presumably related to the excretion of exo-polysaccharides forming a protecting capsule around the cells, a typical

characteristic of representatives of this species (Kwot et al. 2002; Edwards and Fife 1955). Therefore, genetic engineering of *Klebsiella* sp. strain 58 will be promising. After identification of the key enzymes responsible for degradation of EHTG and IOTG, e.g., in strains MT-E3 and MT-I1, and cloning of the respective genes, it would be possible to establish EHTG and IOTG degradation in *Klebsiella* sp. strain 58. Thereby, high resistance towards the wastewater and its components and degradation of these components could be combined yielding a strain which is possibly highly suitable for wastewater treatment and also bioremediation.

Achromobacter sp. strain MT-E3 grew with EHTG and *Pseudomonas* sp. strain MT-I1 with IOTG as sole carbon and energy source. Both strains used compounds of the investigated industrial wastewater for their growth, but perhaps also the less toxic pollutants of the wastewater. Tolerance against the inhibitory effects of the wastewaters toxicity was up to 33% (v/v), which is a much higher tolerance compared to other bacteria. The experiments conducted with pure EHTG and IOTG at concentrations of 1% (v/v) substantiated the assumption that the recently isolated strains are potent in utilizing these esters and their precursors. The cell densities of these bacteria increased during cultivation in minimal salts medium containing the respective OSC as sole carbon and energy source for growth. Therefore, a first essential prerequisite to establish a wastewater treatment for these hazardous contaminative compounds was achieved. Representatives of the genus *Achromobacter* exhibit frequently the capabilities to degrade esterified OSC, e.g., as also described in the past for *Achromobacter* (or *Alcaligenes*) *xylosoxidans* ssp. *denitrificans* (Kim et al. 1997; Lee et al. 2000; Tikhonova et al. 2002; Garcia-Ruiz et al. 2002).

Analyses of the cell-free supernatants of *Achromobacter* sp. strain MT-E3 demonstrated an efficient and significant decrease of EHTG, thus indicating a certain interaction between cells and OSC (Fig. 5). However, detailed examinations identified nearly equal amounts of the ester in the unprocessed culture broth, thereby implying that the OSC was somehow adsorbed at the cell wall or stored inside the cells without being completely catabolized. The results of the cell pellet analysis confirmed these assumptions. During the experiment, concentrations of methylated EH and TG in the cell pellets increased constantly with incubation time. This could well be interpreted as storage of EHTG within the cells of *Achromobacter* sp. strain MT-E3. Because the isolate is independent of an inorganic sulfur source during growth with EHTG, purification of the industrial wastewater employing this strain would not require an external sulfur source.

It is obvious that neither *Pseudomonas* sp. strain MT-I1 nor *Achromobacter* sp. strain MT-E3 degrades the sulfur containing esters or the wastewater in an applicable industrial scale. Instead, the strains were capable to remove remarkable concentrations of the respective thiochemical from the supernatant (Fig. 5). In theory, the separation of the most contaminative pollutants is thus possible with the obtained knowledge, but the process has to be investigated after an extensive upscaling. Therefore, this study about microbial degradation of EHTG and IOTG and the availability of bacteria utilizing or tolerating these compounds provide a basis to establish a microbial wastewater treatment process. However, further experiments to optimize the processes will be necessary. Further studies should include optimization of medium composition and growth conditions, natural adaptation to increasing concentrations of toxic substrate, and strain optimizations by mutation and metabolic engineering.

Acknowledgments The authors thank the company Bruno Bock Chemische Fabrik GmbH & Co KG (Niedermarschacht, Germany) for providing wastewater samples and information about its chemical composition as well as bulk containers of EHTG, IOTG and TG.

References

Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Dominguez-Espinosa R (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol 9:477–485

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Bruland N, Wübbeler JH, Steinbüchel A (2009a) 3-Mercaptopropionate dioxygenase, a cysteine dioxygenase homologue, catalyzes the initial step of 3-mercaptopropionate catabolism in the 3, 3′-thiodipropionic acid degrading bacterium *Variovorax paradoxus*. J Biol Chem 284:660–672

Bruland N, Bathe S, Willems A, Steinbüchel A (2009b) *Pseudorhodoferax soli* gen. nov., sp. nov., and *Pseudorhodoferax caeni* sp. nov., betaproteobacteria belonging to

the family *Comamonadaceae*. Int J Syst Evol Microbiol. doi:10.1099/ijs.0.006791-0

Carbajal-Rodríguez I, Bruland N, Wübbeler JH, Steinbüchel A (2009) Aerobic degradation of mercaptosuccinate by the Gram-negative bacterium *Variovorax paradoxus* strain B4 (in preparation)

Edwards PR, Fife MA (1955) Studies on the Klebsiella-Aerobacter group of bacteria. J Bacteriol 70:382–390

Garcia-Ruiz V, Martin-Otero LE, Puyet A (2002) Transformation of thiodiglycol by resting cells of *Alcaligenes xylosoxidans* PGH10. Biotechnol Prog 18:252–256

Gerhardt P, Murry RGE, Wood WA, Krieg NR (1994) Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 136:557–580

Hollenbach K, Schmidt P, Stremmel D (1972) Animal experiment studies on the effects of thioglycolic acid isooctylester, thioglycolic acid and 2-ethylhexanol on blood pressure. Z Gesamte Hyg 18:481–485

Innis MA, Gelfaud DH, Suinsky JJ, White TJ (1990) PCR-protocol: a guide to methods and applications. Academic Press, San Diego

Kim JW, Rainina EI, Efremenko E, Engler CR, Wild JR (1997) Degradation of thiodiglycol, the hydrolysis product of sulfur mustard, with bacteria immobilized within poly(vinyl)alcohol cryogels. Biotechnol Lett 19:1067–1071

Kwot TT, Chen P, Liu PY, Tang YC, Kong SK, Fung KP, Choy YM (2002) The anti-tumour effect of *Klebsiella pneumoniae* capsular polysaccharides. Biol Signals Recept 10:294–298

Lee TS, Chan SH, Weigand WA, Bentley WE (2000) Biocatalytic transformation of [(2-hydroxyethyl)thio]acetic acid and thiodiglycolic acid from thiodiglycol by *Alcaligenes xyolosoxidans* ssp. xylosoxidans (SH91). Biotechnol Prog 16:363–367

Maidak BL, Olsen GJ, Larsen N, Overbeek R, McCaughey MJ, Woese CR (1997) The RDP (ribosomal database project). Nucleic Acids Res 25:109–111

Mantzavinos D, Kalogerakis N (2005) Treatment of olive oil effluents Part I. Organic matter degradation by chemical and biological processes—an overview. Environ Int 31: 289–296

Marmur J (1961) A procedure for the isolation of desoxyribonucleic acid from microorganisms. J Mol Biol 1: 208–218

Page RDM (1996) Treeview: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12:357–358

Pant D, Adholeya A (2007) Biological approaches for treatment of distillery wastewater: a review. Bioresour Technol 98:2321–2334

Pfennig N (1974) *Rhodopseudomonas globiformis* sp. nov., a new species of the Rhodospirillaceae. Arch Microbiol 100:197–206

Rüdiger A, Rüdiger I, Jurisevic S (2007) Aqua-Biomant: integrated biological and electrochemical oxidation for industrial wastewater treatment. Water Sci Technol 55:195–200

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Laboratory, Cold Spring Harbor, N.Y

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schlegel HG, Kaltwasser H, Gottschalk G (1961) Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch Mikrobiol 38:209–222

Schmidt P, Fox G, Hollenbach K, Rothe R (1974) Zur akuten Toxizität des Thioglykolsäure-2-äthylhexylesters im Tierversuch. Z Gesamte Hyg 20:575–578

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Tikhonova EB, Ermakova IT, Slepen'kin AV, Kashparov KI, Starovoitov II, Boronin AM (2002) The bioutilization of thiodiglycol (a breakdown product of mustard gas): isolation of degraders and investigation of degradation conditions. Microbiologiia 71:247–252

Wübbeler JH, Lütke-Eversloh T, Vandamme P, Van Trappen S, Steinbüchel A (2006) *Tetrathiobacter mimigardefordensis* sp. nov., isolated from compost, a betaproteobacterium capable of utilizing the organic disulfide 3, 3′-dithiodipropionic acid. Int J Syst Evol Microbiol 56:1305–1310

Wübbeler JH, Bruland N, Kretschmer K, Steinbüchel A (2008) A novel pathway for the catabolism of the organic sulfur compound 3, 3′-dithiodipropionic acid via 3-mercaptopropionic acid and 3-sulfinopropionic acid to propionyl-CoA by the aerobic bacterium *Tetrathiobacter mimigardefordensis* strain DPN7. Appl Environ Microbiol 74: 4028–4035

Wübbeler JH, Bruland N, Wozniczka M, Steinbüchel A (2009) Biotechnological applications and microbial utilisation of the organic sulphur compounds 4,4′-dithiodibutyric acid, 3,3′-dithiodipropionic acid and 3,3′-thiodipropionic acid (Submitted)