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ORIGINAL ARTICLE

New insights into the biodegradation of thiodiglycol, the hydrolysis product of Yperite (sulfur mustard gas)

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Abstract

Aims: To isolate thiodiglycol (TDG)-degrading bacteria, the mustard gas hydrolysis product, and to characterize the metabolites formed and the enzymes involved in the degradation.
Methods and Results: Two strains, identified as *Achromobacter xylosoxydans* G5 and *Paracoccus denitrificans* E4, isolated from a petroleum-contaminated soil, utilized TDG as sole carbon and sulfur source. During the degradation of TDG by strain E4 [(2-hydroxyethyl)thio] acetic acid (HETA), thiodiglycolic acid (TDGA) and *bis*-(2-hydroxyethyl)disulfide (BHEDS) were identified by gas chromatography–mass spectrometry analysis, while HETA and TDGA were identified for strain G5. Two-dimensional isoelectric focussing-gel electrophoresis (2-D IEF/SDS–PAGE) maps of protein extracts of *P. denitrificans* E4 grown on TDG showed a spot identified as a methanol dehydrogenase. Increased expression of a putative *iscS* gene, involved in sulfur assimilation, was observed in TDG-grown cells of *A. xylosoxydans* G5.
Conclusions: TDG degradation by *P. denitrificans* E4 occurred through two pathways: one involved cleavage of the C–S bond of HETA, yielding BHEDS and the other, oxidation of the alcoholic groups of TDG, yielding TDGA. The cleavage of the C–S bond of TDGA gave mercaptoacetic acid, further oxidized to acetate and sulfate.
Significance and Impact of the Study: Increased knowledge of TDG-degrading bacteria and the possibility of using them in a tailored-two-stage mustard gas destruction process.

Introduction

Thiodiglycol (TDG), namely *bis*-(2-hydroxyethyl) sulfide, is a chemical used in both private industry (textile dyeing, printing solvents and other industrial applications) and the military sector. TDG is the precursor and the main hydrolysis product of sulfur mustard gas or Yperite, i.e. *bis*-(2-chloroethyl) sulfide, the most produced and stored chemical warfare agent. Yperite is markedly cytotoxic and is also a carcinogenic and mutagenic alkylating agent. Furthermore, it is a vesicant that is slightly soluble in water and, under alkaline conditions, hydrolysable, with the formation of the un-chlorinated water-soluble compound, TDG (Munro *et al.* 1999). Since 1999, the destruction of Yperite has mainly been through incineration or neutralization, both very expensive processes, and, compared with these conventional processes, bioremediation offers a very advantageous alternative method because of its minimal environmental impact and its cost effectiveness. Biodegradation of mustard gas has not been achieved under laboratory conditions probably because of its toxicity to micro-organisms (Munro *et al.* 1999).

A two-step process for the destruction of mustard gas has already been conceived and developed: the first step

lies in the chemical hydrolysis of the gas (Harvey *et al.* 1998) or the chemical treatment of mustard–lewisite mixtures (Boronin *et al.* 2000), and the second step the biodegradation of the detoxification product, namely TDG. Although TDG has a lower toxicity, it has to be destroyed and not left to accumulate in the environment, as specified by the Chemical Weapons Convention of the Organization for the Prohibition of Chemical Weapons (U.S. Arms Control and Disarmament Agency 1993).

In this context, the biodegradation of mustard gas can be closely related to the utilization of TDG by microorganisms. Some organisms such as *Candida rugosa* (Kawashima 1995) and *Alcaligenes xylosoxydans* PGH10 (Garcia-Ruiz *et al.* 2002) transform TDG through oxidation. Other bacteria, such as *Alc. xylosoxydans* ssp. *denitrificans* strain TD2 (Ermakova *et al.* 2002), *Pseudomonas* sp. 8-2 (Medvedeva *et al.* 1998), *Alc. xylosoxydans* ssp. *xylosoxydans* strains SH42 (Harvey and DeFrank 1993) and SH91 (Kim *et al.* 1997; Lee *et al.* 2000) use TDG as sole carbon source. The utilization of TDG by *Alc. xylosoxydans* SH91 gave [(2-hydroxyethyl)thio]acetic acid (HETA) and thiodiglycolic acid (TDGA) as intermediate products, which were further completely degraded (Lee *et al.* 1997, 2000). *Rhodococcus rhodochrous* IGTS8 cultures are instead able to use TDG as the sulfur source for growth (Kilbane and Jakowski 1996).

Recently, a *Pseudomonas* sp. tolerant to the organochlorine substances in mustard gas hydrolysate has been found to be capable of utilizing mustard gas hydrolysis products and of degrading TDG (Medvedeva *et al.* 2007).

The present paper reports an investigation aimed at studying the TDG catabolism pathway in two new bacterial isolates, performed by analysing: (i) the intermediates formed in the course of TDG degradation in the absence and presence of 4-methyl pyrazole, an alcohol dehydrogenase inhibitor; (ii) PCR amplifications of genes coding for enzymes potentially involved in TDG catabolism and in S assimilation; (iii) 2D protein profiles of strains grown on TDG and on succinate. The identification of metabolites led us to the determination of two possible metabolic pathways for TDG degradation by *Paracoccus denitrificans* strain E4.

Materials and methods

Chemicals

TDG, 2,2′-sulfonyldiethanol ($DGSO_2$; 60–65% by wt. aqueous solution), diethylene glycol, ethylene glycol, methanol, ethanol, 1-butanol, hexane, dodecane and hexadecane were purchased from Sigma-Aldrich Chemie (Steinheim, Germany); TDGA, diethyl sulfide, 2-ethoxyethanol, 2-mercaptoethanol (MeET), mercaptoacetic acid (MAA), dimethyl sulfoxide (DMSO) and sulfoacetic acid (SAA) from Fluka Chemie (Buch, Switzerland). Ethyl 2-hydroxyethyl sulfide was purchased from Thermo Fisher Scientific (Geel, Belgium). All other chemicals were reagent grade.

Synthesis of 2,2′-sulfenyldiethanol

2,2′-Sulfenyldiethanol (DGSO), a possible metabolite of TDG biodegradation, was synthesized as follows: an aliquot of 1·65 ml of H_2O_2 was slowly added to a mixture of 1·5 ml TDG and 7·5 ml acetone, maintained at 3–4°C in an ice bath. The mixture was stirred overnight on ice, then the liquid phase was decanted and the formed crystals were purified by precipitation with acetone from a warmed aqueous solution. The DGSO yield was 0·33 g. Analytical data of DGSO are reported in Table 1.

Enrichment, isolation, identification and characterization of TDG-degrading strains

Soil samples taken from a petroleum-contaminated area were used to enrich the bacterial strains capable of utilizing TDG as sole C and S source. Three grams of soil were mixed with 27 ml of sterile minimal elemental (ME) medium supplemented with 4·8 mmol l^{-1} of TDG. Cycloheximide (0·1 g l^{-1}) was added to the medium to inhibit the growth of eukaryotic cells. The cultures were shaken at 150 rev min^{-1} at 30°C for 7 days. The components of ME were as follows (g l^{-1}): NH_4Cl, 2·0; $MgCl_2{\cdot}6H_2O$, 0·2; K_2HPO_4, 4·0; NaH_2PO_4, 4·0; $CaCl_2{\cdot}2H_2O$, 0·001 and $FeCl_3{\cdot}6H_2O$, 0·001. The medium was supplemented with microelements in the following concentrations (mg l^{-1}): $FeCl_2{\cdot}4H_2O$, 1·5; $CoCl_2{\cdot}6H_2O$, 0·19; $CuCl_2{\cdot}2H_2O$, 0·017; H_3BO_3, 0·06; $ZnCl_2$, 0·07; $MnCl_2\ 4H_2O$, 0·1; $Na_2MoO_4{\cdot}2H_2O$, 0·036; and $NiCl_2{\cdot}6H_2O$, 0·024. The final pH of ME was adjusted to 7 with a 1-mol l^{-1} NaOH and the medium was autoclaved at 121°C for 20 min. When necessary, a solution of 6 mmol l^{-1} Na_2SO_4 was added as sulfur source.

The culture was adapted to utilize TDG through seven subsequent passages by re-inoculation of 1-ml cell suspensions in 20 ml of fresh ME medium. The enriched culture was then serially diluted and seeded on 10-fold diluted Tryptic Soy agar (TSA 0·1×; Difco, Detroit, MI, USA). After 8 days of incubation at 30°C, the colonies that appeared on the plates were isolated and tested for capability to grow on TDG.

The growth of the isolates on TDG and on TDG-related or -unrelated substrates was tested in 100-ml flasks containing 20 ml of ME with the appropriate compound added. The flasks were shaken at

Table 1 Analytical data of TDG and related metabolites

	GC* (Rt)	HPLC (Rt)	^{1}H-NMR (DMSO-d_6)	GC–MS or MS (EI)†
TDG	7·02	4·20	4·75 (t, 2H, 2 OH) 3·50 (q, 4H, 2 CH_2-O-) 2·58 (t, 4H, 2 CH_2-S-)	266 (0%), 251 (7%), 191 (7%), 176 (30%), 161 (15%), 130 (20%), 117 (32%), 116 (90%), 103 (65%), 101 (25%), 87 (25%), 75 (42%), 73 (100%)
OTO	2·29	nd	4·69 (m, 1H, O-CH-O) 4·17 and 3·69 (2 m, 2 H, S-C-CH2-O), 2·67-2·45 (m, 3H, O-C-CH-S-CH2), 2·32 (bd, 1H, O-C-CH-S)	192 (32%), 170 (20%), 133 (17%), 119 (85%), 103 (73%), 79 (98%), 77 (75%), 73 (100%)
HETA	7·59	3·76	3·54 (t, 2H, CH_2-O-) 3·24 (s, 2H, S-CH_2-CO) 2·68 (t, 2H, CH_2-S-)	280 (10%), 265 (15%), 204 (7%), 190 (52%), 133 (13%), 117 (18%), 103 (25%), 89 (9%), 73 (100%)
TDGA	8·33	3·56	3·35 (s, -CH_2-S-)	294 (10%), 279 (7%), 204 (55%), 117 (12%), 87 (7%), 79 (2%), 75 (45%), 73 (100%)
BHEDS	9·46	3·68	4·85 (m, 2H, 2 OH) 3·62 (t, 4H, 2 CH_2-O-) 2·79 (t, 4H, 2 CH_2-S-)	298 (30%), 182 (11%), 154 (8%), 133 (28%), 117 (56%), 107 (48%), 103 (28%), 92 (14%), 73 (100%)
DGSO	9·69	3·75	4·95 (t, 2H, 2 OH) 3·80 (bq, 4H, 2 CH_2-O-) 2·90 (m, 4H, 2 CH_2-S-)	EI: 138 (7%) 94 (65%), 76 (100%), 63 (50%) GC–MS: 283 (3%) 267 (45%), 238 (18%), 166 (62%), 135 (30%), 117 (100%), 103 (25%), 87 (7%), 73 (76%), 59 (10%)
$DGSO_2$	9·87	3·62	5·06 (t, 2H, 2 OH) 3·80 (q, 4H, 2 CH_2-O-) 3·26 (t, 4H, 2 CH_2-S-)	298 (0%) 283 (82%), 239 (12%), 133 (7%), 117 (53%), 101 (22%), 88 (12%), 75 (58%), 73 (100%)

*The reported retention times (Rt) are relative to trimethylsilyl derivatives of the indicated products.
†All the reported GC–MS data are relative to trimethylsilyl derivatives. For DGSO are reported also the data for the compound before derivatisation (EI).

150 rev min^{-1} at 30°C. All growth experiments were done at least twice.

TDG, diethylene glycol, ethylene glycol, diethyl sulfide, ethyl 2-hydroxyethyl sulfide, 2-ethoxyethanol, DGSO, $DGSO_2$, MeET, MAA, SAA, DMSO, methanol, ethanol 1-butanol, hexane, dodecane and hexadecane were separately added to the cultures; TDGA sodium salt and succinate were supplied as aqueous solutions. All compounds and solutions were added to the medium after filter sterilization with a 0·45-μm-pore-size membrane filter.

G5 and E4 strains were identified by phylogenetic analyses (neighbour-joining method). Overnight cell suspensions grown on LB medium (100 μl OD_{600} = 2·0) were centrifuged at 13 000 ***g*** for 7 min and the pellet suspended in 100 μl of sterile MilliQ water, 100 μl of 10 mmol l^{-1} Tris–HCl buffer (pH 8·0) and 13 μl of Proteinase K (1 mg ml^{-1}). The mix was incubated for 2 h at 55°C, then boiled for 10 min and centrifuged at 13 000 ***g*** for 5 min. The DNA-containing supernatant was withdrawn and put into sterile microtubes. TDG-degrading strains were identified on the basis of sequence analysis of near-complete 16S rRNA gene, using eubacterial universal primers P27f and P1495r referred to an *Escherichia coli* nucleotide sequence of 16S rRNA gene (Weisburg *et al.* 1991).

TDG degradation experiments

TDG degradation experiments were carried out in triplicate in 500 ml bottles, each containing 90 ml of ME medium. TDG was added to the bottles at the required concentration. Each bottle was inoculated with 10 ml of the cell suspension grown on TDG, then closed with a butyl rubber stopper and incubated under shaking at 150 rev min^{-1} at 30°C; samples were taken at intervals to determine cell density, TDG consumption and metabolite formation.

To test the involvement of an alcohol dehydrogenase activity in the initial breakdown on TDG, the strains were grown in 100 ml bottles containing 20 ml of ME medium containing 10 mmol l^{-1} of TDG in both the presence and absence of 1 mmol l^{-1} 4-methylpyrazole (Sigma-Aldrich), an inhibitor of alcohol dehydrogenase (Brimfield *et al.* 1998). Cell density was measured at appropriate intervals,

while the metabolite concentrations in the culture broth were measured only at the end of the experiments.

Analytical procedures and metabolite extraction

Growth was monitored by recording the increase in $OD_{600\ nm}$ with a model UV-1700 spectrophotometer (Beckman model DU 640). TDG, HETA and TDGA concentrations were monitored by HPLC chromatograph (Merck-Hitachi L6200 Intelligent pump with L4000 UV detector; Japan Servo Co., Japan) equipped with a column Supelco C18 Discovery (25-cm long). A mixture of water–acetonitrile (60 : 40) was the mobile phase and the flow rate was 0·7 ml min^{-1}. The compounds were detected by measuring UV absorbance at 220 nm and their relative retention times (Rt) are reported in Table 1. The concentrations of TDG and TDGA were calculated using standards of known concentrations; that of HETA using samples extracted from culture broths. To identify the metabolites, ^{1}H-NMR analyses of the crude organic extracts were performed with a Bruker Ultrashield 400 using DMSO-d_6 as solvent. The values of the signals are reported in Table 1. After derivatization of crude organic extracts with *bis*-(trimethylsilyl)trifluoroacetamide, gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) analyses were performed. The GC analyses were performed using a DANI 1000 gas-chromatograph equipped with an FID detector (hydrogen 0·9 bar, air 1·0 bar and nitrogen 1·0 bar) and a fused silica capillary column WCOT-CP-Sil 8 CB Chrompack (25 m × 0·32 mm i.d.), carrier helium (0·8 bar), injector temperature 200°C, detector temperature 250°C, oven temperature 90°C (2 min), temperature increase 10°C min^{-1}, final isotherm 250°C, injection volume 2 μl. All chromatograms and data were generated and processed by DANI Data Station ver. 1.7 software (Dani Instruments, Cologno Monzese, Italy). The same column and chromatographic conditions were used for GC–MS, and mass spectra were recorded on a VG 7070 EQ instrument. For the extraction of the metabolites the culture broth, after pH control, was treated with solid NaCl until saturation, and then filtered into a separatory funnel. In the presence of acidic pH, the broth was extracted by ethyl acetate (3 times with a volume of ethyl acetate as 3/5 of the volume of aqueous solution) and the organic layers collected, dried with solid Na_2SO_4 and filtered. The organic solvent was eliminated by evaporation under reduced pressure. In the presence of neutral pH, the broth was extracted as described above and subsequently acidified by adding a 6-mol l^{-1} HCl solution to give a pH of 2–3 and then a second extraction was performed. The obtained crude extracts (from the neutral and acidic aqueous phases) were dissolved separately in 5-ml ethyl acetate; 4 ml of the solution was dried and submitted to ^{1}H-NMR analysis and 1 ml was transferred drop by drop into a glass vial (0·1 ml) under dry nitrogen stream to evaporate the solvent and prepare the sample for the silylation procedure. To the raw material, 100 μl of pyridine and 100 μl of *bis*-(trimethylsilyl)trifluoroacetamide were added and the mixture maintained at 40°C for 30 min, after which it was ready for gas-chromatographic or gas–mass analyses. The Rt values and the spectroscopic properties of TDG and the related metabolites detected in the culture broths are shown in Table 1.

To determine the presence of any sulfates, aliquots of the culture broth (0·5 ml), filtered through a 0·2-μm-pore-size membrane filter, were acidified with 6-mol l^{-1} HCl (pH 2) and 0·5 ml of a 5-mol l^{-1} $BaCl_2$ solution were added to the sample. The precipitation of $BaSO_4$ revealed sulfate formation.

Molecular methods

Genes potentially involved in TDG degradation were searched for by PCR amplification of the following gene fragments: *tbmA* for toluene 2-monoxygenase, responsible for growth on ether and thioether compounds by *Burkholderia cepacia* G4/PR1 (Hur *et al.* 1997); *bdhA* for butanol dehydrogenase in *Aeromonas hydrophila* JMP636 (AF388671; Schmidt and Pemberton, unpublished data). An *iscS* gene for cysteine desulfurase involved in sulfur assimilation process was also searched (Mihara and Esaki 2002). Table 2 shows the primers used. Primers for *iscS* were designed on the consensus region of *Pseudomonas fluorescens* Pf-5 (CP000076) and *Burkholderia pseudomallei* K96243 (BX571965), *Ralstonia pickettii* 12J (CP001068) and *Bradyrhizobium* sp. BTAi1 (NC009485), and primers for *bdhA* on the consensus region of *Aer. hydrophila* JMP636 (AF388671), *Burkholderia pseudomallei* 1655 (DS981341) and of *Rhizobium leguminosarum* bv. *viciae*

Table 2 Oligonucleotide primers used for PCR amplification of genes potentially involved in TDG degradation and in the S assimilation pathway

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Theoretical PCR product size (bp)	References
tbmA	AAGACCTATCCSGARTACGT	GGCTGGATCWGRCCTGCSAGGAA	1200	Cavalca *et al.* (2004)
bdhA	GTCCCTATCTTGTCAAA	CGCCCAGGCTCGCGACCA	710	This work
iscS	AAGCGGATCGAGCTGTGMGC	GYGAAGGCTTCGARGTGACC	720	This work

S is for C + G; R, for A + G; W, for A + T, M, for A + C, Y, for C + T.

3841 (NC008380). The PCR mix consisted of deoxynucleotide triphosphates at 200 μmol l^{-1} each, 0·30 μmol l^{-1} each primer, 2·0 mmol l^{-1} $MgCl_2$, 1× PCR buffer and 1 U of *Taq* DNA polymerase (Invitrogen, Paisley, UK) in a total volume of 50 μl. The annealing temperatures were 55°C for *tbmA*, 51°C for *bdhA* and 52°C for *iscS*.

For the semi-quantitative transcriptional analysis, a reverse transcriptase (RT)-PCR approach was taken. DNAse-treated total RNAs were reverse transcribed to first-strand cDNA using Stratascript Reverse Transcriptase (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions; cDNA aliquots from the RT reactions were used for the amplification of *iscS* gene with *Taq* DNA Polymerase (Invitrogen, UK), using the primers for *iscS* listed in Table 2. The PCR products were separated on 2% agarose gels along with O'gene Ruler 1-kb ladder (Fermentas, Burlington, ON, Canada). As positive control, genomic DNA from the same strain was used as template. As negative control, not-reverse transcribed DNAse-treated RNA was used as template in order to avoid false-positive results.

Total RNA was extracted from *Achromobacter xylosoxydans* G5 cultures after 5-day growth in ME medium supplemented either with TDG (6 mmol l^{-1}) or succinate and Na_2SO_4 (6 mmol l^{-1} each). Samples (10^{10} cells) were spun down (8000 ***g*** for 15 min), the cell pellets were suspended in 3·25-ml lysing buffer (80 mmol l^{-1} Tris–HCl buffer pH 7·6 containing 800 mmol l^{-1} NaCl and 8 mmol l^{-1} EDTA) and digested for 1 h at 50°C with 1 mg proteinase K. RNA was further extracted with phenol: chloroform: isoamyl alcohol (25 : 24 : 1) and chloroform: isoamyl alcohol (24 : 1). RNA was recovered after precipitation with ethanol and treated with RNAse-free DNAse I (GE Healthcare, Stockholm, Sweden).

2-D IEF/SDS–PAGE and image analysis

G5 and E4 strains were grown separately in 100 ml ME medium containing either succinate and Na_2SO_4 (6 mmol l^{-1} each) or TDG (6 mmol l^{-1}). Cells were harvested by centrifugation (15 min at 10 000 ***g*** at 5°C) after 3 days of growth, washed twice with 50 mmol l^{-1} Tris–HCl buffer (pH 7·5) containing 0·3 mol l^{-1} NaCl. Each cell pellet was suspended in 10 ml of lysis buffer [7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 2% CHAPS, 65 mmol l^{-1} 1,4-dithiothreitol (DTT)] and disrupted by sonication (Sonifier 250; Branson, Marchan, Ontario, Canada). The resultant lysate was centrifuged at 13 000 ***g*** for 10 min at 4°C, and the supernatant was collected. The protein concentration was determined using 2D Quant Kit (Amersham Bioscience).

Isoelectric focussing was performed on 7 cm, pH 3–10 linear IPG strips (GE Healthcare, Milan, Italy). The strips were rehydrated overnight in a solution consisting of 7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 2% CHAPS, 65 mmol l^{-1} 1,4-dithiothreitol, 2% IPG buffer pH 3–10 (GE Healthcare, Milan, Italy) containing 50 μg of the protein sample. Strips were focussed at 6500 V-h, with a maximum of 3000 V, at 20°C using the Multiphor II electrophoresis unit (Amersham Biosciences, Milan, Italy). Prior to the second dimension, the strips were incubated in equilibration buffer (375 mmol l^{-1} Tris–HCl, pH 8·8, 6 mol l^{-1} urea, 2% SDS, 20% glycerol) with 65 mmol l^{-1} DTT for 15 min, then with 243 mmol l^{-1} iodoacetamide in the same buffer without DTT for 10 min. The separation was performed in 12% SDS–PAGE gels using a mini-PROTEAN III cell (Bio-Rad, Milan, Italy). Three gel replicates were produced for each sample. The gels were stained with Coomassie Brilliant Blue (CBB), scanned in an Epson Expression 1680 Pro Scanner and analysed with IMAGEMASTER 2-D Platinum Software (GE Healthcare, Milan, Italy).

Protein in-gel digestion and liquid chromatography electrospray ionization tandem mass spectrometry

Protein spot was excised from CBB stained 2-DE gel and in-gel digested as described by Magni *et al.* (2007).

The obtained sample was analysed using a Finnigan LCQ Deca XP MAX IT mass spectrometer equipped with a Finnigan Surveyor (MS Pump Plus) HPLC system (Thermo Fisher Scientific). Chromatography separations were conducted on a BioBasic C18 column (180 μm i.d., 150-mm long, 5-μm particle size), using a linear gradient from 5% to 80% solvent B [solvent A: 0·05% (v/v) formic acid; solvent B: ACN containing 0·05% (v/v) formic acid] with a flow of 2·5 μl min^{-1}. The capillary temperature and the spray voltage were set at 220°C and at 3·0 kV, respectively. For MS/MS scans, the normalized collision energy was set at 35%. Acquisitions were performed in data-dependent MS/MS scanning mode and enabling a dynamic exclusion window of 3 min. Protein identification was conducted by TurboSEQUEST® Bioworks Browser 3·2 (Thermo Fisher Scientific) software through correlation of uninterpreted spectra to the entries of the *P. denitrificans* protein database downloaded from the National Center for Biotechnology Information (NCBI). The software was set to allow two missed cleavages per peptide and to take into account cysteine carboxyamidomethylation and methionine oxidation. The parent ion and fragment ion mass tolerance were set to ±2 and ±1 Da, respectively. In order to identify proteins, only peptides with Xcorr > 1·5 (+1 charge state), >2·0 (+2 charge state), >2·5 (≥3 charge state) and peptide probability $<1 \times 10^{-3}$ were considered. Theoretical molecular weight and pI of the characterized protein were

calculated by processing sequence entry at http://www.expasy.org/tools/pi_tool.html.

Sequence accession numbers

The nucleotide sequences of the 16S rRNA genes of strains E4 and G5 were registered in GenBank/EMBL as accession numbers FM207553 and FM207511, respectively. The nucleotide sequence of putative *iscS* gene has been registered as accession number EU523110.

Results

Isolation, identification and growth characteristics of TDG-degrading bacteria

One enrichment culture capable of growing on 4·8 mmol l^{-1} of TDG was obtained. Thirty colonies from the plates of the culture grown on TSA 0·1× were isolated and re-streaked on TSA 0·1× to obtain pure cultures. Among the isolates, two strains, named E4 and G5, utilized TDG as sole C and S source.

Phylogenetic analysis based on 16S rRNA sequences indicated that the strains E4 and G5 were identified as *P. denitrificans* (99·1% sequence identity to GenBank acc. num. CP000490) and *A. xylosoxydans* (93% sequence identity to GenBank acc. no. AY468369), respectively.

Table 3 shows the different growth characteristics of the strains to assimilate substrates with molecular structure similar to TDG, intermediates of TDG oxidation, and other substrates as alcohol and aliphatic hydrocarbons. They grew on TDG, TDGA, ethylene glycol, ethanol and on 1-butanol, but not on ethyl 2-hydroxyethyl ether, diethylene glycol, diethyl sulfide, DGSO, $DGSO_2$, DMSO and MeET. *Paracoccus denitrificans* E4 grew well on MAA and SAA, possible derivatives of TDG degradation and on methanol, but very poorly on ethyl 2-hydroxyethyl sulfide. On the contrary, G5 scarcely grew on methanol and failed to grow on MAA and SAA. In addition, the growth of G5 on TDG in the presence of MAA was inhibited and the inhibitory effect increased with increasing MAA concentration (data not shown). Finally, *A. xylosoxydans* G5 grew on middle long-chain alkanes. All the results indicated a strain specificity in recognizing as growth substrates only the compounds containing a sulfide bound to oxidized chains as in the TDG molecule and not to alkylic chains as in diethylsulfide.

Table 3 Growth of E4 and G5 strains in ME medium supplemented with various organosulfur compounds and with related compounds

Substrate*	E4	G5
Thiodiglycol (TDG)	+	+
Diethylene glycol	–	–
Diethyl sulfide	–	–
Ethyl 2-hydroxyethyl sulfide	±	–
Ethyl 2-hydroxyethyl ether	–	–
2,2′-Sulfenyldiethanol (DGSO)	–	–
2,2′-Sulfonyldiethanol($DGSO_2$)	–	–
Thiodiglycolic acid (TDGA)	+	+
Dimethyl sulfoxide (DMSO)	–	–
2-Mercaptoethanol (MeET)	–	–
Mercaptoacetic acid (MAA)	+	–
Sulfoacetic acid (SAA)	+	–
Methanol	+	±
Ethanol	+	+
Ethylene glycol	+	+
1-Butanol	+	+
Hexane	–	–
Dodecane	–	+
Hexadecane	–	+

*Each compound was added at a final concentration of 5 mmol l^{-1}. Growth after 4 days of incubation: growth, + ($OD_{600\ nm}$ > 0·3); scarce growth, ± ($OD_{600\ nm}$ 0·10–0·18); absence of growth, – ($OD_{600\ nm}$ < 0·08).

Identification of metabolites in growing cell experiments

Figure 1 shows profiles of pH variation (Fig. 1a), TDG disappearance and $OD_{600\ nm}$ increase (Fig. 1b) of the culture broths of the isolates. During growth and TDG consumption, the pH values of the cultures dropped from 7·2 to 5·2 for strain E4 and from 7·0 to 5·0 for strain G5 in 48 h. The data concerning TDG degradation by *P. denitrificans* E4 are shown in Fig. 2. HETA, TDGA, i.e. the mono and diacid derivatives of TDG, and small amounts of *bis*-(2-hydroxyethyl)disulfide (BHEDS), which is the oxidation product of MeET were detected. At 36 h of incubation, HETA was the dominant metabolite and the sulfate test was negative. HETA and BHEDS disappeared at 72 and 96 h, respectively, while TDGA was still present. The sulfate release became evident when the pH dropped to 5·2.

TDGA was the sole metabolite extracted from the cultures of *A. xylosoxydans* G5. At 24 h of incubation, there was the presence of 3 mmol l^{-1} of TDGA, which became completely degraded at 40 h to coincide with the sulfate release.

Growth of strains E4 and G5 and TDG oxidation were affected differently when 4-methyl pyrazole was added to the cells growing on TDG. A growth inhibitory effect of 78% and 30% for E4 and G5 was observed, respectively (Fig. 3). At the end of the incubation time (192 h), 1,4-oxathian-2-ol (OTO), as the main metabolite, together with traces of HETA and TDGA were detected in *P. denitrificans* E4 cultures. OTO was derived from the cyclization of [(2-hydroxyethyl)thio]acetic aldehyde

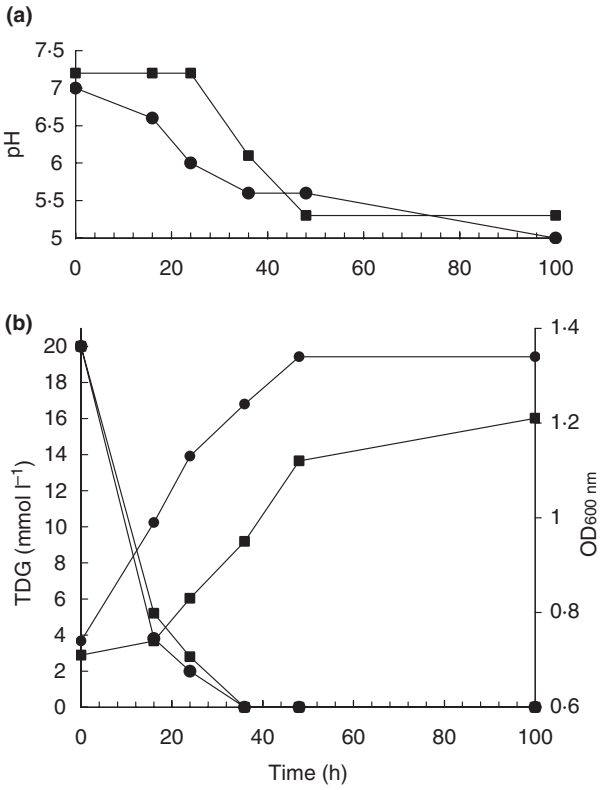

Figure 1 Utilization of 20 mmol l^{-1} of TDG as C and S source by *Paracoccus denitrificans* E4 (■) and *Achromobacter xylosoxydans* G5 (●): (a) pH drop during the growth and (b) TDG consumed and increase of $OD_{600\ nm}$. Each value is the mean of three determinations and the SD was <8%.

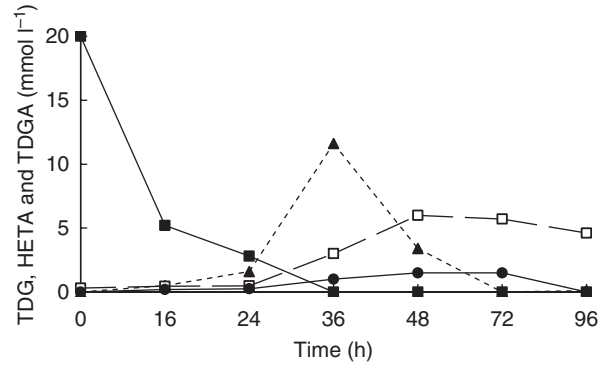

Figure 2 Time course of 20 mmol l^{-1} of TDG degradation (■); HETA (▲), TDGA (□) and BHEDS (●) formation by *Paracoccus denitrificans* E4. Each value is the mean of three determinations and the SD was <8%.

(HETAL). Instead, traces of HETA were detected in the cultures of *A. xylosoxydans* G5. The analytical data of these metabolites are reported in Table 1.

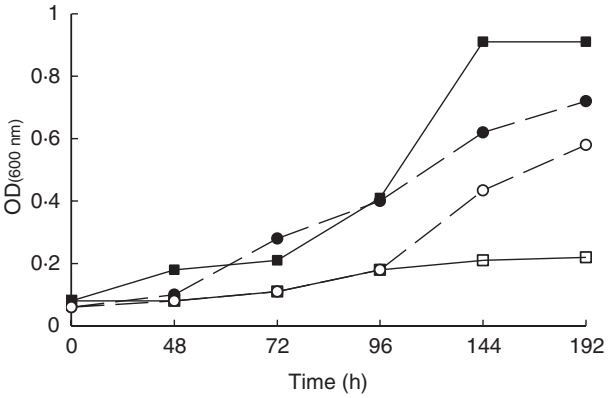

Figure 3 Growth of *Paracoccus denitrificans* E4 (■) and *Achromobacter xylosoxydans* G5 (●) on TDG (10 mmol l^{-1}) in the absence (full symbol) and presence (empty symbol) of 4-methylpyrazole (1 mmol l^{-1}). Each value is the mean of three determinations and the SD was <10%.

Molecular analysis

Molecular determinants for enzymes potentially involved in TDG degradation and sulfur assimilation were searched for by PCR amplification of gene fragments with primers for *tbmA*, *bdhA* and *iscS* (Table 1). None of the strains yielded amplicons related to *tbmA* and *bdhA*. This implies that genes with different nucleotide sequence composition than the known *tbmA* and *bdhA* genes may be present in strains E4 and G5, or alternately these genes may be absent. However, a fragment of 720 bp was amplified with primers for *iscS* in *A. xylosoxydans* G5 grown on TDG, in agreement with the expected size value. The *iscS* nucleotide sequence of *A. xylosoxydans* G5 revealed a similarity of 94% to part of *iscS* gene of *Bordetella parapertussis* 12822 (GenBank acc. no. BX640429). The deduced aminoacidic sequence showed a similarity of 78% with the cysteine desulfurase IscS of *Pseudomonas aeruginosa* PAO1 (Protein acc. no. Q9HXI8).

To determine whether the *iscS* gene was actually transcribed in response to TDG, transcriptional analysis was performed on total RNA isolated from TDG-grown and succinate-grown cells of *A. xylosoxydans* G5 (Fig. 4). With the use of a primer for *iscS*, an expected fragment of 720 bp was amplified from the total RNA extracted from cells grown on TDG, while the product was barely amplified from cells grown on succinate, thus indicating that the growth of *A. xylosoxydans* G5 on TDG led to increasing the expression of the *iscS* gene.

2-D electrophoretic analysis

Total protein extracts from the strains, obtained as described in section 'Materials and methods', were used

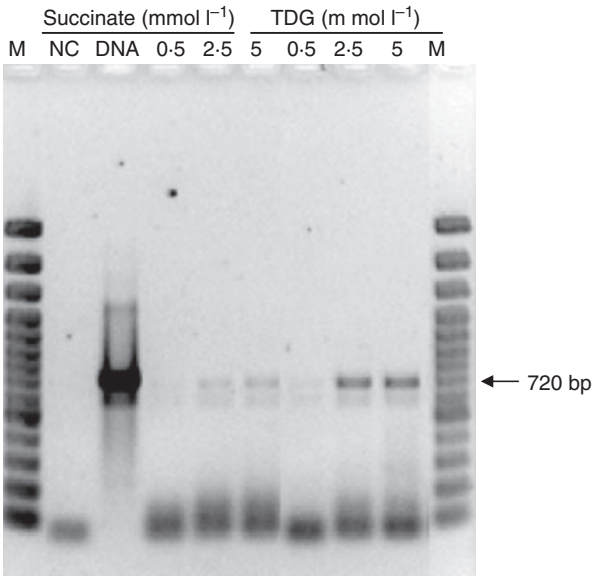

Figure 4 Transcriptional analysis of *iscS* gene for cysteine desulfurase of *Achromobacter xylosoxydans* G5. Total RNA from cells grown on succinate or on TDG was used as template for amplification. NC, negative control; DNA, positive control; M, O'Gene Ruler 1-kb ladder.

to generate two-dimensional isoelectric focusing-gel electrophoresis (2-D IEF/SDS–PAGE) maps (Fig. 5). Globally, a comparison of the maps of the strains grown on succinate or TDG evidenced several differences in the protein profiles. The map of TDG-grown G5 cells denotes a large increase in the amount of one spot around 55 kDa with pI 5·5 and two spots near 45 kDa with pI between 7·5 and 8·5. In regard to strain E4, the most important change in the maps concerns the spot of 70 kDa with pI 5·0. This spot was excised from the gel, digested with trypsin and analysed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), as described in section 'Materials and methods'. The protein was identified as a methanol dehydrogenase [MDH subunit 1 precursor from *P. denitrificans* (acc. no. P12293), with theoretical MW and pI of 69 799 Da and 5·08, respectively] with amino acid coverage of 19·5% (eight peptides).

Discussion

The results presented here demonstrate that micro-organisms of different genera capable of TDG degradation may exist in contaminated sites. Previously, degradation studies of TDG have been mainly focussed on the catabolic pathway (Lee *et al.* 2000; Ermakova *et al.* 2002; Medvedeva *et al.* 2007) and up to now the genes and enzymes involved in TDG breakdown have not yet been characterized.

The initial breakdown of TDG can occur via three possible reactions: (i) oxidation of sulfur of TDG to DGSO and $DGSO_2$, (ii) C–S bond cleavage yielding MeET and (iii) oxidation of the alcoholic groups.

DGSO and as a minor metabolite $DGSO_2$, that can serve as a biological marker for sulfur mustard poisoning (Black and Read 1995), have been reported as

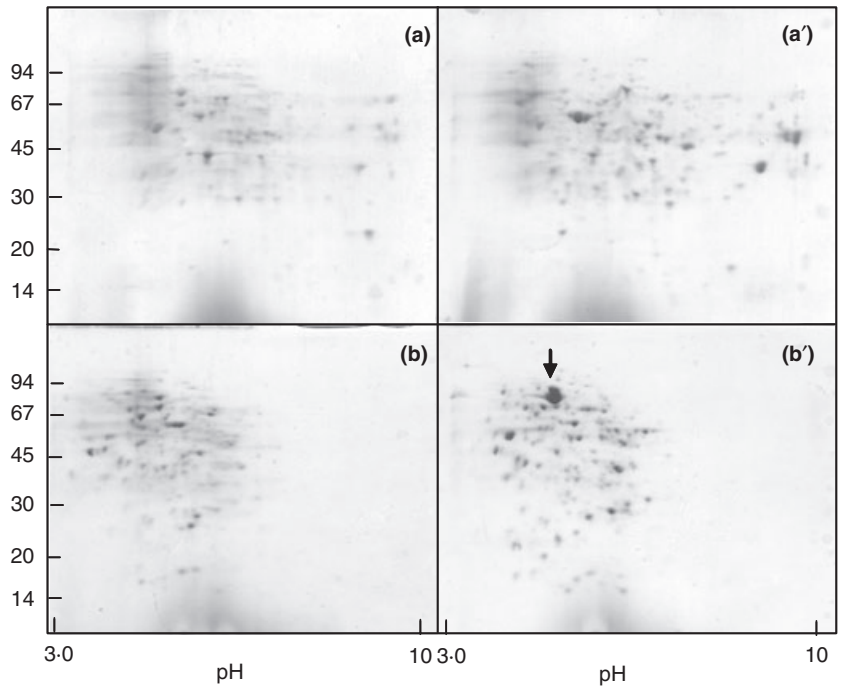

Figure 5 Coomassie blue-stained protein maps of strains: *Achromobacter xylosoxydans* G5 grown on succinate (a) and on TDG (a'); *Paracoccus denitrificans* E4 grown on succinate (b) and on TDG (b'). In panel b' the arrow shows the spot analysed by LC-ESI-MS/MS. Experimental details are given in section 'Materials and methods'.

in vivo metabolites of sulfur mustard in the urine of men and rats, providing evidence of the occurrence of oxidative processes other than the oxidation of the alcoholic group in TDG metabolism. Both the cytochromes, P450 and flavin monooxygenases, have been implicated in this type of transformation (Levi and Hodgson 1988).

DGSO and $DGSO_2$ were never found to be formed by E4 and G5 cells growing on TDG. This result, together with the incapacity of the strains to grow on DGSO and $DGSO_2$ led us to exclude that TDG degradation in these isolates occurred with the initial S oxidation. The C–S bond cleavage of TDG with the formation of MeET has been proposed by Medvedeva *et al.* (2007). *Paracoccus denitrificans* E4 and *A. xylosoxydans* G5 did not utilize MeET as carbon source to grow. These isolates attacked the TDG molecule by oxidizing both the primary alcohol groups to yield TDGA, according to Lee *et al.* (2000) and Ermakova *et al.* (2002). However, in *P. denitrificans* E4 cultures, the transient formation of small amounts of BHEDS, i.e. the oxidation product of MeET, was evidenced (Fig. 2). The transformation of MeET into BHEDS probably served E4 to preserve the MDH activity. This hypothesis can be supported by the results of Leonovich *et al.* (2001) which found that MeET inhibited the alcohol oxidase activity of *Pichia methanolica* growing on different carbon sources, methanol included. BHEDS could be subsequently oxidized to *bis*-(mercaptoacetic acid) disulfide and then cleaved to MAA.

A butanol/thiodiglycol dehydrogenase was proposed to carry out the initial oxidation of TDG by *Alc. xylosoxydans* SH91 (Lee *et al.* 1997). The failure in the amplification of the *bdhA* gene can exclude the involvement of such enzyme in G5 and E4 strains. Moreover, with regard to strain E4, the 2-D IEF/SDS–PAGE maps evidenced an important change of the spot of 70 kDa with pI 5·0 of protein profiles (Fig. 5). This protein spot was identified as an MDH subunit 1 precursor by LC-ESI-MS/MS analysis, thus indicating that MDH oxidizes TDG to HETAL. The capability of *P. denitrificans* E4 to utilize methanol as carbon source strengthened this finding. 4-Methylpyrazole inhibited TDG oxidation in *P. denitrificans* E4 and partially in *A. xylosoxydans* G5, probably suggesting that different dehydrogenases may be responsible for TDG oxidation.

On the basis of the overall experimental results obtained with *P. denitrificans* E4, we suggest for TDG degradation the pathways shown in Fig. 6. According to

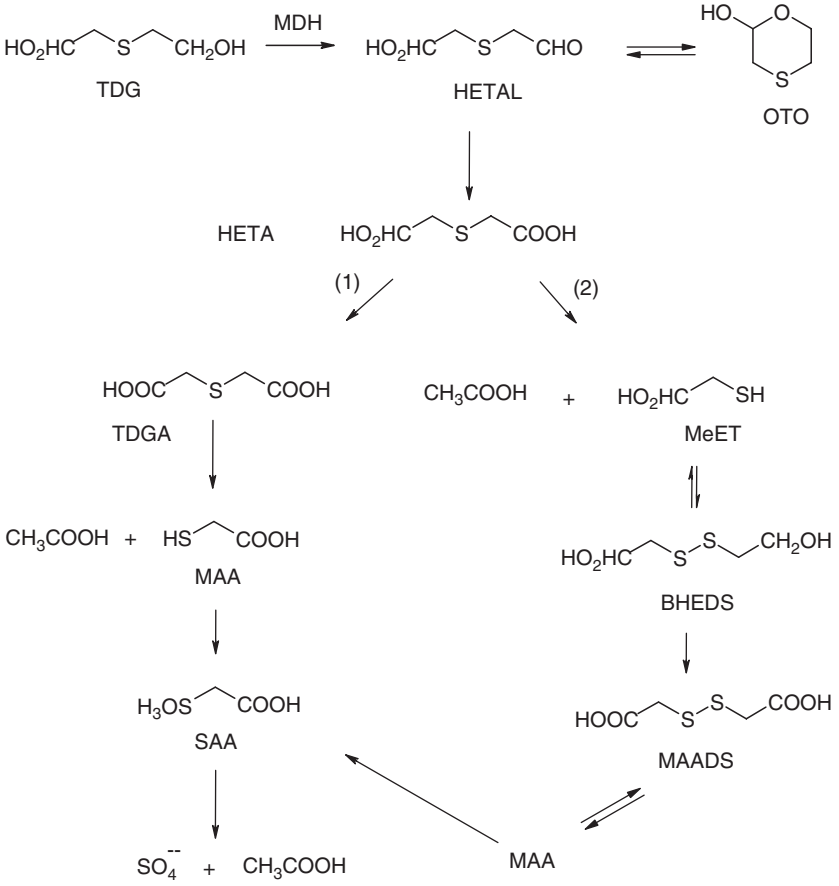

Figure 6 Proposed scheme for TDG degradation by metabolically active cells of *Paracoccus denitrificans* E4.

this scheme, TDG is oxidized to HETAL and then to HETA, whose subsequent oxidation can follow two routes. According to route 1, HETA is oxidized to TDGA with subsequent cleavage of the C–S bond to form MAA and acetic acid. MAA is oxidized to SAA, and the second C–S bond is cleaved forming acetate and sulfate. This pathway is in agreement with the growth characteristics and the identified metabolites. On the contrary, the presence of BHEDS in the culture broths allows us to consider that HETA, or even HETAL, can also be substrates for the enzymatic C–S bond cleavage. According to this possibility, in route 2, which represents a minor pathway, the C–S cleavage leads to the formation of acetic acid and MeET.

In G5, an *iscS* gene that encodes for cysteine desulfurase activity was amplified. Cysteine desulfurase is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that catalyses the conversion of L-cysteine to L-alanine and sulfane sulfur, via the formation of an enzyme-bound persulfide intermediate. However, cysteine desulfurase is not directly involved in the C–S cleavage of TDG because the enzyme requires substrates containing an amino group for binding PLP (Mihara and Esaki 2002). *IscS*-related genes have been found to play a role in the biosynthesis of NAD (Sun and Setlow 1993; Lauhon and Kambampati 2000) and in the mobilization of sulfur from cysteine to construct and repair Fe–S clusters in protein substrates that, in turn, catalyse essential redox reactions in critical metabolic pathways (Lauhon and Kambampati 2000; Tantalean *et al.* 2003). A high NAD-requirement to oxidize the two TDG alcoholic groups might explain the higher expression level of the *iscS* gene in TDG-growing, rather than in succinate-growing, cells of *A. xylosoxydans* G5. Besides, alcohol dehydrogenase of some micro-organisms utilizes NAD as cofactor and as coenzyme (Arfman *et al.* 1997; Zachariou *et al.* 1986).

In conclusion, two new bacterial strains that successfully degraded TDG, the product of Yperite hydrolysis, have been described in this study. The strains can serve as powerful agents for the bioremediation of TDG-contaminated soils as well as for two-stage-tailored Yperite destruction processes. Furthermore, in bioaugmentation processes, growth and activity of *P. denitrificans* E4 and *A. xylosoxydans* G5 can be monitored by the detection of functional markers easily identifiable.

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