Isolation of a *Xanthobacter* sp. degrading dichloromethane and characterization of the gene involved in the degradation

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Abstract A bacterial strain able to degrade dichloromethane (DCM) as the sole carbon source was isolated from a wastewater treatment plant receiving domestic and pharmaceutical effluent. 16S rDNA studies revealed the strain to be a *Xanthobacter* sp. (strain TM1). The new isolated strain when grown aerobically on DCM showed Luong type growth kinetics, with μ_{max} of 0.094 h^{-1} and S_m of 1,435 mg l^{-1}. Strain TM1 was able to degrade other aromatic and aliphatic halogenated compounds, such as halobenzoates, 2-chloroethanol and dichloroethane. The gene for DCM dehalogenase, which is the key enzyme in DCM degradation, was amplified through PCR reactions. Strain TM1 contains type A DCM dehalogenase (*dcmA*a), while no product could be obtained for type B dehalogense (*dcmA*b). The sequence was compared against 12 *dcmA*a from other DCM degrading strains and 98% or 99% similarity was observed with all other previously isolated DCM dehalogenase genes. This is the first time a *Xanthobacter* sp. is reported to degrade DCM.

Introduction

Dichloromethane (DCM) is one of the most abundant pollutants in waters and in the atmosphere, with a yearly industrial production in the US of around 90 million tons (Ames et al. 2000). It is used as a solvent in the production of chemicals, refrigerants and as paint remover (Howard 1990). DCM is highly toxic and has carcinogenic and mutagenic effects; concentrations as low as 10 mg l^{-1} can disturb biological processes in human bodies (Trotsenko and Doronina 2003). Furthermore, due to its very low boiling point, it easily escapes into the atmosphere where it destroys the ozone layer (Trotsenko and Doronina 2003). Since it is very soluble in water, it is a major pollutant in both wastewaters and communal waters despite the efforts made to decreasing its production (Line et al. 1997).

Bacterial strains able to mineralize DCM aerobically (Doronina et al. 1995; Janssen et al. 1991; Nikolausz et al. 2005; Scholtz et al. 1988; Stucki et al. 1981) and anaerobically (Mägli et al. 1996) have been isolated from soil or water contaminated with DCM or from wastewater treatment plants (WWTP). DCM degrading consortia have also been isolated from estuarine water (Krausova et al. 2006).

In the presence of reduced glutathione (GSH), these bacteria convert DCM to the intermediate *S*-chloromethylglutathione which is then supposedly spontaneously converted to formaldehyde and GSH (Evens et al. 2000). Formaldehyde is then further degraded by formaldehyde dehydrogenase to formate, a central metabolite of methylotrophic growth.

There are two known types of DCM dehalogenases—group A dehalogenases, which yield low catalytic activity and will upon induction by DCM constitute about 20% and 50% of total suspended protein for batch and chemostat cultures, respectively (Gisi et al. 1998). Group B dehalogenases, which are almost six times more active than group A dehalogenases (Vuilleumier and Leisinger 1996) and reach significantly lower percentage of total suspended protein, only 9% and 20% for batch and chemostat cultures, respectively (Gisi et al. 1998). Most known bacterial strains degrading DCM contain group A dehalogenases and all show almost identical nucleotide sequences of the gene (Vuilleumier et al. 2001). Only one bacterial strain containing type B dehalogenase, *Methylophilus* sp. DM11, has been isolated (Scholtz et al. 1988), and dehalogenase from this strain shows only about 56% similarity with type A dehalogenases on a nucleotide sequence level (Bader and Leisinger 1994). Despite the large difference between the two types of dehalogenases, gene hybridization studies suggest them to be related, presenting subunits with equal molecular weight, and both requiring GSH for dehalogenation (Scholtz et al. 1988).

In this study, a bacterium able to degrade DCM as its sole source of carbon was isolated from a WWTP receiving pharmaceutical and domestic effluent. Growth properties of the strain and substrate metabolic versatility were assessed and the dehalogenase gene was sequenced and compared with other existing dehalogenase gene sequences.

Materials and methods

Enrichment and isolation of DCM degrading strain

Different sources of inoculum, including soil samples collected at the vicinity of an industrial pharmaceutical plant, activated sludge from a domestic WWTP that also received pharmaceutical wastewater, and activated sludge from a municipal WWTP receiving domestic and industrial effluents, were used for the enrichment of DCM degrading bacteria. Each type of inoculum was well-mixed and added to independent 250 ml flasks containing minimal medium (MM) (Freitas dos Santos and Livingston 1993). DCM was added directly to sterilized MM and was allowed to dissolve for at least 24 h before inoculation. The flasks were placed on a rotary shaker (100 rpm) at 25°C. When there was indication of DCM degradation, which was detected by monitoring of chloride release, diluted samples of the culture were plated onto nutrient agar (NA) plates. Isolated strains recovered from the NA plates were re-inoculated into liquid MM containing DCM at a concentration of 50 mg l^{-1} in flasks sealed with rubber stoppers faced with a teflon layer to minimize adsorption losses. When growth was observed, indicated by an increase in the optical density and by chloride release, the culture was plated onto NA plates to verify its purity. One pure strain (TM1) able to degrade DCM as a sole carbon source was isolated.

Identification of strain TM1

The 16S rRNA gene of strain TM1 was amplified by PCR using the primer set f27-r1492 (Lane 1991), (30 cycles of 1 min at 92°C, 1 min at 55°C and 1 min at 72°C) with Taq DNA polymerase (Promega, USA). The template DNA was obtained by boiling washed cell suspension for 5 min in 50 µl sterile distilled water and using 2 µl of the supernatant. The amplified fragments were cloned into the pGEM T-Easy vector (Promega, USA) and sequenced by STAB Vida, Portugal (Taq DyeDeoxy Terminator Cycle Sequencing and Model 373A gel apparatus, Applied Biosystems) using vector primers.

Phylogenetic analysis

The sequences were aligned using the program Greengenes according to DeSantis et al. (2006) available at http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi.

Phylogenetic analyses were performed using the ARB program package (Ludwig et al. 2004) and a

phylogenetic tree was constructed in ARB using the neighbor joining method (Saitou and Nei 1987).

Growth kinetics of strain TM1 on DCM

Growth kinetics of strain TM1 at DCM concentrations up to 1,500 mg l^{-1} were assessed in batch reactors. Samples of strain TM1 from a pre-grown inoculum were added to batch reactors containing different DCM concentrations in MM. Samples were periodically taken for determination of DCM, chloride and biomass concentrations. Experiments were repeated at least three times. For each DCM concentration, the average of the growth rates was determined.

Metabolic versatility tests

The utilization of various chlorinated and fluorinated compounds by strain TM1 was tested in 100 ml serum flasks filled with MM to one-fourth of their volume and inoculated with a TM1 culture pre-grown on DCM. The compounds tested are listed in Table 1.

Each carbon source was supplied to a final concentration of 50 mg l^{-1}. Samples were taken during a 20-day period and analyzed for biomass and for chloride or fluoride release. Tests were run in duplicate.

Table 1 Utilization of various aromatic and aliphatic compounds by strain TM1

Substrate	Growth	Halide release (%)
Chloroacetic acid	+	100
Fluoroacetic acid	−	0
Dichloroethane	+	60
Dichloromethane	+	100
Trichloroethylene	−	0
2-Chloroethanol	+	100
2-Fluorobenzoate	+	80
3-Fluorobenzoate	+	100
4-Fluorobenzoate	+	51
2-Chlorobenzoate	+	35
4-Chlorobenzoate	+	52
4-Fluorophenol	−	0
Fluorobenzene	−	0
Methanol	+	N/A

Genetic analysis of gene *dcmA*a

To study the DCM delahlogenase gene (*dcmA*a) PCR was performed using primer DMforout and DMrevout according to Vuilleumier et al. (2001). Template DNA was obtained by harvesting strain TM1 during exponential growth and using a genomic DNA isolation kit (MoBio, USA). The amplified fragments were sequenced by STAB Vida, Portugal (Taq DyeDeoxy Terminator Cycle Sequencing and model 373 A gel apparatus, Applied Biosystems) using the primers DMforout and DMrevout.

Analytical methods

The concentration of fluoride ion was measured with a fluoride electrode as previously described (Carvalho et al. 2002) and the concentration of chloride ion was measured using a colorimetric method (Iwasaki et al. 1956). DCM was analyzed using Gas Chromatography, for which 1 ml of liquid samples was added to a 2 ml vial containing 0.5 g of NaCl, which was subsequently sealed tightly. Each sample was left to equilibrate for 10 min and the gas phase injected into a Varian CP 3800 equipped with CP-wasc 52 CB capillary column (Chrompack International B.V., The Netherlands) at a constant temperature of 50°C for 5 min. The OD was measured at 600 nm using a spectrophotometer (Helios gamma, Unicam Instruments, UK).

Reagents

All chemicals used were of analytical grade and were obtained from Sigma-Aldrich Chemie (Germany) or Merck (Germany).

Results

Enrichment of DCM degrading strains

Degradation of DCM was detected in the enrichment reactor supplied with an inoculum derived from activated sludge collected from the domestic WWTP receiving pharmaceutical effluent. Strain TM1 was isolated from the DCM degrading consortium and was able to utilize DCM as a sole source of carbon and energy. When plated, small yellow colonies

appeared and when analyzed under a light microscope gram-negative rod shaped cells were seen. Strain TM1 was classified by 16S rRNA gene analysis and was affiliated with the *Xanthobacter* sp. group. From BLAST searches and subsequent phylogenetic analysis (Fig. 1), it became evident that the rRNA sequence of TM1 clusters with that of *Xanthobacter tegetidis* (Acc. No X99469), which was isolated from compost and is able to degrade substituted thiophenes (Padden et al. 1997), and those of an alpha proteobacteria, strain Z2A-6A (Acc. No AJ224615) and an alpha proteobacteria, strain Z4A-2 (Acc. No AJ224616), which are both thiosulphate oxidizing bacteria isolated form rice field soil (Stubner et al. 1998). The partial 16S rDNA sequence from TM1 has been submitted to the genebank with the accession number EF626696. 16S sequences from other DCM degrading strains were also included in the phylogenetic tree (shown in bold) and, among them, strain DM13 is the closest related to strain TM1. Strain DM13 (Acc. No AF004845) is a *Methylorhabdus multivorans* isolated in Switzerland on DCM-agar from an enrichment culture established with DCM contaminated groundwater (Doronina et al. 1995). Other distinct clusters observed for DCM degraders are the *Hyphomicrobium* spp., including strain GJ21 (Acc. No AJ854108), KDM4 (Acc. No AJ854110), KDM2 (Acc. No AJ854109), and DM1869 (Acc. No AJ854111). Strain DCM5b (Acc. No AF430123) and DCM7b (Acc. No AF430125) are both *Pseudomonas* spp. and are, together with strain DCM5a (Acc. No AF430124), the furthest away from strain TM1 in the tree. These three strains all belong to the class gamma proteobacteria while the other DCM degraders present in the tree belong to the class alpha proteobacteria.

Growth kinetics of strain TM1

TM1 growth kinetics was characterized using batch cultures; a typical growth curve is shown in Fig. 2. Stoichiometric liberation of chloride and decrease in DCM concentrations were observed from the beginning of the experiment, indicating complete

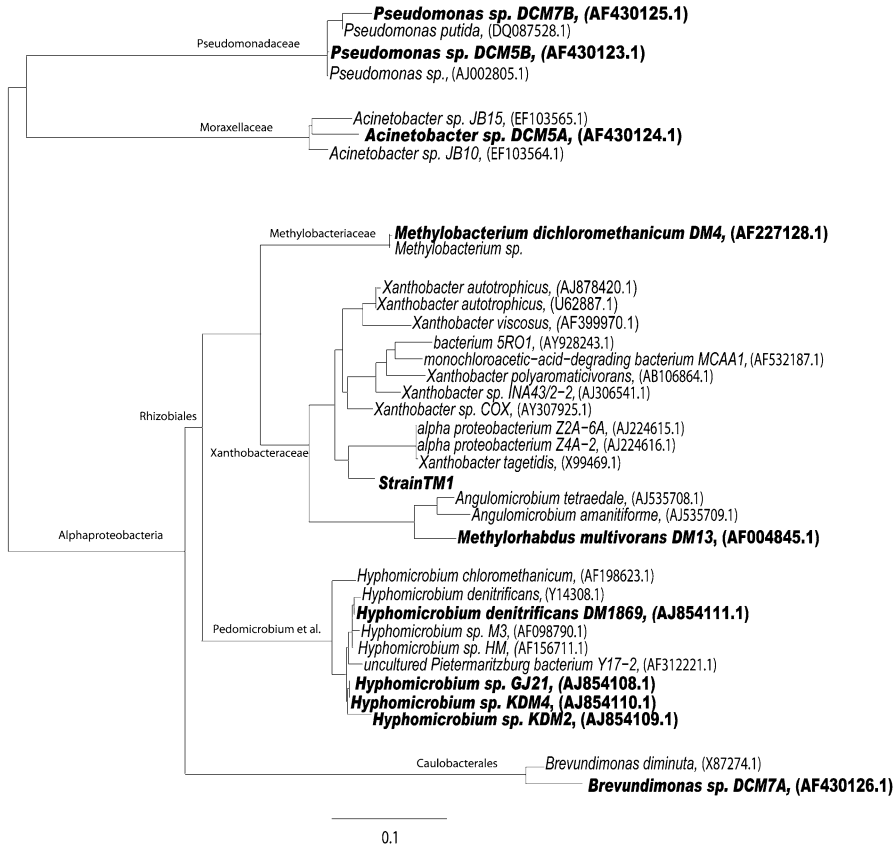

Fig. 1 Phylogenetic tree constructed with 16S rRNA sequences from strain TM1 and its closest phylogenetic relatives, including some DCM degrading strains (in bold). Genbank accession numbers are given for reference sequences

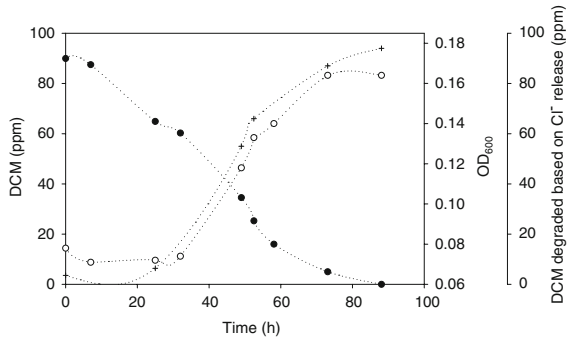

Fig. 2 Growth of strain TM1 on 100 mg l^{-1} DCM, showing DCM concentration (●), DCM degraded based on chloride release (+) and biomass measured as optical density at 600 nm (○) over time are shown

dechlorination of the parent compound by strain TM1. A concomitant increment of biomass was observed, no degradation occurred in non inoculated controls and no growth occurred in medium without the carbon source. The growth kinetics of strain TM1 on DCM is shown in Fig. 3. The mean of the standard error obtained was 10% and is represented at the different concentrations investigated with a 95% interval confidence. An inhibitory effect with increasing DCM concentrations was observed at concentrations higher than 400 mg l^{-1} (Fig. 3). Several kinetic models, including Monod (1949), Haldane-Andrews (Andrews 1968), Edwards (1970), Luong (1987) and Wayman and Tseng (1976) were tested to the describe growth rate pattern of strain TM1. The Luong model, shown in Eq. 1, resulted in

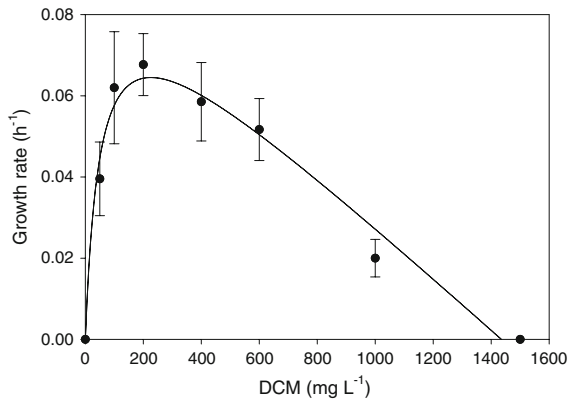

Fig. 3 Growth kinetics of strain TM1 on DCM. The solid line shows Luong equation plotted with $\mu_{max} = 0.094$, $K_s = 52$ mg l^{-1} and $S_m = 1{,}435$ mg l^{-1}. Growth rate (●) represented with 95% confidence interval

the highest R^2 value (0.92) and was therefore chosen to describe the degradation behavior of strain TM1 growing on DCM. Application of the models resulted in the following kinetic parameters: maximum growth rate (μ_{max}) = 0.094 h^{-1}, substrate saturation constant (K_s) = 52 mg l^{-1}, and maximum substrate concentration above which growth is completely inhibited (S_m) = 1,435 mg l^{-1}.

$$\mu = \frac{\mu_{max} S}{K_s + S}\left[1 - \frac{S}{S_m}\right] \quad (1)$$

Metabolic versatility of strain TM1

Strain TM1 was tested for the ability to dehalogenate a range of chlorinated and fluorinated organic compounds in batch cultures (Table 1). Halogenated aromatic compounds were also included in the test since their degradation would require different types of degradation pathways. Different substrates were added to TM1 cultures pre-grown on DCM. The strain was capable of completely mineralizing DCM, chloroacetic acid, 2-chloroethanol, and 3-fluorobenzoate and also showed significant degradation activity towards 1,2-dichloroethane, 2-fluorobenzoate, 4-fluorobenzoate, 2-chlorobenzoate, and 4-chlorobenzoate. The strain did not show any activity for fluorobenzene, 4-fluorophenol or fluoroacetate.

DCM dehalogenase of strain TM1

In order to study the genes involved in DCM degradation by strain TM1, the DCM dehalogenase gene was amplified through PCR. Primers specific for *dcmA*a genes were used in PCR experiments and a product of approximately 1.5 kb was obtained. The gene sequence has been submitted to the genebank with the accession number EF626697. Primers specific for the type B dehalogenase (*dcmA*b) were also tested but no PCR product was obtained, even when lowering the annealing temperature or increasing the $MgCl_2$ concentration. The results attained after comparing the *dcmA*a gene sequence of strain TM1 with other *dcmA*a sequences available in literature are shown in Table 2. The highest number of mismatches between *dcmA*a from TM1 and sequences from other bacteria was 14, coding 9 differences in amino acids.

Table 2 Comparison of gene sequences for gene *dcmA*a for strain TM1 and other DCM degrading strains

Gene accession no.	Strain	Base/amino acid differences	Identity (%)	Strain references
AJ271133.1 (Vuilleumier et al. 2001)	*Methylorhabdus multivorans* DM13	3/1	99	Doronina et al. (1995)
AJ271137.1 (Vuilleumier et al. 2001)	Unidentified waste water sludge bacterial sample S1	7/5	99	Vuilleumier et al. (2001)
M32346.1 (La Roche and Leisinger 1990)	*Methylobacterium* sp. DM4	8/7	99	La Roche and Leisinger (1990)
AJ271131.1 (Vuilleumier et al. 2001)	Dichloromethane degrading bacterium DM1	8/7	99	Schmid-Appert et al. (1997)
AJ271138.1 (Vuilleumier et al. 2001)	Bacterial enrichment culture S3-E1	9/7	99	Vuilleumier et al. (2001)
AJ854046 (Nikolausz et al. 2005)	*Hyphomicrobium denitrificans* DSM1869	9/8	99	Nikolausz et al. (2005)
AJ271132.1 (Vuilleumier et al. 2001)	*Hyphomicrobium* sp. DM2	9/8	99	Stucki et al. (1981)
AJ271136.1 (Vuilleumier et al. 2001)	Dichloromethane degrading culture LZ	10/7	99	Zuber et al. (1997)
AJ271135.1 (Vuilleumier et al. 2001)	Bacterial enrichment culture S2-E1	10/7	99	Vuilleumier et al. (2001)
AJ854044.1 (Nikolausz et al. 2005)	*Hyphomicrobium* sp. KDM2	13/9	98	Nikolausz et al. (2005)
AJ854045.1 (Nikolausz et al. 2005)	*Hyphomicrobium* sp. KDM4	13/9	98	Nikolausz et al. (2005)
AJ271134.1 (Vuilleumier et al. 2001)	*Hyphomicrobium* sp. GJ21	14/9	98	Ottengraf et al. (1986), Janssen et al. (1991)

The lowest number of differences observed was only three, coding one different amino acid.

Discussion

From a DCM degrading consortium enriched using an inoculum collected from a WWTP receiving pharmaceutical effluent containing that compound, one bacterial strain able to utilize DCM as a sole source of carbon and energy was isolated. Analysis of the 16S rRNA gene in comparison with other sequences in the GeneBank database revealed that the organism was a *Xanthobacter* sp. The sequence showed high similarity (99%) to the 16S rRNA gene in strain *X. tegetidis* (Acc. No X99469). There are no previous reports for DCM degradation by a *Xanthobacter* sp. However, this group of bacteria have been reported to degrade other halogenated hydrocarbons, such as 1,2-dichloroethane, 2-chloroethanol (Janssen et al. 1985), and chloroacetic acid (Song et al. 2004). A recent study (Torz et al. 2007) reports that *Xanthobacter autotrophicus* strain GJ10, which is a well known dichloroethane degrader, also degrades several other halogenated C2 and C1 compounds, such as dibromomethane, bromochloromethane and 1-bromo-2-chloroethane. In the same study DCM was also tested as growth substrate for strain GJ10, and no dehalogenation was detected; however, dibromomethane and dibromoethane were both partially dehalogenated by strain GJ10.

The growth kinetics of strain TM1 was best described by the Luong model, presenting a μ_{max} of 0.094 h^{-1}. This model has also been proposed for describing the growth kinetics of bacterial strains growing on other volatile organic compounds, such as 1,2-dichloroethane (Ferreira Jorge and Livingston 1999) and fluorobenzene (Carvalho et al. 2005). DCM was found to be inhibitory at concentrations higher than 400 mg l^{-1}. Several volatile organic compounds, such as toluene (Woldringh 1973), chlorobenzene (Ferreira Jorge and Livingston

1999) and fluorobenzene (Carvalho et al. 2002), have demonstrated a negative effect on cell growth at similar concentrations. TM1 shows a maximum growth rate similar to other DCM degrading strains, such as *Hyphomicrobium* sp. DM2, which has a μ_{max} of 0.07 h^{-1} (Stucki et al. 1981) and *Methylobacterium* sp. strain DM4, which showed a μ_{max} of 0.09 h^{-1} (Gälli and Leisinger 1985). Two other DCM degrading *Hyphomicrobium* sp., strain KDM2 and strain KDM4, were reported to have lower μ_{max}, 0.028 and 0.051 h^{-1}, respectively (Nikolausz et al. 2005). On the other hand, DCM degrading strain DM11, a *Methylobacterium* sp. containing type B DCM dehalogenase, was shown to have a significantly higher μ_{max} of 0.22 h^{-1}. The type B enzyme from strain DM11 was also reported to have much higher specific activity (2.5-fold) than type A enzymes, but it also constitutes a smaller portion of total soluble enzymes inside the cells in comparison with DCM dehalogenases type A (Scholtz et al. 1988). In another study it was shown that when microbial cultures containing type A enzymes were grown in continuous culture the level of expression of the enzyme increased two to threefold in comparison to when grown in batch conditions and that the μ_{max} for strains increased significantly, while for strain DM11, which contains type B enzyme, the same μ_{max} was obtained in batch and continuous cultures. It was suggested that strain DM11 had been exposed to selective conditions so to maximize its growth rate (Gisi et al. 1998).

Strain TM1 showed degradation activity towards other aliphatic halogenated compounds, such as 1,2-dichloroethane. DCM degrading strain DM2, a *Hyphomicrobium* sp., was also reported to degrade 1,2-dichloroethane, while other chlorinated alkanes, such as chloropropane, 1,1-dichloroethane, 1,4-dichlorobutane, and dichloroacetic acid, exhibited an inhibitory effect towards the enzyme DCM dehalogenase in that strain (Kohler-Staub and Leisinger 1985). Apart from several aliphatic compounds, strain TM1 also demonstrated degradation activity toward several aromatic halogenated compounds, such as halobenzoates. Strain TM1 should contain a diverse genetic set-up since it is able to degrade both aliphatic and aromatic compounds.

Several *Xanthobacter* sp. have previously been reported to degrade a range of aromatic compounds, such as polycyclic aromatic hydrocarbons (Hirano et al. 2004) and 1,4-dichlorobenzene (Spiess et al. 1995). A *Xanthobacter* sp. strain py2 containing an alkene monooxygenase, which is closely related to aromatic monooxygenases, oxidizes benzene, toluene, and phenol and completely mineralizes phenol and catechol (Zhou et al. 1999).

The TM1 DCM dehalogenase gene was amplified through PCR and was found to be a type A dehalogenase. The *dcmA*a sequence from TM1 was compared with 12 *dcmA*a sequences from the NCBI databases belonging to other DCM degrading strains. The *dcmA*a from TM1 was almost identical to all the other existing sequences. The sequence displaying more differences relative to strain TM1 was that of strain GJ21, a *Hyphomicrobium* sp. isolated in The Netherlands (Ottengraf et al. 1986; Janssen et al. 1991), showing 14 base differences encoding nine amino acid differences (Table 2). The strain with the most similar *dcmA*a to TM1 was strain DM13, which has three different nucleotides and only one amino acid difference on a protein level (Table 2). DM13 was also the strain that clustered closest with strain TM1 in the phylogenetic tree (Fig. 1). A large portion of the differences in nucleotide sequences yielded differences in amino acid sequences, i.e., few synonymous changes (changes that does not result in translation of a different amino acid) were observed at the third, wobble position of the codon, which was also noted previously in other reports (Vuilleumier et al. 2001). In a few cases, different DCM utilizing strains contain completely identical DCM dehalogenase genes, such as strain DM1, which has not been well characterized, and strain DM4, which is a *Methylobacterium*; strains DSM1869 and DM2, which are both *Hyphomicrobium* sp. (Vuilleumier et al. 2001), and strains KDM2 and KDM4, which are also both *Hyphomicrobium* sp. (Nikolausz et al. 2005). Very conserved gene sequences have been observed for other known dehalogenases. For 1,2-dichloroethane degrading strains, several different bacteria contain identical haloalkane dehalogenases (van den Wijngaard et al. 1992). Triazine dehalogenases from different bacteria show very high gene sequence similarity (De Souza et al. 1998), and the 1,2-dibromoethane degrading organism *Mycobacterium* sp. strain GP1 contains a haloalkane dehalogenase gene ($dhlA_f$) that is almost identical to *dhaA* genes found in several other species (Poelarends et al. 2000).

There are more examples of much conserved sequences for dehalogenases and it has been suggested that there are few possibilities for different degradation pathways for these compounds in nature, and therefore horizontal transfer of the genes actually occur faster than development of new pathways. In fact, dehalogenases often contain insertion fragments and they are often found on plasmids, which are both indications of horizontal gene transfer (Janssen et al. 2005). A further development of this work includes the investigation into whether the gene *dcmA*a is strain TM1 is situated on the genome or on a plasmid.

Conclusions

Bacterial strain TM1, the first *Xanthobacter* sp. reported to degrade DCM, was isolated from a WWTP. The strain showed growth properties similar to other existing DCM degraders and, although the strain is a *Xanthobacter* sp., the DCM dehalogenase gene was almost identical to other DCM dehalogenase genes belonging to either *Hyphomicrobium* sp. or *Methylobacterium* sp. Furthermore, strain TM1 shows promising degradation activity towards several other halogenated compounds, both aliphatic and aromatic.

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