Degradation of 2,4-dichlorophenoxyacetic acid by haloalkaliphilic bacteria

Olga Maltseva, Catherine McGowan, Roberta Fulthorpe† and Patrick Oriel

Author for correspondence: Olga Maltseva. Tel: +1 517 353 7858. Fax: +1 517 353 2917.
e-mail: 23142ovm@msu.edu

Center for Microbial Ecology and Department of Microbiology, Michigan State University, East Lansing, Michigan 48824, USA

Three 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacterial isolates were obtained from the highly saline and alkaline Alkali Lake site in southwestern Oregon contaminated with 2,4-D production wastes. While similar in most respects, the three isolates differed significantly in 2,4-D degradation rates, with the most active strain, I-18, demonstrating an ability to degrade up to 3000 mg 2,4-D l⁻¹ in 3 d. This strain was well adapted to the extreme environment from which it was isolated, growing optimally on 2,4-D at pH 8.4–9.4 and at sodium ion concentrations of 0.6–1.0 M. According to its optimum salt concentration and pH for growth, this isolate was a moderately halophilic, alkaliphilic bacterium. The 16S rRNA gene sequence (303 nt) was identical for all three isolates and most closely resembled those of the moderately halophilic eubacteria of the family Halomonadaceae (91% identity). Biochemical and genetic examination revealed strain I-18 utilizes the same 2,4-D degradation pathway as most of the 2,4-D-degrading bacteria from non-extreme environments. Hybridization data and comparison of the partial sequences of the *tfdA* gene from the Alkali Lake isolates with those of bacteria from non-extreme environments suggested a common genetic origin of the 2,4-D degradation pathway in the two groups of micro-organisms.

Keywords: *Halomonadaceae*, 2,4-dichlorophenoxyacetic acid, degradation of chloroaromatic compounds, halophilic bacteria, alkaliphilic bacteria

INTRODUCTION

The critical role of micro-organisms in the degradation of organic pollutants is well known. Although the micro-organisms capable of degradation of organic pollutants and their catabolic pathways have been investigated intensively, information on the microbial degradation of xenobiotics in environments of high salinity and alkalinity is still very limited (Oren *et al.*, 1992). Comparison of analogous catabolic enzymes and pathways of phylogenetically diverse bacteria isolated from extreme and moderate

environments should provide information regarding their metabolic diversity. The study of xenobiotic-degrading extremophiles may also help in evaluating their use in bioremediation of contaminated saline and/or alkaline environments.

The 2,4-dichlorophenoxyacetic acid (2,4-D) degradation pathway is one of the best understood, and provides a good model for such investigations. This herbicide has been released into the environment for more than 40 years, and is degraded in soils fairly rapidly. Many bacteria of different genera are able to completely degrade 2,4-D (Don & Pemberton, 1981; Chaudhry & Chapalamadugu 1991; Häggblom, 1992). Degradation of 2,4-D via oxidative cleavage of the ether bond with subsequent chlorophenol hydroxylation followed by the modified *ortho*-cleavage pathway of chlorocatechols has been demonstrated for most of these isolates. This pathway has been most extensively studied using *Alcaligenes eutrophus* strain JMP134(pJP4) (Don & Pemberton, 1981, 1985). The enzymes participating in the 2,4-D degradation pathway

†Present address: Division of Physical Sciences Scarborough Campus, University of Toronto, 1265 Military Trail, Scarborough, Ontario M1C 1A4, Canada.

Abbreviation: 2,4-D, 2,4-dichlorophenoxyacetic acid.

The GenBank accession numbers for the partial nucleotide sequences of the *tfdA* gene and 16S ribosomal RNA from the Alkali Lake isolates reported in this paper are U22499 and U22836, respectively. (The two sequences were identical in the three isolates studied.)

Fig. 1. Pathway for the degradation of 2,4-D in *A. eutrophus* strain JMP134.

have been purified and characterized, and genes that code for these enzymes (*tfdA*, *tfdB*, *tfdCDEF*) have been sequenced (Ghosal & You, 1988, 1989; Perkins *et al.*, 1990; Kuhm *et al.*, 1990; Schlömann *et al.*, 1990; Häggblom, 1992; Fukumori & Hausinger, 1993; Seibert *et al.*, 1993). The pathway is summarized in Fig. 1.

We now report on the isolation and characterization of 2,4-D-degrading bacteria of the family *Halomonadaceae* as a first example of moderately halophilic bacteria capable of complete mineralization of chloroaromatic compounds.

METHODS

Site description and sample collection. Alkali Lake and West Alkali Lake are hypersaline alkaline lakes in the high desert of southwestern Oregon. The groundwater in the vicinity of the lakes has a salinity near 10% due to sodium chloride and sodium carbonate, and a pH of 9.5–10 (Pankow *et al.*, 1984). Between 1969 and 1971 the area separating Alkali Lake and West Alkali Lake was used as a waste storage area. In 1976 approximately 6000 tonnes 2,4-D herbicide production waste were released into the site by the crushing of waste drums in shallow covered trenches. The groundwater in this site is within about 1 m of the surface and is now contaminated with high levels of

monochloro-, dichloro- and trichlorophenols, and also with 2,4-D, 4-methyl-2-chlorophenoxyacetic acid and related haloaromatic compounds (Pankow *et al.*, 1984; Johnson *et al.*, 1985). Samples used in this study were taken from soil just above groundwater level across the contaminant plume gradient in April 1993 in cooperation with the State of Oregon Department of Environmental Quality.

Medium. The alkaline mineral medium 1 (AMM1), pH 9.5, was prepared by mixing sterile component A (20 g Na₂CO₃ and 20 g NaHCO₃ in 300 ml H₂O), component B [40 g NaCl, 3 g KCl, 0.5 g NH₄NO₃, 0.3 g (NH₄)₂SO₄, 0.4 g MgSO₄, 0.3 g NaH₂PO₄ and trace elements (Imhoff & Trüper, 1977) in 700 ml H₂O] and vitamins (Wolin *et al.*, 1963). MgSO₄, NaH₂PO₄ and vitamins were made as 1000× stock solutions and sterilized separately. Alkaline mineral medium 2, pH 9.4, with decreased carbonate concentration (AMM2) was used in some experiments. It contained 5 g Na₂CO₃ and 5 g NaHCO₃ in component A. AMM agar was prepared by adding 20 g agar to component B before sterilization. Prior to mixing, all solutions were cooled to room temperature or, in the case of AMM agar media, at least to about 45 °C. The carbon sources and their concentrations were 2,4-D (50–3000 mg l⁻¹) and yeast extract (50–500 mg l⁻¹).

Enrichment. Soil samples collected at six points across the contaminant plume gradient were combined. Subsamples (5 g) of the mixture was added to 200 ml AMM1 in Erlenmeyer flasks and incubated aerobically at 30 or 37 °C. Cultures were grown statically or on a rotary shaker at 200 r.p.m. 2,4-D (50 mg l⁻¹) was used as the only carbon source. In the case of complete disappearance of 2,4-D from enrichment cultures, repeated additions (from two to five) were used to supply microorganisms with growth substrate. The ability to degrade 2,4-D was evaluated by HPLC and by measurement of the ¹⁴CO₂ evolution from ¹⁴C-labelled 2,4-D (see below). Enrichments demonstrating 2,4-D degradation (5 ml) were transferred to fresh AMM1 (50 ml) containing 50 mg yeast extract l⁻¹ and 200 mg 2,4-D l⁻¹. These enriched samples were plated onto AMM1 agar plates containing 500 mg 2,4-D l⁻¹. For purification of isolates, single colonies were selected and transferred onto AMM1 plates containing 500 mg 2,4-D l⁻¹. Confirmation of 2,4-D degradation was carried out by inoculating each isolate into liquid AMM1 containing 50 mg yeast extract l⁻¹ and 200 mg 2,4-D l⁻¹ and determination of both increase of OD₅₅₀ and disappearance of herbicide. Liquid AMM1 with the same concentrations of yeast extract and 2,4-D, but without microbial cultures, was used as a control.

Catabolism of ¹⁴C-labelled 2,4-D. For isotopic experiments, the reaction mixture contained in a 1.5 ml microcentrifuge tube 0.25 ml AMM1, 0.25 ml enrichment cultures and 50 mg l⁻¹ ring-labelled 2,4-D [Sigma, specific activity 20.2 mCi mmol⁻¹ (747 MBq mmol⁻¹), 0.05 µCi per sample]. A control tube contained only labelled 2,4-D and AMM1 without enrichment cultures. The tubes were inserted into scintillation vials containing on the bottom 1 ml phenethylamine as a trapping agent. Vials were closed with caps and incubated on a rotary shaker at 150 r.p.m. for 3 d. At the end of the incubation period the microcentrifuge tubes were removed from the scintillation vials and 10 ml of the Bio-Safe II scintillation cocktail was added to the trapping agent. Radioactivity was measured using an LKB 1211 Rackbeta liquid scintillation counter with appropriate correction for quenching.

High performance liquid chromatography (HPLC). This was performed with a Hewlett-Packard series 1050 chromatograph equipped with a multiple wavelength detector set at 230 nm. Separation was achieved on a reversed-phase Lichrosorb RP-18 column (Merck) of internal diameter 4 mm and length 250 mm.

The flow rate was 1.5 ml min⁻¹. Compounds were detected at 230 nm. The mobile phase was an aqueous solution of 40% (v/v) methanol and 0.1% (w/v) H₃PO₄.

Utilization of other aromatic compounds as sources of carbon and energy. Growth on aromatic compounds was measured in AMM with benzoic, 3-hydroxy- and 4-hydroxybenzoic acids supplied at 300 mg l⁻¹; 2-chloro-, 3-chloro- and 4-chlorobenzoic acids, and 2-methyl-4-chlorophenoxyacetic acid supplied at 150 mg l⁻¹; and monochlorophenols, 2,4-dichloro-, 2,6-dichloro- and 2,4,6-trichlorophenol, and 2,4,5-trichlorophenoxyacetic acid supplied at 30 mg l⁻¹. Cultures were analysed for increase in OD and removal of the aromatic compound from the medium.

Determination of optimal growth conditions. The specific growth rates under various conditions were analysed during exponential growth by linear regression of the logarithm of OD₅₅₀ with time. Salt dependency of growth was determined in AMM1 and AMM2 containing various concentrations of NaCl (0–1.8 M) in component B. To determine the pH optima for growth, media pH was varied by mixing component B of AMM with various buffer systems: 40 mM HEPES, pH 6.8–7.5; 40 mM Tris, pH 7.1–8.9; and 200 mM Na₂CO₃/NaHCO₃ buffer, pH 8.6–10.6. The pH values reported here are the initial ones, but they varied less than 0.2 units during growth of the culture. All media were supplemented with NaCl to keep the sodium ion concentration near 0.8 M.

Fatty acid analysis. The fatty acid methyl ester analysis of lipids was performed by MIDI Laboratories (Newark, DE). Isolate I-18 was precultured on AMM1 agar supplemented with 2 g 2,4-D l⁻¹ and streaked onto plates of tryptic soy agar (TSA) containing 10% (w/v) NaCl. Plates were incubated for 4 d. Saponification, methylation and extraction were performed as described by Sasser & Wichman (1991).

Preparation of cell-free extracts and enzyme assay. Strain I-18 was grown on AMM1 containing 2000 mg 2,4-D l⁻¹ and 50 mg yeast extract l⁻¹ for 36 h, then harvested by centrifugation and washed twice with 50 mM Tris/HCl buffer, pH 8.0 containing 0.1 mM dithiothreitol and 2 mM MnSO₄. After disruption of the cells by sonication for 2 min at 0 °C, cell-free extracts were separated from whole cells and cell debris by centrifugation at 25000 g for 40 min at 5 °C.

Activities of the modified *ortho*-cleavage pathway enzymes were measured spectrophotometrically using a Perkin-Elmer Coleman 124 instrument (USA). One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 µmol product min⁻¹ at room temperature. Activities of chlorocatechol 1,2-dioxygenase (EC 1.13.11.1), chloromuconate cycloisomerase (EC 5.5.1.7) and dienelactone hydrolase (EC 3.1.1.45) were determined as described previously (Dorn & Knackmuss, 1978; Maltseva *et al.*, 1994).

Protein concentrations were determined using a Bio-Rad Protein Assay Kit with bovine serum albumin as a standard.

DNA extraction and Southern hybridization. Strain I-18 was cultured with 2,4-D as growth substrate as described above. Strains P-3 and I-17 were grown on AMM1 containing 1 g sodium pyruvate l⁻¹ and 200 mg 2,4-D l⁻¹. The miniprep method of Ausubel *et al.* (1987) was used to extract total genomic DNA. *Eco*RI-digested DNA was separated by gel electrophoresis on an 0.8% (w/v) agarose gel and blotted onto Hybond-N nylon membranes (Amersham). The probes were internal segments of the *tfdA*, *tfdB*, *tfdC* and *tfdD* genes of *A. eutrophus* JMP134(pJP4) (Holben *et al.*, 1992). All probes were labelled

with digoxigenin dUTP using a DNA labelling kit (Boehringer-Mannheim) according to the manufacturer's instructions. Hybridizations were performed at three different stringencies as described by Fulthorpe *et al.* (1995).

PCR amplification and sequencing of PCR products. The 16S ribosomal RNA genes were amplified using rD1 and fD1 primers (Weisburg *et al.*, 1991). The *tfdA* genes were amplified using primers TV1 and TV2 designed by Tatiana Vallaes and Alice Wright and synthesized at the Macromolecular Facility, Michigan State University (Vallaes *et al.*, 1996). The PCR reaction mixtures were prepared according to the manufacturer's protocol (Perkin Elmer Cetus). Thermal cycling was done in a Perkin Elmer 9700 Thermal Cycler using the following conditions: melting at 92 °C for 1 min; cycling 35 times at 92 °C for 1 min 10 s, 55 °C for 30 s and 72 °C for 2 min 10 s; followed by a final extension at 72 °C for 6 min 10 s. Amplified products were purified using a Gene Clean kit (BIO 101). Sequencing was carried out at Michigan State University Sequencing Facility using the Applied Biosystems Model 373A automatic sequencer (Perkin Elmer Cetus) and fluorescently labelled dye termination. The sequencing primer used for 16S was 519R (5'-GTA TTA CCG CGG CTG CTG G-3') (Lane *et al.*, 1985). Partial sequences were compared to data in GenBank using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (Altschul *et al.*, 1990) and also the database of the Ribosomal Database Project (Larsen *et al.*, 1993).

Chemicals. Catechol was purchased from Sigma and 4-chlorocatechol from Helix Biotech Corporation. 3-Chlorocatechol and 3,5-dichlorocatechol, and *cis*-dienelactone were kind gifts from M. Schlömann, Universität Stuttgart, and W. Reineke, Bergische Universität-Gesamthochschule Wuppertal, respectively. Chloromuconic acids were prepared as described by Kuhm *et al.* (1990) using partially purified chlorocatechol dioxygenase kindly supplied by M. Schlömann and M. Vollmer, Universität Stuttgart.

RESULTS

Characterization of isolates

Three strains able to degrade 2,4-D were isolated from the enrichments and designated P-3, I-17 and I-18. On AMM1 agar supplemented with 1 g yeast extract l⁻¹ and 1 g Casamino acids l⁻¹ these bacteria initially produced small transparent colonies, progressing into yellow colonies with tan pigmentation in the centre and a lobate edge. The isolates grew well at 30 °C, but no growth was observed at 37 °C. All strains were Gram-negative, motile rods. Electron microscopy revealed cells of isolate I-18 to be about 1.7–1.9 µm in length and about 0.6–0.7 µm in diameter (data not shown).

The sequences of approximately 303 nucleotides, corresponding to the *E. coli* 16S RNA gene sequence from nt 211 to 513, were identical for all three isolates and showed strong similarity to 16S RNA sequences of the moderately halophilic eubacteria. The highest identity was found with *Halomonas elongata* ATCC 33173 (91.4%), *Halovibrio variabilis* DSM 3051 (91.1%), *Deleya halophila* DSM 4770 (89.8%), *Halomonas meridiana* DSM 5425 (89.8%) and *Deleya marina* ATCC 25374 (89.8%).

The major fatty acids of isolate I-18 and their concentration ranges were 10:0 (3.56–4.52%), 12:0 3-OH (2.81–3.58%), 14:0 (1.94–2.40%), 16:0 (8.46–9.06%), 16:1 *cis* 9 (12.24–15.48%), 17:0 cyclo (4.90–5.90%) and

Fig. 2. Influence of yeast extract on 2,4-D degradation by strain I-18. Cells were grown on AMM1, pH 9.5, containing 2,4-D (500 mg l⁻¹) (■) or 2,4-D (500 or 3000 mg l⁻¹) plus yeast extract (50 mg l⁻¹) (●, ○).

Fig. 3. Degradation of 2,4-D by Alkali Lake isolates I-18 (▲), P-3 (○) and I-17 (■) in AMM1, pH 9.5, supplemented with 50 mg yeast extract l⁻¹.

an unresolved mixture of 18:1 (54.54–56.57%) and 19:0 cyclo 11-12 (4.41–6.38%).

Dynamics of 2,4-D degradation

Although the Alkali Lake isolates were able to grow on minimal alkaline medium with 2,4-D as single carbon and energy source, addition of a trace of yeast extract greatly increased the rate of 2,4-D degradation (Fig. 2; data for isolates I-17 and P-3 not shown). While similar in appearance and colony morphology, the three isolates demonstrated significant differences in ability to metabolize 2,4-D (Fig. 3). The growth range and degradation

ability of the most active isolate I-18 were examined in more detail. When supplemented with 50 mg yeast extract l⁻¹, strain I-18 demonstrated an ability to degrade 3000 mg 2,4-D l⁻¹ over a 3 d period. This strain was able to utilize other aromatic compounds including benzoic acid, *m*-hydroxy- and *p*-hydroxybenzoic acid, 3-chlorobenzoic acid and, less efficiently, 4-chlorophenol and 2,4-dichlorophenol (data not shown). Growth was not observed with 2-chloro- or 4-chlorobenzoic acid, 2-chloro- or 3-chlorophenol or 2,4,5-trichlorophenoxyacetic acid.

Salt effects on the growth of strain I-18

Different NaCl optima were observed during growth of isolate I-18 in AMM1 and AMM2 containing different amounts of sodium carbonates (Fig. 4a). When specific growth rates were plotted against total sodium ion concentration, however, almost coincident curves were obtained, indicating the strong growth response to sodium ions and lesser dependence of growth on carbonate or chloride concentration (Fig. 4b). This organism was able to grow over a wide range of salt concentrations on both yeast extract and 2,4-D. However, the use of complex media supported growth over a wider range of NaCl concentrations than did defined media. The optimal sodium ion concentration for growth was the same on both defined and complex media (0.6–1.0 M), and no growth was observed below 0.1 M.

pH effects on the growth of strain I-18

This was studied in media of different pH and a sodium ion concentration of approximately 0.8 M. The bacterium was able to grow on yeast extract over a wide pH range (6.5–10.5) and had a broad pH optimum (8.2–9.6) (Fig. 5). When grown on defined medium with 2,4-D, a more narrow pH range of the growth was found (7.4–9.8) with the optimum between 8.4 and 9.4.

Degradation pathway of isolate I-18

High activities of catechol 1,2-dioxygenase, muconate cycloisomerase and dienelactone hydrolase were detected in cell-free extracts of isolate I-18 grown on 2,4-D (Table 1). Activities of catechol 1,2-dioxygenase and muconate cycloisomerase were higher with chlorinated than with unsubstituted substrates. The data suggest that haloalkaliphilic strain I-18, like the well-studied strain *A. eutrophus* JMP134(pJP4), uses the modified *ortho*-cleavage pathway for 2,4-D degradation.

Genetic comparison of the 2,4-D degradation pathway in bacteria from extreme and non-extreme environments

No hybridization of the DNA from the Oregon isolate I-18 with the pJP4 genes *tfdA*, *tfdB*, *tfdC* and *tfdD* encoding the first four steps of the 2,4-D degradation pathway in *A. eutrophus* JMP134 was found at high stringency, but positive signals were observed with *tfdA*, *tfdB* and *tfdD* probes at medium stringency and very weak hybridization

Fig. 4. Influence of sodium chloride (a) and sodium ion concentration (b) on growth of isolate I-18 on yeast extract (500 mg l⁻¹; open symbols) and on 2,4-D (500 mg l⁻¹) plus yeast extract (50 mg l⁻¹) (filled symbols). Cells were grown in AMM1, pH 9.5, containing 20 g Na₂CO₃ l⁻¹ and 20 g NaHCO₃ l⁻¹ (□, ■) or in AMM2, pH 9.4, containing 5 g Na₂CO₃ l⁻¹ and 5 g NaHCO₃ l⁻¹ (○, ●). Both media were supplemented with different concentrations of sodium chloride.

Fig. 5. Influence of pH on growth of isolate I-18 on yeast extract (500 mg l⁻¹; open symbols) or on 2,4-D (500 mg l⁻¹) plus yeast extract (50 mg l⁻¹) (filled symbols). The pH values of the media were adjusted with the following buffer systems: 40 mM HEPES/NaOH (pH 6.8–7.5) (△), 40 mM Tris/HCl (pH 7.1–8.9) (○, ●), and 200 mM Na₂CO₃/NaHCO₃ (pH 8.6–10.6) (□, ■). All media were supplemented with NaCl to keep the sodium ion concentration near 0.8 M.

Table 1. Enzyme activities in cell extracts of 2,4-D-grown cells of isolate I-18

Enzyme	Substrate	Specific activity [mU (mg protein) ⁻¹]	Relative activity (%)*
Catechol 1,2-dioxygenase	Catechol	110	100
	3-Chlorocatechol	61	55
	4-Chlorocatechol	81	74
	3,5-Dichlorocatechol	188	171
Muconate cycloisomerase	<i>cis, cis</i> -Muconate	17	100
	2-Chloro- <i>cis, cis</i> -muconate	43	252
	3-Chloro- <i>cis, cis</i> -muconate	56	329
	2,4-Dichloro- <i>cis, cis</i> -muconate	33	194
Dienelactone hydrolase	<i>cis</i> -Dienelactone	65	

*Relative activities of catechol 1,2-dioxygenase and muconate cycloisomerase are expressed as a percentage of the value with catechol and muconate, respectively.

with *tfdC* was found at low stringency (Fig. 6). Hybridization patterns for all three Alkali Lake isolates were identical (data for isolates I-17 and P-3 not shown). The sizes of all DNA restriction fragments of these isolates hybridizing with *tfdA-D* differed from those of pJP4.

The *tfdA* genes from the Alkali Lake isolates were PCR-amplified and the products sequenced. The sequences of

approximately 317 nucleotides, corresponding to the *A. entrophus* JMP134 *tfdA* gene sequence from nt 449–765, were identical for all three isolates. The partial sequences of these isolates showed strong similarity to *tfdA* sequences of *Burkholderia* sp. strain RASC (formerly *Pseudomonas* sp. TFD3) and *A. entrophus* JMP134 (94% and 77% identity, respectively) (Perkins *et al.*, 1990; Suwa

Fig. 6. Low stringency hybridization of pJP4-derived probes *tfdA* (lanes 1 and 2), *tfdB* (lanes 3 and 4), *tfdC* (lanes 5 and 6) and *tfdD* (lanes 7 and 8) with DNA from Alkali Lake isolate I-18 (lanes 1, 3, 5 and 7) and *A. eutrophus* JMP134(pJP4) (lanes 2, 4, 6 and 8) digested with *Eco*RI.

et al., 1994) and were identical to that of strains B6-9 and TFD31 (nucleotide accession number U43196) which were tentatively identified as *Rhodospirillum rubrum* (Fulthorpe *et al.*, 1995).

DISCUSSION

Three bacterial isolates capable of complete mineralization of 2,4-D were obtained from the highly saline and alkaline Alkali Lake site. The most active strain, I-18, was well-adapted to the extreme environment from which it was isolated, growing optimally at a sodium ion concentration near 0.6–1.0 M and at pH 8.4–9.4. According to the classification of Kushner & Kamekura (1988), this isolate is a moderately halophilic bacterium. Since microorganisms with pH optima for growth in excess of pH 8 are defined as alkaliphiles (Grant & Tindall, 1986), strain I-18 should be regarded as a moderately halophilic, alkaliphilic bacterium.

The partial 16S RNA sequences of the three 2,4-D degrading isolates from the Alkali Lake site indicate that they are members of the gamma subdivision of the *Proteobacteria* and belong to the family *Halomonadaceae*, which unifies moderately halophilic eubacteria (Dobson *et al.*, 1993; Franzmann & Tindall, 1990; Franzmann *et al.*, 1988). The 16S RNA sequences obtained for the Alkali Lake isolates had cytosine at position 486 (*Escherichia coli* numbering), as have all the other members of this family examined so far, which is an extremely rare attribute among the prokaryotes (Dobson *et al.*, 1993). It has been shown recently that the three genera of the family

Halomonadaceae (*Halomonas*, *Deleya* and *Halovibrio*) cannot be resolved on the basis of phylogenetic, chemotaxonomic or phenotypic data and unification of these genera in a single genus has been proposed (Dobson *et al.*, 1993). Until the taxonomy of this group is clarified it is not feasible to assign these bacteria to a species or genus. The close taxonomic relationship of the Alkali Lake isolates and the *Halomonadaceae* indicated by the 16S RNA sequencing data is consistent with the finding that isolate I-18 is a moderately halophilic bacterium. In addition the major fatty acids of isolate I-18 (16:0, 16:1, 17:0 cyc, 18:1 and 19:0 cyc), representing 90% of the total fatty acids, are the same as for other members of the *Halomonadaceae* (Franzmann & Tindall, 1990).

The alkaliphilicity of *Halomonadaceae* strain I-18 is not surprising as it is known that some bacteria of this family are capable of growth at pH 9 and even pH 10 (Del Moral *et al.*, 1988; Vreeland *et al.*, 1980). Recently, it was shown that two groups of alkaliphilic isolates from Kenyan Soda Lakes also belong to the *Halomonadaceae* (Jones *et al.*, 1994).

The Alkali Lake isolates are the first known aerobic, halophilic bacteria able to use chloroaromatic compounds as sources of carbon and energy. Enzyme activities of strain I-18 suggest that it utilizes the same pathway of 2,4-D degradation as the majority of 2,4-D-degrading bacteria from non-extreme environments (Häggblom, 1992). Since the contamination of the Alkali Lake site with chloroaromatic compounds is recent, the origin of the 2,4-D degradation pathway in the haloalkaliphilic bacteria is of interest. The DNA from all three Alkali Lake isolates hybridized to pJP4 genes *tfdA*, *tfdB*, *tfdC* and *tfdD*, responsible for 2,4-D degradation in the well-known 2,4-D-degrading bacterium *A. eutrophus* JMP134. Moreover, the partial sequences of the *tfdA* genes from the Alkali Lake isolates were only slightly different from the sequence of the *tfdA* gene of *A. eutrophus* JMP134 and identical to that of the 2,4-D-degrading strains *R. fermentans* B6-9 and TFD31 isolated from non-extreme environments in Ontario and Southern Saskatchewan, respectively (Fulthorpe, 1991; Tonso *et al.*, 1995). These data suggest a common origin of the genes encoding 2,4-D degradation in the haloalkaliphilic bacteria and bacteria from non-extreme environments.

An interesting characteristic of moderately halophilic eubacteria is their mode of osmoadaptation. In contrast to the extremely halophilic Archaea that have high intracellular potassium and sodium ion concentrations and enzymes adapted to this environment (Hochstein, 1988), moderately halophilic bacteria can extensively accumulate organic osmolytes called 'compatible solutes' which do not interfere with cell metabolism and may even contribute to enzyme stability (Galinski, 1993). It is not clear whether the enzymes of moderately halophilic eubacteria have special adaptive changes to function optimally in an environment of high osmolarity (Kushner & Kamekura, 1988). The data obtained in this investigation indicate that very similar TfdA enzymes can function efficiently in both halophilic and non-halophilic eubacteria. Complete

sequencing of the genes encoding 2,4-D degradation in strain I-18, and study of catabolic gene transfer between halophilic and non-halophilic eubacteria should more firmly establish the compatibility of the enzymes from both groups of micro-organisms and the requirements for engineering xenobiotic-degrading halophilic eubacteria.

ACKNOWLEDGEMENTS

This work was supported by National Science Foundation Science and Technology Center Grant No. BIR 9120006. We wish to thank Brian McClure of the Oregon Department of Environmental Quality for assistance in sample collection. We are grateful to Helen Gorlew for performing the FAME analysis and to Jacqueline Wood of the MSU Center for Electron Optics for the scanning electron microscopy. We thank Tatiana Valleys and Alice Wright for designing the primers used to amplify DNA from *tfdA* genes. We are grateful to M. Schlömann and M. Vollmer for supplying 3-chlorocatechol, 3,5-dichlorocatechol and partially purified chlorocatechol 1,2-dioxygenase and to W. Reineke for *cis*-dienelactone. We also thank Eva Top and Cindy Nakatsu for helpful advice.

REFERENCES

- Altschul, S. F., Gish, W., Miller, M., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: John Wiley.
- Chaudhry, G. R. & Chapalamadugu, S. (1991). Biodegradation of halogenated organic compounds. *Microbiol Rev* **55**, 59–79.
- Del Moral, A., Prado, B., Quesada, E., García, T., Ferrer, R., & Ramos-Comenzana, A. (1988). Numerical taxonomy of moderately halophilic Gram-negative rods from an inland saltern. *J Gen Microbiol* **134**, 733–741.
- Dobson, S. J., McMeekin, T. A. & Franzmann, P. D. (1993). Phylogenetic relationships between some members of the genera *Deleya*, *Halomonas*, and *Halovibrio*. *Int J Syst Bacteriol* **43**, 665–673.
- Don, R. H. & Pemberton, J. M. (1981). Properties of six pesticide degradation plasmids isolated from *Alcaligenes eutrophus* and *Alcaligenes paradoxus*. *J Bacteriol* **145**, 681–686.
- Don, R. H. & Pemberton, J. M. (1985). Genetic and physical map of the 2,4-dichlorophenoxyacetic acid-degradative plasmid pJP4. *J Bacteriol* **161**, 466–468.
- Dorn, E. & Knackmuss, H.-J. (1978). Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate grown pseudomonad. *Biochem J* **174**, 73–84.
- Franzmann, P. D. & Tindall, B. J. (1990). A chemotaxonomic study of the members of the family *Halomonadaceae*. *Syst Appl Microbiol* **13**, 142–147.
- Franzmann, P. D., Wehmeyer, U. & Stackebrandt, E. (1988). *Halomonadaceae* fam. nov., a new family of the class *Proteobacteria* to accommodate the genera *Halomonas* and *Deleya*. *Syst Appl Microbiol* **11**, 16–19.
- Fukumori, F. & Hausinger, R. P. (1993). Purification and characterization of 2,4-dichlorophenoxyacetate/ α -ketoglutarate dioxygenase. *J Biol Chem* **268**, 24311–24317.
- Fulthorpe, R. R. (1991). *Survival, activity and transfer of a bacterial catabolic transposon in an aquatic ecosystem*. PhD thesis, Carleton University, Ottawa.
- Fulthorpe, R. R., McGowan, C., Maltseva, O. V., Holben, W. H., & Tiedje, J. M. (1995). 2,4-Dichlorophenoxyacetic acid degrading bacteria are mosaics of catabolic genes. *Appl Environ Microbiol* **61**, 3274–3281.
- Galinski, E. A. (1993). Compatible solutes of halophilic eubacteria: molecular principles, water-solute interaction, stress protection. *Experientia* **49**, 487–496.
- Ghosal, D. & You, I.-S. (1988). Nucleotide homology and organization of chlorocatechol oxidation genes of plasmids pJP4 and pAC27. *Mol Gen Genet* **211**, 113–120.
- Ghosal, D. & You, I.-S. (1989). Operon structure and nucleotide homology of the chlorocatechol oxidation genes of plasmids pJP4 and pAC27. *Gene* **83**, 225–232.
- Grant, W. D. & Tindall, B. J. (1986). The alkaline saline environment. In *Microbes in Extreme Environments*, pp. 25–54. Edited by R. A. Herbert & G. A. Cood. London: Academic Press.
- Häggblom, M. M. (1992). Microbial breakdown of halogenated aromatic pesticides and related compounds. *FEMS Microbiol Rev* **103**, 29–72.
- Hochstein, L. I. (1988). The physiology and metabolism of the extremely halophilic bacteria. In *Halophilic Bacteria*, vol. 2, pp. 67–79. Edited by F. Rodriguez-Valera. Boca Raton, FL: CRC Press.
- Holben, W. E., Schroeter, B. M., Calabrese, V. G. M., Olsen, R. H., Kukor, J. K., Biederbeck, V. O., Smith, A. E. & Tiedje, J. M. (1992). Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. *Appl Environ Microbiol* **58**, 3941–3948.
- Imhoff, J. F. & Trüper, H. G. (1977). *Ectothiorhodospira halochloris* sp. nov. a new extremely halophilic phototrophic bacterium containing bacteriochlorophyll b. *Arch Microbiol* **114**, 115–121.
- Johnson, R. L., Brillante, S. M., Isabelle, L. M., Houck, J. E. & Pankow, J. F. (1985). Migration of chlorophenolic compounds at the chemical waste disposal site at Alkali Lake, Oregon. 2. Contaminant distribution, transport, and retardation. *Ground Water* **23**, 652–666.
- Jones, B. E., Grant, W. D., Collins, N. C. & Mwatha, W. E. (1994). Alkaliphiles: diversity and identification. In *Bacterial Diversity and Systematics*, pp. 195–230. Edited by F. G. Priest, A. Ramos-Cormenzana & B. J. Tindall. New York & London: Plenum Press.
- Kuhm, A. E., Schlömann, M., Knackmuss, H.-J. & Pieper, D. (1990). Purification and characterization of dichloromucate cycloisomerase from *Alcaligenes eutrophus* JMP134. *Biochem J* **266**, 877–883.
- Kushner, D. J. & Kamekura, M. (1988). Physiology of halophilic bacteria. In *Halophilic Bacteria*, vol. 1, pp. 109–138. Edited by F. Rodriguez-Valera. Boca Raton, FL: CRC Press.
- Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. A., Sogin, M. L. & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* **82**, 6955–6959.
- Larsen N., Olsen, G. J., Madak, B. L., McCaughey, M. J., Overbeek, R., Make, T. J., Marsh, T. L. & Woese, C. R. (1993). The ribosomal database project. *Nucleic Acids Res* **21**, 3021–3023.
- Maltseva, O. V., Solyanikova, I. P., Golovleva, L. A., Schlömann, M. & Knackmuss, H.-J. (1994). Dienelactone hydrolase from *Rhodococcus erythropolis* 1CP: purification and properties. *Arch Microbiol* **162**, 368–374.
- Oren, A., Gurevich, P., Azachi, M. & Henis, Y. (1992). Microbial degradation of pollutants at high salt concentration. *Biodegradation* **3**, 387–398.
- Pankow, J. F., Johnson, R. L., Houck, J. E., Brillante, S. M. & Bryan, W. J. (1984). Migration of chlorophenolic compounds at the

chemical waste disposal site at Alkali Lake, Oregon. 1. Site description and ground-water flow. *Ground Water* **22**, 593–601.

Perkins, E. J., Gordon, M. P., Caceres, O. & Lurquin, P. F. (1990). Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. *J Bacteriol* **172**, 2351–2359.

Sasser, M. & Wichman, M. D. (1991). Identification of microorganisms through use of gas chromatography and high performance liquid chromatography. In *Manual of Clinical Microbiology*, pp. 11–118. Edited by W. J. Hausler, K. L. Herrmann, H. D. Isenberg & H. J. Shadony. Washington, DC: American Society for Microbiology.

Schlömann, M., Pieper, D. H. & Knackmuss, H.-J. (1990). Enzymes of haloaromatics degradation: Variations of *Alcaligenes* on a theme by *Pseudomonas*. In *Pseudomonas: Biotransformation, Pathogenesis, and Evolving Biotechnology*, pp. 185–196. Edited by S. Silver, A. M. Chakrabarty, B. Iglevsky & S. Kaplan. Washington, DC: American Society for Microbiology.

Seibert V., Stadler-Fritzsche, K. & Schlömann, M. (1993). Purification and characterization of maleylacetate reductase from *Alcaligenes eutrophus* JMP 134 (pJP4). *J Bacteriol* **175**, 6745–6754.

Suwa, Y., Holben, W. E. & Forney, L. J. (1994). Cloning of a novel

2,4-D catabolic gene isofunctional to *tfdA* from *Pseudomonas* sp. TFD3. In *Abstracts of the 94th General Meeting of the American Society for Microbiology*. Washington, DC: American Society for Microbiology.

Tonso, N. L., Matheson, V. G. & Holben, W. E. (1995). Polyphasic characterization of a suite of bacterial isolates capable of degrading 2,4-D. *Microb Ecol* **30**, 1–22.

Vallaes, T., Fulthorpe, R. R., Wright, A. M. & Soulas, G. (1996). The metabolic pathway of 2,4-dichlorophenoxyacetic acid degradation involves different families of *tfdA* and *tfdB* genes according to PCR-RFLP analysis. *FEMS Microbiol Ecol* (in press).

Vreeland, R. H., Litchfield, C. D., Martin, E. L. & Elliot, E. (1980). *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria. *Int J Syst Bacteriol* **30**, 485–495.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1963). Formation of methane by bacterial extracts. *J Biol Chem* **238**, 2882–2886.

Received 12 September 1995; revised 22 December 1995; accepted 10 January 1996.