APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1997, p. 3707–3710
0099-2240/97/$04.00+0

Vol. 63, No. 9

Purification and Characterization of a Haloalkane Dehalogenase of a New Substrate Class from a γ-Hexachlorocyclohexane-Degrading Bacterium, *Sphingomonas paucimobilis* UT26

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Received 30 December 1996/Accepted 10 June 1997

The *linB* gene product (LinB), 1,3,4,6-tetrachloro-1,4-cyclohexadiene halidohydrolase, which is involved in the degradation of γ-hexachlorocyclohexane in *Sphingomonas paucimobilis* UT26 (Y. Nagata, T. Nariya, R. Ohtomo, M. Fukuda, K. Yano, and M. Takagi, J. Bacteriol. 175:6403–6410, 1993), was overproduced in *E. coli* and purified to homogeneity. The molecular mass of LinB was deduced to be 30 kDa by gel filtration chromatography and 32 kDa by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel, indicating that LinB is a monomeric enzyme. The optimal pH for activity was 8.2. Not only monochloroalkanes (C_3 to C_{10}) but also dichloroalkanes, bromoalkanes, and chlorinated aliphatic alcohols were good substrates for LinB, suggesting that LinB is a haloalkane dehalogenase with a broad range of substrate specificity. These results indicate that LinB shares properties with another haloalkane dehalogenase, DhlA (S. Keuning, D. B. Janssen, and B. Witholt, J. Bacteriol. 163:635–639, 1985), which shows significant similarity to LinB in primary structure (D. B. Janssen, F. Pries, J. van der Ploeg, B. Kazemier, P. Terpstra, and B. Witholt, J. Bacteriol. 171:6791–6799, 1989) but not in substrate specificity. Principal component analysis of substrate activities of various haloalkane dehalogenases suggested that LinB probably constitutes a new substrate specificity class within this group of enzymes.

γ-Hexachlorocyclohexane (γ-HCH; also called BHC or lindane) is a halogenated organic insecticide which has been used worldwide. Because of its toxicity and long persistence in soil, most countries have prohibited the use of γ-HCH; however, many contaminated sites still remain. Moreover, some countries are currently using γ-HCH for economic reasons, and new sites are continually being contaminated by γ-HCH.

Because γ-HCH is a highly chlorinated compound which has six chlorine atoms per molecule, dechlorination is a very significant step toward its degradation. In fact, we found that two different types of dechlorination reactions are involved in the degradation of γ-HCH in *Sphingomonas* (*Pseudomonas*) *paucimobilis* UT26 (13). The first reaction is dehydrochlorination of γ-HCH to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN) via γ-pentachlorocyclohexene (γ-PCCH). The second reaction is hydrolytic dechlorination of 1,4-TCDN to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) via 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL). Finally, 2,5-dichlorohydroquinone (2,5-DCHQ), which is produced from 2,5-DDOL by 2,5-DDOL dehydrogenase (LinC), is mineralized. We have cloned and sequenced three genes encoding the enzymes which catalyze these three reactions and named them *linA* (6), *linB* (14), and *linC* (16), respectively.

In the previous study, γ-HCH dehydrochlorinase (LinA) was overproduced in *Escherichia coli* and then purified to homogeneity (15). Degradation assays of various halogenated compounds by purified LinA showed that the substrate specificity of LinA is very narrow. Because no significant sequence homologous to that of the *linA* gene has been found yet, its origin is unknown. LinA is thought to be a unique dehydrochlorinase, and its reaction mechanism of dehydrochlorination is of great interest.

Unlike LinA, the deduced amino acid sequence of LinB showed significant similarity to three types of α,β-hydrolase fold enzymes, haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10 (9), haloacetate dehalogenase (DehH1) from *Moraxella* sp. strain B (10), and serine hydrolases represented by 2-hydroxymuconic semialdehyde hydrolase (DmpD) from *Pseudomonas* sp. strain CF600 (17). Because DhlA showed the highest level of similarity to LinB among these three types of hydrolases, it has been suggested that LinB belongs to the family of haloalkane dehalogenase enzymes that catalyze dehalogenation by a hydrolytic mechanism (14). In a previous study, we showed that not only 1-chlorobutane but also 1-chlorodecane and 2-chlorobutane, which are poor substrates for DhlA, were good substrates for resting *E. coli* cells overproducing LinB (14).

In this study, the *linB* gene from *Sphingomonas paucimobilis* UT26 was highly expressed in *E. coli*, and the *linB* product, 1,4-TCDN halidohydrolase (LinB), was purified to homogeneity and characterized. The position of this halidohydrolase, LinB, within a family of haloalkane dehalogenases is discussed.

Purification of LinB overproduced in *E. coli*. In purification steps, LinB activity was routinely assayed with 1-chlorobutane (1-CB) as a substrate. A suitable amount of enzyme was incubated with 50 to ~100 mM 1-CB in 50 mM potassium phosphate buffer (pH 7.5) at 30°C. One unit of enzyme activity was defined as the amount of enzyme required for the release of 1 μmol of chloride ion per min under these conditions. The amount of chloride ion released was measured spectrophotometrically at 460 nm with mercuric thiocyanate and ferric ammonium sulfate by the method of Iwasaki et al. (7). The amount of protein was determined by the protein assay kit

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TABLE 1. Purification of LinB

Step	Amt of total protein (mg)	Total activity (10^{-3} U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	575	1,622	2.8	1	100
$(NH_4)_2SO_4$	332	1,488	4.5	1.6	92
Butyl Toyopearl 650M	64.6	732	11.3	4.0	45
DEAE Sephadex A-50	22.1	305	13.8	4.9	19
DEAE Toyopearl 650M	11.9	292	24.5	8.8	18
Sephadex G-75	3.4	177	52.1	18.6	11

(Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin as a standard. To overproduce LinB in *E. coli*, pMYLB1 (14) was used. In the plasmid, *linB* is transcribed by the *tac* promoter (P*tac*) under the control of *lacI*^{q}. *E. coli* MV1190 containing pMYLB1 was cultured in 2 liters of Luria broth without sodium chloride at 30°C. Cells were harvested (10 g [wet weight]) after induction with 5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), washed with 50 mM potassium phosphate buffer [pH 7.5], and resuspended in the buffer (50 mM potassium phosphate buffer [pH 7.5] containing 1 mM 2-mercaptoethanol and 10% glycerol). The cells were disrupted by sonication (Sonifier 250; Branson, Danbury, Conn.). After centrifugation at 100,000 × *g* for 1 h, the supernatant was used as crude extract. Fifty to eighty percent of ammonium sulfate precipitate fraction was further purified on a butyl Toyopearl 650M column (Tosho, Tokyo, Japan), a DEAE-Sephadex A-50 column (Pharmacia, Uppsala, Sweden), a DEAE-Toyopearl 650M column (Tosho), and a Sephadex G-50 column (Pharmacia). The purification scheme for LinB overproduced in *E. coli* is summarized in Table 1. The enzyme was purified 18.6-fold, with an overall yield of 11%. Only one protein band was observed on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after five steps of purification (Fig. 1).

Molecular weight determination. The molecular weight of denatured protein was determined by SDS-polyacrylamide gel electrophoresis by the method of Laemmli (12). Phosphorylase *b* (97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400) were used as reference proteins. The molecular weight of the native enzyme was estimated by gel filtration on a Superdex 200R 10/30 column (Pharmacia). The column was calibrated with thyroglobulin (670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B_{12} (1,350). The molecular mass of LinB was deduced to be 30 kDa by gel filtration chromatography and 32 kDa by electrophoresis on an SDS-polyacrylamide gel, indicating that LinB is a monomeric enzyme.

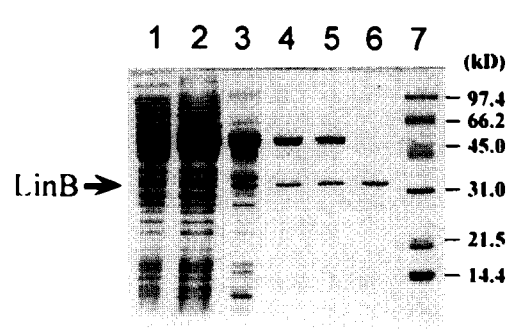

FIG. 1. SDS-polyacrylamide gel electrophoresis of proteins in purification steps. Lanes: 1, crude extract; 2, $(NH_4)_2SO_4$; 3, butyl Toyopearl 650M; 4, DEAE Sephadex A-50; 5, DEAE Toyopearl 650M; 6, Sephadex G-75; 7, molecular mass markers (Bio-Rad).

Optimal pH of LinB. To determine the optimal pH of LinB, potassium phosphate buffer (pH 6 to 8), tricine-NaOH buffer (pH 7.4 to 8.6), and glycine-NaOH buffer (pH 8.6 to 10) were used. Although the optimal pH for LinB activity was 8.2, LinB showed the same level of activities at pH 8.0 to 8.6.

Substrate specificity of purified LinB. The dehalogenation activity of LinB against various substrates was assayed by measuring the rates of halide release. For determination of substrate specificity, a 20 mM concentration of substrates and glycine-NaOH buffer (pH 8.6) was used, and the relative activity of each substrate for 1-CB was determined. Because DhlA from *X. autotrophicus* GJ10 (9) shows significant level of similarity to LinB, the substrate specificity of DhlA was compared with that of LinB (Table 2). LinB had a broad substrate specificity. Not only monochloroalkanes (C_3 to C_{10}) but also dichloroalkanes, bromoalkanes, and chlorinated aliphatic alcohols were good substrates for LinB, indicating that LinB is a haloalkane dehalogenase with a broad range of substrate specificities. However, multihalogenated alkanes and chlorinated aliphatic acids were not dehalogenated by LinB.

Statistical analysis. Principal component analysis (PCA) (24) was applied to compare the substrate specificity of LinB with the activities of the halidohydrolases isolated from other strains. PCA extracts and visualizes systematic patterns or trends in large data matrices. Clustering of the enzymes based on their activities for 19 various halogenated substrates (Table 2) has been investigated with score and loading plots from PCA. Score plots of the first and third principal components are shown in Fig. 2. LinB more closely resembles the enzymes of *Rhodococcus* sp. strain HA1 (19), *Rhodococcus erythropolis* Y2 (18), *Rhodococcus* sp. strain m15-3 (26), *Acinetobacter* sp. strain GJ70 (8), *Corynebacterium* sp. strain m2C-32 (25), and *Rhodococcus* sp. strain CP9 (2) than it resembles those of *X. autotrophicus* GJ10 (11) and *Pseudomonas* sp. strain E4M (23) in the horizontal direction, and it is separated from other enzymes in the vertical direction. Separation of the dehalogenases in the direction of the horizontal axis clearly distinguishes the GJ10 and E4m dehalogenases from all others. This result reflects the fact that GJ10 and E4m dehalogenases prefer short-chain substrates, while the remaining dehalogenases prefer long-chain ones. The difference in the vertical direction reflects the results that 1-chloropropane and 1-bromobutane are good substrates for LinB but not for the HA1, Y2, m15-3, GJ70, m2C-32, and CP9 enzymes. Separate clustering of LinB may indicate that it is a representative of a new substrate specificity class within the known halidohydrolase family. The dehalogenation activity data for all dehalogenases except LinB and detailed description of the statistical analysis are being published elsewhere (2).

Kinetics of LinB activity. The activity of halidohydrolases for α,ω-dihalogenated ethanes is very important for characterizing these functionally related groups of enzymes. All halidohydrolases known to date have been shown to easily dehalogenate 1,2-dibromoethane, while only a few of them can attack 1,2-dichloroethane. We have critically tested both dichlorinated and dibrominated ethanes and 1-chloro-2-bromoethane as substrates of LinB (Table 3). Michaelis-Menten kinetic constants with several saturated and unsaturated halogenated compounds (Table 3) were determined by initial-velocity measurements by using gas chromatography to determine the dissolved substrate and product concentrations. The concentration range of the substrates used for determination

TABLE 2. Substrate ranges of purified LinB and DhlA

Substrate	Reaction rate[a] of: LinB	DhlA[b]
Monochloroalkanes		
1-Chloropropane[c] (C_3)	135	166
1-CB[c] (C_4)	100	100
1-Chloropentane[c] (C_5)	120	ND
1-Chlorohexane[c] (C_6)	145	
1-Chloroheptane[c] (C_7)	161	
1-Chlorooctane[c] (C_8)	139	
1-Chlorononane[c] (C_9)	70	
1-Chlorodecane (C_{10})	27	
Laurylchloride (C_{12})	ND	
2-Chlorobutane (C_4)	101	
2-Chlorooctane (C_8)	47	
3-Chlorohexane (C_6)	19	
Dichloroalkanes		
Dichloromethane	ND	ND
1,2-Dichloroethane[c]	ND	323
1,3-Dichloropropane[c]	63	258
1,4-Dichlorobutane	84	
1,6-Dichlorohexane[c]	83	ND
1,9-Dichlorononane[c]	100	ND
1,1-Dichloroethane	ND	ND
Multihalogenated alkanes		
1,1,1-Trichloroethane	ND	
1,1,2,2-Tetrachloroethane	ND	
Halogenated alkanes		
1-Bromoethane[c]	257	77
1-Bromobutane[c]	234	
1,2-Dibromoethane[c]	355	303
1,2-Dibromopropane[c]	241	
Chlorinated aliphatic alcohols		
2-Chloroethanol[c]	13	ND
3-Chloropropanol[c]	ND	
4-Chlorobutanol	90	
6-Chlorohexanol	116	
Chlorinated aliphatic acids		
Chloroacetic acid[c]	ND	ND
2-Chloropropionic acid[c]	ND	ND
3-Chloropropionic acid	ND	ND
2-Chlorobutyric acid	ND	
3-Chlorobutyric acid	ND	

[a] Relative to the rate for 1-CB. ND, not detectable.
[b] Data from reference 11.
[c] Substrate used for PCA.

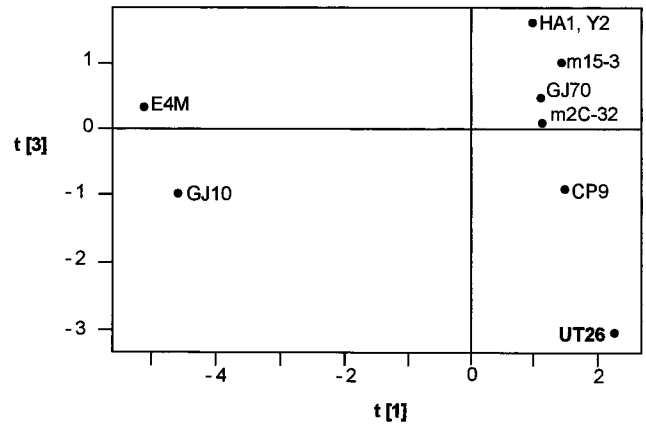

FIG. 2. Score plot of first (t [1]) and third (t [3]) latent variables from PCA. The clustering of the haloalkane halidohydrolases in space is based on their relative activities towards various halogenated compounds (2). The enzymes positioned close to each other are likely to be similar in their substrate specificities (see the text for a more detailed explanation). UT26, *S. paucimobilis* UT26 (this study); GJ10, *X. autotrophicus* GJ10 (11); E4M, *Pseudomonas* sp. strain E4M (23); GJ70, *Acinetobacter* sp. strain GJ70 (8); HA1, *Rhodococcus* sp. strain HA1 (19); Y2, *Rhodococcus erythropolis* Y2 (18); m15-3, *Rhodococcus* sp. strain m15-3 (26); m2C-32, *Corynebacterium* sp. strain m2C-32 (25); CP9, *Rhodococcus* sp. strain CP9 (2).

of the Michaelis-Menten constants was approximately 0.02 to 6 mM depending on the affinity of the enzyme for a particular substrate. The substrates with no reported activity were tested at higher concentrations (up to 100 mM). No activity has been observed with 1,2-dichloroethane, and very high activity has been observed with the brominated analog. LinB was shown to attack 1-chloro-2-bromoethane with somewhat higher K_m and lower V_{max} constants than 1,2-dibromoethane. The chloroethanol was identified by using gas chromatography as a reaction product of the 1-chloro-2-bromoethane dehalogenation (data not shown). This result suggested that it could be an intrinsic property of the halogen substituent which is removed (and not the other one) of α,ω-dihalogenated ethanes determining whether dehalogenation will proceed.

An effort was also made to determine the kinetic constants of two groups of analogous chlorinated-brominated/saturated-unsaturated compounds. Short-chain chlorinated compounds, recalcitrant in the saturated form, became relatively good substrates upon obtaining the unsaturated bond, while the opposite phenomenon was observed for brominated compounds—saturated ones which were relatively good substrates compared to unsaturated ones (Table 3). These results indicate that qualitatively similar activity has been obtained for crude extracts of *R. erythropolis* Y2 (3) and purified LinB.

Conclusion. We purified the LinB overproduced in *E. coli* to homogeneity. LinB was a monomeric enzyme, and its optimal pH was 8.2. Not only monochloroalkanes (C_3 to C_{10}) but also dichloroalkanes, bromoalkanes and chlorinated aliphatic alcohols were good substrates for LinB. These results indicate that LinB shares properties with DhlA (11) except for the substrate specificity (Table 2). In brief, LinB prefers long-chain haloalkanes as substrates, while DhlA prefers short-chain haloalkanes. A hypothetical three-dimensional structure of LinB enzyme has been predicted by homology modeling (1) based on

TABLE 3. Kinetic parameters of LinB

Compound	V_{max} (10^{-9} mol liter^{-1} s^{-1})	K_m (mM)
1,2-Dichloroethane	–[a]	–
1,2-Dibromoethane	0.4	1.9
1-Chloro-2-bromoethane	0.3	3.9
1,2-Dichloropropane	–	–
1,2-Dibromopropane	0.2	0.9
1-Chloro-2-methylpropane	–	–
1-Bromo-2-methylpropane	0.03	0.05
2,3-Dichloropropene	0.01	0.7
2,3-Dibromopropene	–	–
3-Chloro-2-methylpropene[b]	0.35	0.3
3-Bromo-2-methylpropene	–	–

[a] –, Not detectable (detection limit, 60 μM).
[b] Constants for 3-chloro-2-methylpropene are qualitative only, due to problems with the analytical part of the measurement.

its significant sequence similarity with DhlA, whose three-dimensional structure is known from X-ray crystallography (5, 21, 22). The deduced active-site residue of DhlA (Asp-124, which is a nucleophilic residue essential for catalysis) is also conserved in LinB (Asp-108), and other residues around the putative active site are highly conserved between the two enzymes, suggesting that the reaction mechanism of at least the first step (nucleophilic bimolecular substitution) of the dehalogenation reaction is the same for both enzymes. One of the residues around the putative active site, Trp-175 in DhlA numbering, is probably changed to Phe (or Gln) in LinB. The importance of this Trp residue in DhlA for its catalysis of 1,2-dichloroethane dehalogenation was examined by quantum chemistry calculations (4). The change of Trp to Phe increased the reaction barrier of the first reaction step, which suggests that this is one of the very important differences between these two enzymes, partly explaining the difference in substrate specificity. To confirm this speculation, further analysis (such as site-directed mutagenesis of LinB) is necessary.

We used the easy and conventional method to measure the dehalogenation activity. This method seems to be the best method to compare the substrate specificity of LinB with other dehalogenases, because the substrate specificities of other known dehalogenases were measured by similar methods. The low solubility of some of the substrates could be a problem with this type of assay. Nevertheless, Scholtz et al. performed experiments with dehalogenation in dimethyl sulfoxide or isopropanol as a solvent and concluded that the presence of solvent in the enzyme assay inhibited the reaction but did not alter the relative rates of reaction for the different compounds (19). For the kinetic analysis presented in this study, we can exclude the problem of solubility, because we measured the actual concentration of tested substrates by gas chromatographic analysis.

We applied PCA for comparison of the substrate activities between our dehalogenase and the other enzymes of the halidohydrolase family. This analysis showed the activity of the LinB dehalogenase for most of the studied substrates to be similar to the activities published for the dehalogenases of HA1, Y2, m15-3, GJ70, m2C-32, and CP9 (Fig. 2). Slater et al. divided haloalkane dehalogenase into two classes (20). Class 3R, from gram-negative bacteria, shows a fairly restricted range of substrate specificity, while class 3B, from gram-positive bacteria, shows much broader substrate specificities. Although LinB has been placed in class 3R, we showed that because of its substrate specificity it resembles class 3B instead. Furthermore, LinB also showed significant differences for several substrates (for example, 1-chloropropane and 1-bromobutane). This result suggests that LinB forms a special substrate specificity class within the halidohydrolase family. It would certainly be useful to compare these three classes of enzymes in order to better understand the structure-function relationships within halidohydrolases.

We thank T. Omori and his colleagues of The University of Tokyo, Tokyo, Japan, for helpful advice and for supplying haloalkanes.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan. This work was performed in the facilities of the Biotechnology Research Center, The University of Tokyo, and the Research Centre for Atmospheric Chemistry (RECETOX), Masaryk University, Brno, Czech Republic.

REFERENCES

1. **Damborsky, J., A. T. Bull, and D. J. Hardman.** 1995. Homology modelling of haloalkane dehalogenase of *Sphingomonas paucimobilis* UT26. Biologia **50:** 523–528.
2. **Damborsky, J., M. G. Nyandoroh, M. Nemec, I. Holoubek, A. T. Bull, and D. J. Hardman.** Some biochemical properties and the classification of a range of bacterial haloalkane dehalogenases. Biotechnol. Appl. Biochem., in press.
3. **Damborsky, J., K. Manová, A. Berglund, M. Sjöström, M. Nemec, and I. Holoubek.** 1997. Biotransformation of chloro- and bromoalkenes by crude extracts of *Rhodococcus erythropolis* Y2. Adv. Environ. Res. **1:**50–57.
4. **Damborsky, J., M. Kuty, M. Nemec, and J. Koca.** Molecular modelling study of the catalytic mechanism of haloalkane dehalogenase. I. Quantum chemical study of the first reaction step. J. Chem. Inf. Comput. Sci., in press.
5. **Franken, S. M., H. J. Rozeboom, K. H. Kalk, and B. W. Dijkstra.** 1991. Crystal structure of haloalkane dehalogenase: an enzyme to detoxify halogenated alkanes. EMBO J. **10:**1297–1302.
6. **Imai, R., Y. Nagata, M. Fukuda, M. Takagi, and K. Yano.** 1991. Molecular cloning of a *Pseudomonas paucimobilis* gene encoding a 17-kilodalton polypeptide that eliminates HCl molecules from γ-hexachlorocyclohexane. J. Bacteriol. **173:**6811–6819.
7. **Iwasaki, I., S. Utsumi, and T. Ozawa.** 1952. New colorimetric determination of chloride using mercuric thiocyanate and ferric ion. Bull. Chem. Soc. Jpn. **25:**226.
8. **Janssen, D. B., J. Gerritse, J. Brackman, C. Kalk, D. Jager, and B. Witholt.** 1988. Purification and characterization of a bacterial dehalogenase with activity toward halogenated alkanes, alcohols, and ethers. Eur. J. Biochem. **171:**67–92.
9. **Janssen, D. B., F. Pries, J. van der Ploeg, B. Kazemier, P. Terpstra, and B. Witholt.** 1989. Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. J. Bacteriol. **171:**6791–6799.
10. **Kawasaki, H., K. Tsuda, I. Matsushita, and K. Tonomura.** 1992. Lack of homology between two haloacetate dehalogenase genes encoded on a plasmid from *Moraxella* sp. strain B. J. Gen. Microbiol. **138:**1317–1323.
11. **Keuning, S., D. B. Janssen, and B. Witholt.** 1985. Purification and characterization of hydrolytic haloalkane dehalogenase from *Xanthobacter autotrophicus* GJ10. J. Bacteriol. **163:**635–639.
12. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
13. **Nagasawa, S., R. Kikuchi, Y. Nagata, M. Takagi, and M. Matsuo.** 1993. Aerobic mineralization of γ-HCH by *Pseudomonas paucimobilis* UT26. Chemosphere **26:**1719–1728.
14. **Nagata, Y., T. Nariya, R. Ohtomo, M. Fukuda, K. Yano, and M. Takagi.** 1993. Cloning and sequencing of a dehalogenase gene encoding an enzyme with hydrolase activity involved in the degradation of γ-hexachlorocyclohexane in *Pseudomonas paucimobilis*. J. Bacteriol. **175:**6403–6410.
15. **Nagata, Y., T. Hatta, R. Imai, K. Kimbara, M. Fukuda, K. Yano, and M. Takagi.** 1993. Purification and characterization of γ-hexachlorocyclohexane (γ-HCH) dehydrochlorinase (LinA) from *Pseudomonas paucimobilis*. Biosci. Biotechnol. Biochem. **57:**1582–1583.
16. **Nagata, Y., R. Ohtomo, K. Miyauchi, M. Fukuda, K. Yano, and M. Takagi.** 1994. Cloning and sequencing of a 2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase gene involved in the degradation of γ-hexachlorocyclohexane in *Pseudomonas paucimobilis*. J. Bacteriol. **176:**3117–3125.
17. **Nordlund, I., and V. Shingler.** 1990. Nucleotide sequences of the *meta*-cleavage pathway enzymes 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase from *Pseudomonas* CF600. Biochim. Biophys. Acta **1049:**227–230.
18. **Sallis, P. J., S. J. Armfield, A. T. Bull, and D. J. Hardman.** 1990. Isolation and characterization of a haloalkane halidohydrolase from *Rhodococcus erythropolis* Y2. J. Gen. Microbiol. **136:**115–120.
19. **Scholtz, R., T. Leisinger, F. Suter, and A. M. Cook.** 1987. Characterization of 1-chlorohexane halidohydrolase, a dehalogenase of wide substrate range from an *Arthrobacter* sp. J. Bacteriol. **169:**5016–5021.
20. **Slater, J. H., A. T. Bull, and D. J. Hardman.** 1995. Microbial dehalogenation. Biodegradation **6:**181–189.
21. **Verschueren, K. H. G., F. Siljée, H. J. Rozeboom, K. H. Kalk, and B. W. Dijkstra.** 1993. Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase. Nature (London) **363:**693–698.
22. **Verschueren, K. H. G., S. M. Franken, H. J. Rozeboom, K. H. Kalk, and B. W. Dijkstra.** 1993. Refined X-ray structures of haloalkane dehalogenase at pH 6.2 and pH 8.2 and implications for the reaction mechanism. J. Mol. Biol. **232:**856–872.
23. **Vienravi, V.** 1993. Ph.D. thesis. University of Kent at Canterbury, Canterbury, England.
24. **Wold, S., K. Esbensen, and P. Geladi.** 1987. Principal component analysis. Chemometrics Intelligent Lab. Syst. **2:**37–52.
25. **Yokota, T., H. Fuse, T. Omori, and Y. Minoda.** 1986. Microbial dehalogenation of haloalkanes mediated by oxygenase of halidohydrolase. Agric. Biol. Chem. **50:**453–460.
26. **Yokota, T., T. Omori, and T. Kodama.** 1987. Purification and properties of haloalkane dehalogenase from *Corynebacterium* sp. strain m15-3. J. Bacteriol. **169:**4049–4054.