Protein Expression and Purification **17,** 299–304 (1999)
Article ID prep.1999.1143, available online at http://www.idealibrary.com on IDEAL®

Construction and Characterization of Histidine-Tagged Haloalkane Dehalogenase (LinB) of a New Substrate Class from a γ-Hexachlorocyclohexane-Degrading Bacterium, *Sphingomonas paucimobilis* UT26

Yuji Nagata,*[,1] Kamila Hynková,† Jiří Damborský,† and Masamichi Takagi*
**Department of Biotechnology, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; and †Laboratory of Biomolecular Structure and Dynamics, Masaryk University, Kotlarska 2, 611 37 Brno, Czech Republic*

Received July 27, 1999, and in revised form August 22, 1999

The *linB* gene product (LinB), which is involved in the degradation of γ-hexachlorocyclohexane in *Sphingomonas paucimobilis* UT26, is a member of haloalkane dehalogenases with a broad range of substrate specificity. Elucidation of the factors determining its substrate specificity is of interest. Aiming to facilitate purification of recombinant LinB protein for site-directed mutagenesis analysis, a 6-histidyl tail was added to the C-terminus of LinB. The His-tagged LinB was specifically bound with Ni-NTA resin in the buffer containing 10 mM imidazole. After elution with 500 mM imidazole, quantitative recovery of protein occurred. The steady-state kinetic parameters of the His-tagged LinB for four substrates were in good agreement with that of wild-type recombinant LinB. Although the His-tagged LinB expressed in an average of 80% of the activity of the wild type LinB for 10 different substrates, the decrease was very similar for different substrates with the standard deviation of 5.5%. The small activity reduction is independent of the substrate shape, size, or number of substituents, indicating that the His-tagged LinB can be used for further mutagenesis studies. To confirm the suitability of this system for mutagenesis studies, two mutant proteins with substitution in putative halide binding residues (W109 and F151) were constructed, purified, and tested for activity. As expected, complete loss in activity of W109L and sustained activity of F151W were observed.

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 throughout the world (1). Moreover, some countries are presently using γ-HCH for economic reasons, and thus new sites are continuously being contaminated (2).

Because γ-HCH is a highly chlorinated compound which has six chlorine atoms per molecule, dechlorination is a very significant step for its degradation. In fact, we have revealed that three different types of dechlorination reactions are sequentially involved in the degradation of γ-HCH in *Sphingomonas* (formerly *Pseudomonas*) *paucimobilis* UT26 (3–5). 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) (3). 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) (4). Finally, the third reaction is reductive dechlorination of 2,5-dichlorohydroquinone (2,5-DCHQ), which is produced from 2,5-DDOL by dehydrogenase (LinC) (6), to hydroquinone (5). We have cloned four genes (*linA*, *linB*, *linC*, and *linD*) which are involved in the above-mentioned reactions (3–6).

The deduced amino acid sequence of LinB showed significant similarity to haloalkane dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10 (7), suggesting that LinB belongs to the family of haloalkane dehalogenase enzymes catalyzing the dehalogenation by hydrolytic mechanism (4). It was revealed that LinB has broader specificity than DhlA. For example, 1-chlorodecane and 2-chlorobutane, which are poor substrates for DhlA, were good substrates for resting *Esch-*

[1] To whom correspondence should be addressed. Fax: +81-3-5841-8015. E-mail: aynaga@hongo.ecc.u-tokyo.ac.jp.

1046-5928/99 $30.00

erichia coli cells overproducing LinB (4). Furthermore, the *linB* gene from *S. paucimobilis* UT26 was highly expressed in *E. coli*, and the LinB was purified to homogeneity and characterized (8). Principal component analysis of substrate activities of various haloalkane dehalogenases suggested that LinB constitutes a new substrate specificity class within this group of enzymes (8). It would certainly be useful to analyze reaction mechanism of LinB to better understand the structure–function relationships within halidohydrolases. LinB belongs to the family of α/β-hydrolases (9) which employ a catalytic triad, i.e., nucleophile–histidine–acid, during the catalytic reaction. First, the position of the catalytic triad within the sequence of LinB was probed by a site-directed mutagenesis. The catalytic triad residues of the haloalkane dehalogenase LinB are proposed to be D108, H272, and E132 (10).

For further site-directed mutagenesis analysis, facilitating purification process of recombinant LinB protein is desirable. In this study, a 6-histidyl tail was added to the C-terminus of LinB. The resulting His-tagged LinB was purified and characterized.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI). The enzymes used for DNA manipulations were obtained from Takara Shuzo Co. (Kyoto, Japan) and TOYOBO Co. (Osaka, Japan). The oligonucleotides were synthesized by Espec-oligo Service Co. (Tsukuba, Japan). The strain used in this study is *E. coli* HB101 (11).

Construction of plasmid for expression of His-tagged LinB. The oligo nucleotides used as primers are as follows: 5′-GCC GAA TTC TAA GGA GGA ATA TCG ATG AGC CTC-3′ (33-mer) and 5′-GCC AAG CTT GGA TTA GTG ATG GTG ATG GTG ATG TGC TGG GCG CAA TCG-3′ (48-mer). The former is designed for the introduction of *Eco*RI site and for change of SD sequence from AAGGAG to TAAGGAGG which interacts with the 3′ end of the 16S rRNA during translational initiation in *E. coli* (12). The latter is designed for the introduction of *Hin*dIII site and 6-histidyl tail into the C-terminus of the LinB. The PCR was performed by using these primers, KOD polymerase (TOYOBO), and pMYLB1 (4) as a template. After digestion with *Eco*RI and *Hin*dIII, the amplified fragment was introduced into multiple cloning site of pUC18 (13). The resulting plasmid was named pULBH6. All of the nucleotide sequences of the amplified fragment were confirmed by the dideoxy-chain termination method with an automated DNA sequencer (LI-COR Model 4000L, Aloka Co., Tokyo, Japan). An overexpression plasmid, pMLBH6 (Fig. 1), was constructed from pAQN, which has the same structure as pAQI (14) except for differences in the aqualysin I-coding region. The plasmid pAQN was digested with *Eco*RI and *Hin*dIII to replace the 1.8-kb aqualysin I-coding fragment with the 0.9-kb *Eco*RI–*Hin*dIII fragment from pULBH6. The resulting plasmid (pMLBH6) contains the origin of replication from pUC18 and *lacI*^{q} gene and expressed the His-tagged *linB* gene under control of the *tac* promoter.

Site-directed mutagenesis. Mutagenesis of His-tagged LinB was performed by using LA PCR *in vitro* mutagenesis kit (TaKaRa Shuzo Co., Kyoto, Japan), according to the provided protocol except for using Pyrobest DNA polymerase (TaKaRa Shuzo Co.) whose fidelity is very high. All of the nucleotide sequences of mutants were confirmed by the dideoxy-chain termination method with ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

The oligo nucleotides used are as follows: W109L (5′-GGC CTG CAA CAG ATC GCG-3′), F151W (5′-GGC CTG CCA CAG ATC GCG-3′).

Expression and purification of the His-tagged LinB. *E. coli* HB101 containing pMLBH6 were cultured in 2 liters of Luria broth (LB) (11) at 30°C. When the culture reached an optical density of 0.6 at 660 nm, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Cells were harvested (10 g wet wt) after 3 h incubation, washed by 50 mM potassium phosphate buffer [pH 7.5], and resuspended in 50 ml of 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, CT). After centrifugation at 100,000*g* for 1 h, the supernatant was used as crude extract. Almost all of the crude extract was further purified by a 20-ml volume of Ni-NTA agarose (QIAGEN) at 4°C. The His-tagged LinB was allowed to bind to the resin in the wash buffer (20 mM potassium phosphate buffer [pH 7.5] containing 0.5 M sodium chloride and 10 mM imidazole), and then was eluted by the elution buffer (20 mM potassium phosphate buffer [pH 7.5] containing 0.5 M sodium chloride and 500 mM imidazole). The purified enzyme was stored in the buffer (50 mM potassium phosphate buffer [pH 7.5] containing 1 mM 2-mercaptoethanol and 10% glycerol) at concentration about 1 mg/ml at 0–4°C.

Circular dichroism spectra. Circular dichroism (CD) spectra were measured with a JASCO J-720 spectropolarimeter at room temperature. The cells used were 1- and 0.1-cm light paths for wavelengths between 250–320 and 190–250 nm, respectively. Enzyme (0.45 mg/ml) in 50 mM potassium phosphate buffer (pH 7.5) was used.

Michaelis–Menten kinetics. Michaelis–Menten kinetic constants were determined by initial-velocity measurements as described previously (10). K_M and k_{cat} values with their standard deviations were calculated

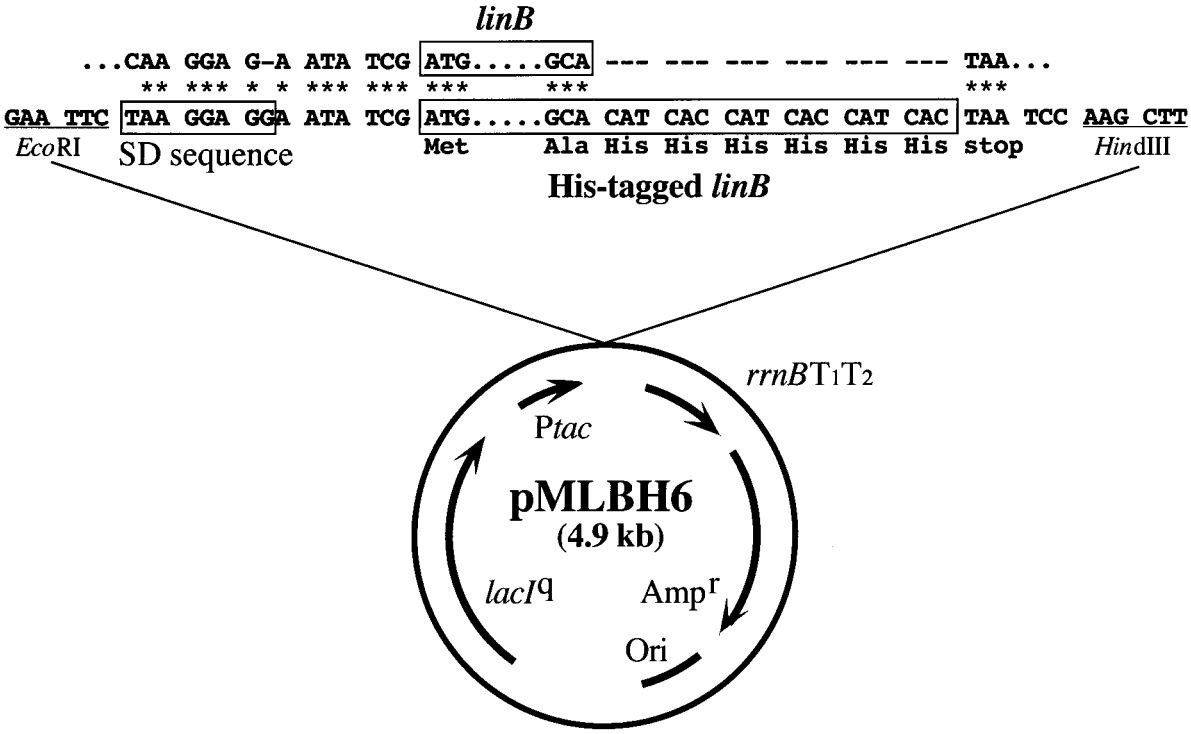

FIG. 1. Construction of His-tagged haloalkane dehalogenase (LinB) expression vector, pMLBH6. The SD sequence was changed, and the 6-histidyl tail was introduced into the C-terminus of LinB. See text for detail.

by means of the program Leonora (15) by the method of least squares with relative weighting.

Activity measurements. 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 glycine buffer 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 minute 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.* (16).

Dehalogenation reaction rates were determined for 10 different substrates using the method of gas chromatography as described previously (10). The reaction rates were quantified by a slope of the relationship between the product concentration and time. Dehalogenation activity was expressed in percentages, where the rate of reaction of wild-type enzyme is 100% for each substrate.

RESULTS

Construction and purification of His-tagged LinB. For expression of the His-tagged LinB, the plasmid pMLBH6 was constructed (Fig. 1). *E. coli* HB101 harboring pMLBH6 was cultured and induced with IPTG as described under Materials and Methods. His-tagged LinB was produced to about 15% of the total cell protein. Because the *linB* gene has rare codons in *E. coli,* such as AGG (12), a higher level of expression of LinB may be possible by coexpression of tRNAs for such rare codons or by site-directed mutagenesis of *linB.* The *E. coli* cells producing LinB were harvested and disrupted. After centrifugation, the supernatant was used as crude extract. The crude extract showed a specific activity of 0.12 U/mg protein. The specific activity of crude extract was increased about two times as a result of improving the SD sequence (data not shown). The His-tagged LinB in the crude extract was further purified by a Ni-NTA resin (QIAGEN). Only one protein band was observed on SDS–polyacrylamide gel electrophoresis after this treatment (Fig. 2). The purification scheme for the His-tagged LinB is summarized in Table 1. The specific activity of the purified His-tagged LinB was 1.18 U/mg protein, indicating that the enzyme was purified approximately 10-fold by the one step.

The integrity of the structure of the purified His-tagged LinB was tested by CD spectroscopy. The CD spectra of the wild-type and His-tagged protein are shown in Fig. 3. Like the wild-type LinB, His-tagged protein had the double ellipticity minimum at 210 and 220 nm typical of α-helical content. The CD spectra of the wild-type and His-tagged proteins are essentially

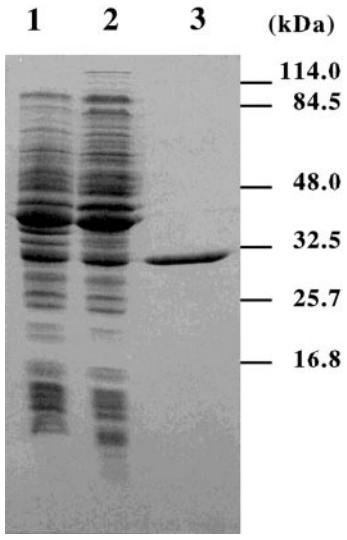

FIG. 2. Protein patterns during the His-tagged LinB purification. The samples were analyzed by SDS–polyacrylamide gel electrophoresis. Lanes: 1, total cell proteins; 2, crude extract; 3, His-tagged LinB.

the same, indicating that the backbone polypeptide chain of the constructed protein had a very similar conformation. It was concluded that the His-tag did not disturb the overall structure of the protein.

Activities of His-tagged LinB. The purified wild-type and His-tagged enzymes were examined for their kinetic characteristics. The steady-state kinetic parameters of the His-tagged LinB for four substrates (1-chlorobutane, 1-bromopropane, 1-bromo-2-methylpropane, and 2-bromo-1-chloropropane) were in good agreement with those of wild-type LinB (Table 2).

The relative activity of His-tagged LinB was determined with the group of 10 different substrates to test whether there is a difference in substrate specificity between wild-type and His-tagged LinB. The reaction rates were quantified by a slope of the plot of the product concentration and time. The rates obtained with the His-tagged LinB are expressed as the percentage activity of the wild-type enzyme in Table 3. Although the His-tagged LinB expressed an average of

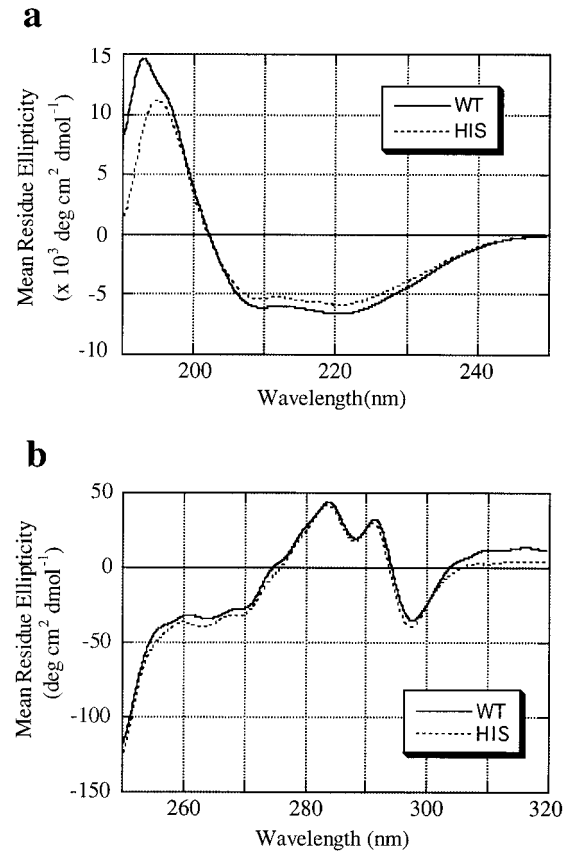

FIG. 3. Circular dichroism spectra for wavelengths between 190 and 250 nm (a) and between 250 and 320 nm (b) of wild-type haloalkane dehalogenase LinB and His-tagged LinB.

80% of the activity of the wild-type LinB for 10 different substrates, the decrease was very similar for different substrates with the standard deviation of 5.5%. The small activity reduction is independent of the substrate shape, size, or number of substituents.

Activities of His-tagged mutant proteins. To confirm the validity of this His-tagged system, we constructed two mutants, W109L and F151W, by site-directed mutagenesis and purified to homogeneity (Fig. 4). Both residues are expected to participate in binding and stabilization of halogen atom during dehalogenation reaction (17). Replacement of these residues by a nonaromatic amino acid should result in significant loss of activity, while at least some activity was expected with the mutant carrying a conservative substitution. Specific activities of W109L and F151W mutants toward 1-chlorobutane were determined spectrophotometrically. No activity was observed for W109L, while F151W showed the same level of activity (1.16 U/mg) as wild-type His-tagged enzyme (1.18 U/mg).

TABLE 1

The Purification Scheme for the His-Tagged LinB

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification factor
Crude extract	918	110	0.12	100	
Ni-NTA	50	59	1.18	54	9.8

TABLE 2

Kinetic Parameters of Wild-Type and His-Tagged LinB

Substrate	Wild-type			His-tagged		
	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M
1-Chlorobutane	0.18 ± 0.02	1.6 ± 0.06	8.8	0.16 ± 0.01	1.0 ± 0.03	6.5
1-Bromopropane	0.23 ± 0.04	5.5 ± 0.54	24	0.31 ± 0.02	6.2 ± 0.18	20
1-Bromo-2-methylpropane	0.05 ± 0.01	1.6 ± 0.15	34	0.06 ± 0.02	1.8 ± 0.17	28
2-Bromo-1-chloropropane	0.55 ± 0.05	1.4 ± 0.06	2.5	0.49 ± 0.05	2.2 ± 0.09	4.4

DISCUSSION

Because the substrate specificity of LinB is relatively broad, this enzyme is interesting for bioremediation purposes. The important pesticides, 1,2-dichloropropane and 2-chloropropane, are recalcitrant under aerobic conditions. Although LinB cannot efficiently catalyze the dehalogenation reaction of these pesticides (no other dehalogenase known to date can), modification of the geometry and composition of an active LinB site could possibly lead to more efficient binding and catalysis of these chlorinated propanes. The catalysis of 1,2-dichloropropane and 2-chloropropane by hydrolytic dehalogenases is possible from the point of view of the reaction mechanism as indicated by quantitative structure–biodegradability relationships studies (18,19).

In this study, we constructed and characterized the His-tagged haloalkane dehalogenase LinB. The His-tagged LinB was highly expressed in *E. coli* and was easily purified by Ni-NTA resin. The CD spectra of the wild-type and His-tagged proteins are essentially the same, indicating that the backbone polypeptide chain of constructed protein had a very similar conformation. At least, the His-tag did not disturb the overall structure of the protein. The K_M and k_{cat} kinetic parameters of the His-tagged LinB for four substrates were in good agreement with those of the wild-type LinB. Although the His-tagged LinB expressed an average of 80% of the relative activity of the wild-type LinB for 10 different substrates, the decrease was very similar for different substrates with the standard deviation of 5.5%. From these results, it was concluded that the His-tagged LinB has enough features similar to those of wild-type protein for further analysis of its reaction mechanism and for the mutagenesis studies. Consistent substrate specificity of the wild-type and His-tagged dehalogenase makes this system suitable also for the quantitative structure–biodegradability studies which require large volumes of the protein material for experimental testing.

Furthermore, we prepared two mutants, W109L and F151W, and confirmed that the His-tagged system we constructed here is working well. W109L lost the dehalogenase activity toward 1-chlorobutane completely, while F151W retained the activity at the same level as the wild-type enzyme. W109 and F151 are proposed to be the residues which are involved in halide stabilization during dehalogenation reaction. For their func-

TABLE 3

Relative Activity of His-Tagged LinB with Different Substrates

Substrate	Relative activity[a] of His-tagged LinB (%)
1-Chlorobutane	77
1,3-Dibromopropane	75
1,3-Dichloropropane	73
1-Bromopropane	75
1-Bromo-3-chloropropane	91
1-Chloropropane	80
1,2-Dibromopropane	81
1-Bromo-2-methylpropane	76
1-Bromohexane	80
1-Bromocyclohexane	89
Average	80
Standard deviation	5.5

[a] Activity expresses the percentage of the rates obtained with the wild-type LinB.

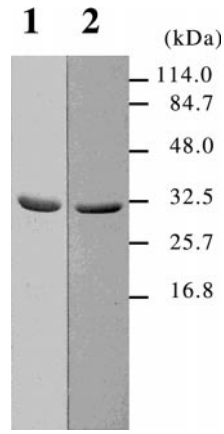

FIG. 4. SDS–polyacrylamide gel electrophoresis of purified LinB mutants, W109L and F151W. Lanes: 1, W109L; 2, F151W.

tion, partial positive charge is essential. Since replacement of tryptophan to leucine diminishes the positive charge of the residue, loss of the dehalogenase activity of W109L is reasonable. Replacement of phenylalanine by tryptophan does not diminish halide stabilization which is consistent with the observed activity of the F151W mutant.

ACKNOWLEDGMENTS

This work was financially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan and by the Czech Grant Agency (Postdoc 203/97/P149), and the Czech Ministry of Education (ME276). A part of this work was performed using the facilities of the Biotechnology Research Center, University of Tokyo.

REFERENCES

1. Iwata, H., Tanabe, S., Sakai, N., and Tatsukawa, R. (1993) Distribution of persistent organochlorines in the oceanic air and surface seawater and the role of ocean on their global transport and fate. *Environ. Sci. Technol.* **27,** 1080–1098.
2. Blais, J. M., Schindler, D. W., Muir, D. C. G., Kimpe, L. E., Donald, D. B., and Rosenberg, B. (1998) Accumulation of persistent organochlorine compounds in mountains of western Canada. *Nature* **395,** 585–588.
3. Imai, R., Nagata, Y., Fukuda, M., Takagi, M., and Yano, K. (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.
4. Nagata, Y., Nariya, T., Ohtomo, R., Fukuda, M., Yano, K., and Tagagi, M. (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.
5. Miyauchi, K., Suh, S.-K., Nagata, Y., and Takagi, M. (1998) Cloning and sequencing of a 2,5-dichlorohydroquinone reductive dehalogenase gene whose product is involved in degradation of γ-hexachlorocyclohexane by *Sphingomonas paucimobilis. J. Bacteriol.* **180,** 1354–1359.
6. Nagata, Y., Ohtomo, R., Miyauchi, K., Fukuda, M., Yano, K., and Takagi, M. (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.
7. Janssen, D. B., Pries, F., van der Ploeg, J., Kazemier, B., Terpstra, P., and Witholt, B. (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.
8. Nagata, Y., Miyauchi, K., Damborsky, J., Manova, K., Ansorgova, A., and Takagi, M. (1997) Purification and characterization of haloalkane dehalogenase of a new substrate class from a γ-hexachlorocyclohexane-degrading bacterium, *Sphingomonas paucimobilis* UT26. *Appl. Environ. Microbiol.* **63,** 3707–3710.
9. Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G., and Goldman, A. (1992) The α/β hydrolase fold. *Protein Eng.* **5,** 197–211.
10. Hynkova, K., Nagata, Y., Takagi, M., and Damborsky, J. (1999) Identification of the catalytic triad in the haloalkane dehalogenase from *Sphingomonas paucimobilis* UT26. *FEBS Lett.* **446,** 177–181.
11. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
12. Makrides, S. C. (1996) Strategies for achieving high-level expression of genes in *Escherichia coli. Microbiol. Rev.* **60,** 512–538.
13. Vieira, J., and Messing, J. (1987) Production of single-stranded plasmid DNA. *Methods Enzymol.* **153,** 3–11.
14. Terada, I., Kwon, S.-T., Miyata, Y., Matsuzawa, H., and Ohta, T. (1990) Unique precursor structure of an extracellular protease, Aqualysin I, with NH_2 and COOH-terminal pro sequences and processing in *E. coli. J. Biol. Chem.* **265,** 6576–6581.
15. Cornish-Bowden, A. (1995) "Analysis of Enzyme Kinetic Data," Oxford Univ. Press, Oxford.
16. Iwasaki, I., Utsumi, S., and Ozawa, T. (1952) New colorimetric determination of chloride using mercuric thiocyanate and ferric ion. *Bull. Chem. Soc. Japan* **25,** 226.
17. Damborsky, J., and Koca, J. (1999) Analysis of the reaction mechanism and substrate specificity of haloalkane dehalogenases by sequential and structural comparisons. *Protein Eng.* **11,** in press.
18. Damborsky, J., Manova, K., and Kuty, M. (1996) A mechanistic approach to deriving quantitative structure biodegradability relationships, *in* "Biodegradability Prediction" (Peijnenburg, W. J. G. M., and Damborsky, J., Eds.), pp. 75–92, Kluwer Academic Publishers, Dordrecht.
19. Damborsky, J., Berglund, A., Kuty, M., Ansorgova, A., Nagata, Y., and Sjöström, M. (1998) Mechanism-based structure–biodegradability relationships for hydrolytic dehalogenation of chloro- and bromo-alkenes. *Quant. Struct. Act. Rel.* **17,** 450–458.
20. Verschueren, K. H. G., Siljée, F., Rozeboom, H. J., Kalk, K. H., and Dijkstra, B. W. (1993) Crystallographic analysis of the catalytic mechanism of haloalkane dehalogenase. *Nature* **363,** 693–698.