The Journal of Microbiology, June 2003, p.89-94 Vol. 41, No. 2
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Structural Analysis of the *fcbABC* Gene Cluster Responsible for Hydrolytic Dechlorination of 4-Chlorobenzoate from pJS1 Plasmid of *Comamonas* sp. P08

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(Received February 14, 2003 / Accepted March 13, 2003)

Bacterial strain No. P08 isolated from wastewater at the Cheongju industrial complex was found to be capable of degrading 4-chlorobenzoate under aerobic condition. P08 was identified as *Comamonas* sp. from its cellular fatty acid composition and 16S rDNA sequence. The *fcb* genes, responsible for the hydrolytic dechlorination of 4-chlorobenzoate, were cloned from the plasmid pJS1 of *Comamonas* sp. P08. The *fcb* gene cluster of *comamonas* sp. P08 was organized in the order *fcbB-fcbA-fcbT1-fcbT2-fcbT3-fcbC*. This organization of the *fcb* genes was very similar to that of the *fcb* genes carried on the chromosomal DNA of *Pseudomonas* sp. DJ-12. However, it differed from the *fcbA-fcbB-fcbC* ordering of *Arthrobacter* sp. SU. The nucleotide sequences of the *fcbABC* genes of strain P08 showed 98% and 53% identities to those of *Pseudomonas* sp. DJ-12 and *Arthrobacter* sp. SU, respectively. This suggests that the *fcb* genes might have been derived from *Pseudomonas* sp. DJ-12 to form plasmid pJS1 in *Comamonas* sp. P08, or that the *fcb* genes in strain DJ-12 were transposed from *Comamonas* sp. P08 plasmid.

Key words: *fcb* genes, pJS1 plasmid, hydrolytic dechlorination, 4-chlorobenzoate, *Comamonas* sp. P08

Large quantities of artificially chlorinated aromatic compounds were released into the environment during the last century (Häggblom, 1992), and the majority are now recognized as serious environmental pollutants due to their recalcitrant and toxic properties, which are attributed to the presence of covalently bound chlorine substitution (Reineke, 1988; Higson, 1992; Park *et al.*, 2001). Therefore, dechlorination is a key requirement of the microbial degradation of chlorinated compounds (Arensdorf and Focht, 1995; Kikuchi *et al.*, 1994).

Chlorinated benzoates are common intermediates, and are produced in the degradative pathway from various kinds of herbicides and polychlorinated biphenyls (Cork and Krueger, 1991; Furukawa, 1994). A variety of 4-chlorobenzoate (4CBA) degrading bacterial strains have been isolated from soil (Dunaway-Mariano and Babbitt, 1994; Yi *et al.*, 2000). These bacteria first convert 4CBA to 4-hydroxybenzoate (4HBA), which is then further catabolized via the benzene ring-cleavage pathway (Dunaway-Mariano and Babbitt, 1994). The hydrolytic dechlorination pathway of 4CBA was first characterized in *Pseudomonas* sp. CBS2 (Thiele *et al.*, 1987). It consists of three steps, which are catalyzed by 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase (Chang *et al.*, 1991; Scholten *et al.*, 1992)

The *fcbABC* genes coding for these three enzymes are organized as an operon, which was reported to be located within the chromosomal DNA in some 4CBA-degrading bacteria (Chae and Kim, 1997; Savard *et al.*, 1986), but to be carried on a plasmid in others (Layton *et al.*, 1992; Schmitz *et al.*, 1992). The nucleotide sequences of the *fcbABC* genes responsible for the hydrolytic dechlorination of 4CBA have been reported in *Pseudomonas* sp. CBS3 (Babbitt *et al.*, 1992) and *Pseudomonas* sp. DJ-12 (Chae *et al.*, 2000). The three genes are commonly organized in the order *fcbB-fcbA-fcbC*. However, there are three open reading frames (orfs) inserted between *fcbA* and *fcbC* in strain DJ-12 (Chae *et al.*, 2000), which play a role in transport of 4CBA. On the other hand, the three genes are located on a plasmid in *Arthrobacter* sp. SU and organized in the order *fcbA-fcbB-fcbC* as an operon (Schmitz *et al.*, 1992).

P08 is a natural isolate and was identified by Youn *et al.* (1992), and can grow on 4CBA as the sole carbon and energy source under aerobic conditions. P08 hydrolyti-

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cally dechlorinates 4CBA- releasing chloride ions. In this study, P08 was identified as *Comamonas* sp. on the basis of its cellular fatty acid profile, Biolog assay, and 16S rDNA sequence analysis. The *fcb* genes responsible for the hydrolytic dechlorination of 4CBA were found in the pJS1 plasmid of *Comamonas* sp. P08 and their genetic structure was determined by comparison with the *fcb* genes carried in the chromosomal DNA of *Pseudomonas* sp. DJ-12.

Materials and Methods

Bacterial strains and dechlorination assay

Comamonas sp. P08 is a bacterial isolate capable of degrading and utilyzing 4-chlorobenzoate as a carbon and energy source under aerobic conditions. *Comamonas* sp. P08 and *Escherichia coli* JM109 transformants were cultivated in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml) at 30°C and 37°C, respectively. To select transformant cells with 4CBA dechlorination activity, we used chloride free minimal medium described by Chae and Kim (1997).

Chloride ions released by the dechlorination of 4CBA were quantified colorimetrically by measuring the absorbance at 453 nm after reacting the supernatant of a *Comamonas* sp. P08 culture with 0.069% $Hg(SCN)_2$ and 0.25 M ferric ammonium sulfate, as described by Bergmann and Sanik (1957).

Fatty acid analysis and Biolog assay

Total cellular fatty acid methyl esters (FAMEs) from strain P08 were determined using the Microbial Identification System (MIDI; Microbial ID Inc., Newark, DE). Results of the MIDI were compared with Trypticase soy broth agar (TSBA) culture profiles of strain P08 using an environmental database and blood culture profiles with a clinical database. The Biolog assay was performed as described by Johnsen *et al.* (1996). Results were analyzed using MicroLog TM 3 computer software (Biolog Inc., USA).

Analysis of 16S ribosomal DNA

The 16S ribosomal DNA of strain P08 was analyzed by PCR using the eubacterial primers 27f and 1492r (Eden *et al.*, 1991). PCR mixtures (100 μl) contained 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, a 200 μM concentration of each deoxynucleotide triphosphate, 50 pM of each primer, 2.5 U of *Taq* DNA polymerase (Posco Chem, Korea) and ~10 ng of DNA template. PCR amplification was performed using a Programmable Thermal Controller (MJ Research, Inc., USA) programmed as follows: 45 sec of denaturation at 95°C, followed by 35 cycles of; 95°C for 45°C sec, 55°C for 45 sec, 72°C for 1 min 30 sec, and a final extension at 72°C for 7 min. The amplified 1.5 kb PCR products were excised from a 1% agarose gel and purified using a Quantum Prep[TH] Freeze 'N Squeeze DNA Gel extraction spin column (Bio-Rad, USA), according to the manufacturer's instructions. Partial DNA sequences obtained using 27f and 1492r were determined directly from the purified PCR products by automated fluorescent *Taq* cycle sequencing using an ABI 373A Sequencer (Applied Biosystems, USA). Partial 16S rDNA sequences of strain P08 were analyzed using the Ribosomal Database Project Sequence Match and Similarity Matrix programs (Hitachi version 7.0) to identify the closely matching species.

Plasmid curing

Plasmid curing of *Comamonas* sp. P08 was performed according to the procedures described by Miller (1992). Acridine orange was added to a final concentration of 10 μg/ml to LB medium inoculated with the diluted cell culture of the early stationary phase. After 24 h of incubation at 30°C, the culture was diluted with saline and plated out onto LB agar plates. The individual colonies appearing on the plates were transferred to MM2 plates supplemented with 0.5 mM 4CBA to investigate their ability to degrade aromatics.

Cloning, sequencing, and analysis of the fcb genes

The plasmid preparation from strain P08 was performed by the alkaline lysis method (Sambrook *et al.*, 1989). DNA fragments were transferred from electrophoresed gel to a nylon membrane using 1 N NaOH, as described by Koetsier *et al.* (1993). Hybridization was performed using the ECL direct nucleic acid labeling and detection systems (Amersham, USA), according to the manufacturer's instructions. The DNA fragments used as probes were from the UltraCleanTM 15 kit (MO BIO, USA).

The plasmid pBluescript SK(+) and *E. coli* JM109 were used as vector and host strain, respectively. Plasmid DNA of strain P08 and pBluescript SK(+) vector digested with *Not*I and *Cla*I, respectively, were ligated for 16 h at 16°C. Ligation mixtures were transformed using the calcium chloride procedure described by Sambrook *et al.* (1989). Several clones were constructed using various endonucleases.

The nucleotide sequence was determined by dideoxychain termination method (Sanger *et al.*, 1977). Sequen cing was performed using a ThermoSequenase kit (Amersham Life Science, USA) and LongRealR 4200 sequencer. The nucleotide sequences obtained were analyzed by using DNASIS, PROSIS, and ClustalW software.

Results and Discussion

Identification of Comamonas sp. P08

The cellular fatty acid profiles of P08 are shown in Table 1, and showed 59% similarity with that of *Comamonas acidovorans*. Also, the biochemical characteristics of

Table 1. Composition of major cellular fatty acids of the isolate No. P08

Fatty acid	Content (%)
C10:0 3OH	4.02
C12:0	2.54
C14:0	1.14
C16:1 w7c/15 iso 2OH	38.26
C16:0	30.27
C17:0 Cyclo	4.83
C16:1 2OH	1.72
C16:0 2OH	1.78
C18:1 w7c	13.59
Similarity	*Comamonas acidovorans* (59%)

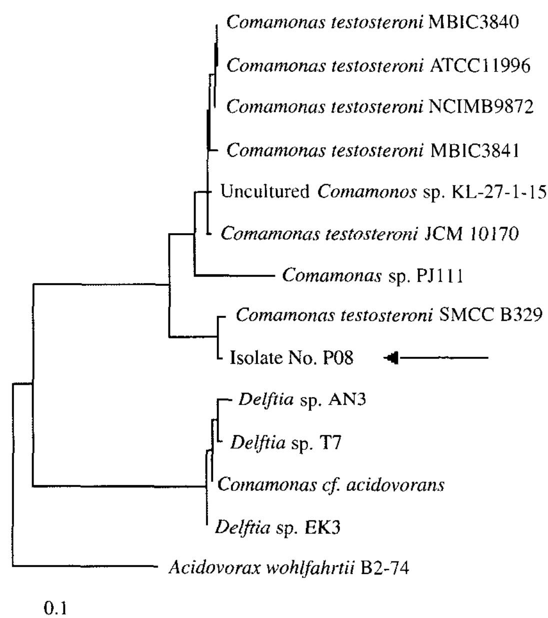

Fig. 1. Phylogenetic tree of complete 16S rDNA of isolate No. P08. The bar indicates a 10% difference.

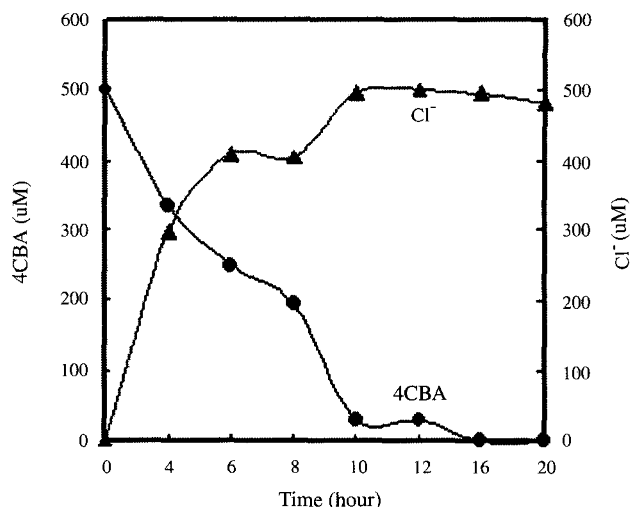

Fig. 2. Degradation of 4CBA causing chloride ion release by isolate No. P08. Dechlorination activity was detected using resting cells in 50 mM potassium phosphate buffer containing 0.5 mM 4-chlorobenzoate colorimetrically.

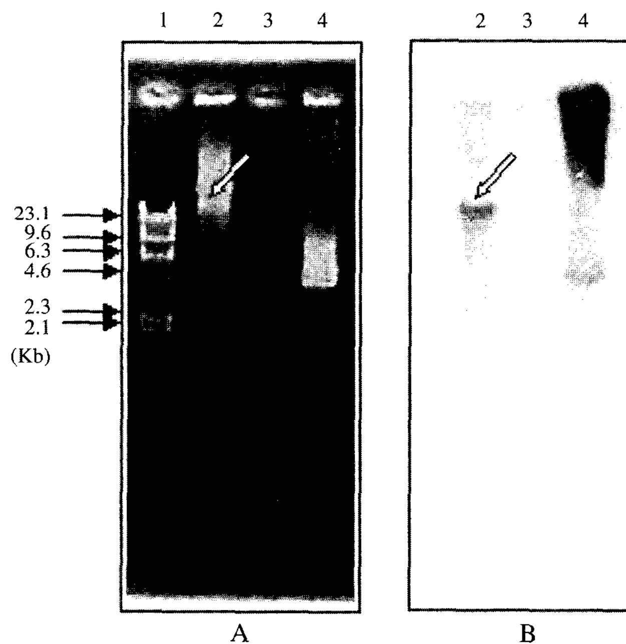

Fig. 3. Electrophoretic (A) and Southern hybridization (B) patterns of plasmid DNA from *Comamonas* sp. P08 and its cured strain P08-49. The 3.4 kb-*Bam*HI fragment of pSE1 was used as a DNA probe. The arrow indicates the plasmid pJS1. Lanes: 1, size marker (Lambda DNA -*Hin*dIII); 2, plasmid DNA of *Comamonas* sp. P08; 3, plasmid DNA of cured strain P08-49; 4, plasmid DNA of *E. coli* SE1 containing the *fcbB* gene.

strain P08, determined by the Biolog test showed 56% similarity with those of *Comamonas acidovorans* (data not shown). The 16S rDNA sequence of P08 placed the strain among several *Comamonas* species, as shown in Fig. 1 and the16S rDNA sequence of P08 showed about 99% homology with *Comamonas testosteroni* SMCC B329. On the basis of these results, strain P08 was identified as *Comamonas* sp. and is referred to *Comamonas* sp. P08 hereafter.

Location of fcb genes in a plasmid

Comamonas sp. P08 degraded 0.5 mM 4CBA completely within 20 h, releasing the corresponding amount of chloride ions, as shown in Fig. 2. The production of chloride ions increased, whereas 4CBA was degraded as a function of reaction time. This result confirmed that *Comamonas* sp. P08 degrades 4CBA to the chloride ion and 4HBA via a hydrolytic dechlorination mechanism, as reported for *Pseudomonas* sp. DJ-12 (Chae *et al.*, 2000). Hydrolytic dechlorination of 4CBA was also studied in *Pseudomonas* sp. CBS3 (Loffler *et al.*, 1991; Babbitt *et al.*, 1992), and *Arthrobacter* sp. SU (Schmitz *et al.*, 1992). In these studies, it was reported that the chloride substituent of the

chloroaromatics was replaced by a hydroxyl group derived from water by consecutive reactions with 4CBA-CoA ligase, 4CBA-CoA dechlorinase, and 4HBA-CoA thioesterase, encoded by *fcbA*, *fcbB*, and *fcbC*, respectively.

In order to confirm that the *fcb* genes are located on a plasmid, Southern hybridization experiments were carried out using a DNA probe. A horse-radish peroxidase-labelled 3.4 kb fragment containing the *fcbB* gene was used as a probe. As shown in Fig. 3, the probe hybridized only to the plasmid pJS1 of *Comamonas* sp. P08, and not to the DNA of the cured strain P08-49. Moreover, the cured strains did not exhibit dechlorination activity to 4-chlorobenzoate. This result indicates that the *fcb* genes of strain P08 were located on the pJS1 plasmid. The genes responsible for 4-chlorobenzoate dechlorination were found in the chromosomal DNA in *Pseudomonas* sp. CBS3 (Babbitt *et al.*, 1992), *Pseudomonas* sp. DJ-12 (Chae *et al.*, 2000), and Arthrobater sp. strain TM1 (Gartemann and Eichenlaub, 2001). However, the *fcb* genes of *Comamonas* sp. P08 were carried on the plasmid pJS1, as are the *fcb* genes in a plasmid of *Arthrobacter* sp. SU (Schmitz *et al.*, 1992).

Structural characteristics of the fcbABC gene cluster

The *fcb* genes responsible for the hydrolytic dechlorination of 4CBA were cloned from the plasmid DNA of *Comamonas* sp. P08 to obtain several clones, i.e., pJS1, pJS2, and pJS3. The physical maps of which are shown in Fig. 4. The total nucleotide sequences (5824 bp) of the *fcbABC* gene cluster were determined from each insert in pJS1, pJS2, and pJS3. Sequence analysis revealed that the *fcb* genes consisted of six orfs designated as; *fcbA*, *fcbB*, *fcbT1*, *fcbT2*, *fcbT3*, and *fcbC*. The six orfs were found to be organized in the order *fcbB-fcbA-fcbT1-fcbT2-fcbT3-fcbC*, as seen in Fig. 4. The order is identical to that of the *fcb* genes of *Pseudomonas* sp. DJ-12 (Chae *et al.*, 2000). A promoter-like sequence (-35 and -10 region) was also found at the 35 bp position upstream of the start codon of the *fcbB* gene.

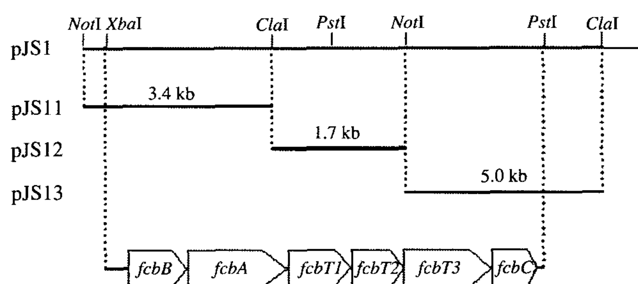

Fig. 4. Physical map of the *fcb* genes from *Comamonas* sp. P08. *fcbA*, 4CBA-CoA ligase gene; *fcbB*, 4CBA-CoA dehalogenase gene; *fcbC*, 4HBA-CoA thioesterase gene; *fcbT1T2T3*, 4CBA transporter genes.

The main characteristics of the *fcbA, fcbB, and fcbC* genes are summarized in Table 2, and the *fcbABC* genes of strain P08 were of 1522, 816, and 432 bp, respectively. The nucleotide sequence of *fcbA* encoding 4CBA-CoA ligase showed 99%, 59%, 54%, and 54% identities to those of the corresponding genes from *Pseudomonas* sp. DJ-12 (Chae *et al.*, 2000), *Pseudomonas* sp. CBS3 (Babbitt *et al.*, 1992), *Arthrobacter* sp. SU (Schmitz *et al.*, 1992), and *Arthrobacter* sp. TM1 (GenBank accession No. AF042490), respectively. And, the nucleotide sequence of *fcbB* encoding 4CBA-CoA dechlorinase exhibited 98%, 76%, 59%, and 59% identities with those of the corresponding genes from the above four 4CBA-dechlorinating strains. Those of *fcbC* encoding 4HBA-CoA thioesterase showed 99%, 65%, 48%, and 47% identities to those of the corresponding genes from the same four strains.

These results indicate that the *fcb* gene order of *Comamonas* sp. P08 is quite different from the *fcbA-fcbB-fcbC* gene order of *Arthrobacter* sp. SU (Schmitz *et al.*, 1992), even though the genes are located in a plasmid like those in *Arthrobacter* sp. SU. However, the sequences of the *fcb* genes from *Comamonas* sp. P08 exhibited high similarities to those of the corresponding genes located within the chromosomal DNA in *Pseudomonas* sp. DJ-12 (Chae *et al.*, 2000). The *fcbT1T2T3* genes of strain P08 were also located between *fcbA* and *fcbC*, and showed high nucleotide sequence homology with strain DJ-12 (Chae *et al.*, 2000). Structural analysis of the *fcbABC* genes from *Comamonas* sp. P08 revealed that they are closely related with the *fcbABC* genes of *Pseudomonas* sp. DJ-12, with only minor differences in nucleotide sequence, as shown in Table 3. Both strains were isolated from the same wastewater by Youn *et al.* (1992). These results suggest that the *fcb* genes in *Comamonas* sp. P08 might be derived from a bacterial strain like *Pseudomonas* sp. DJ-12 to form the pJS1 plasmid or that the *fcb* genes in strain

Table 2. Characteristics of *fcbABC* genes from *Comamonas* sp. P08 and their identity with those from other strains.

Gene	No. of nucleotide (bp)	G+C (%)	No. of amino acid (aa)	Predicted molecular mass (kDa)	Identity (%) of nucleotide sequence			
					DJ-12	CBS3	SU	TM1
fcbA	1522	62	506	54	99	59	54	54
fcbB	816	62	271	30	98	76	59	59
fcbC	432	58	143	16	99	65	48	47

DJ-12, *Pseudomonas* sp. DJ-12; CBS3, *Pseudomonas* sp. CBS3; SU, *Arthrobacter* sp. SU; TM1, *Arthrobacter* sp. TM1.

Table 3. Structural and functional differences of the *fcbABC* genes between *Comamonas* sp. P08 and *Pseudomonas* sp. DJ-12.

Characteristic	*Comamonas* sp. P08	*Pseudomonas* sp. DJ-12
Location of *fcb* genes	plasmid	chromosome
Order of *fcb* genes	*fcbB-fcbA-fcbT1-fcbT2-fcbT3-fcbC*	*fcbB-fcbA-fcbT1-fcbT2-fcbT3-fcbC*
fcbA gene		
Nucleotide number	1522 bp	1519 bp
Different nucleotides	(1404)CGCAAGTG(1411)	(1404)CAGTG(1408)
Amino acid residue	506 aa	505 aa
Different amino acids	(467)SASE(470)	(467)SSE(469)
fcbB gene		
Nucleotide number	816 bp	810 bp
Different nucleotides	(764)CGAACACAGAG(774), (810)TGGTT(814)	(764)CGATAAGG(771), (807)TT(808)
Amino acid residue	271 aa	269 aa
Different amino acids	(255)GEHRA(259), (269)GFG(271)	(255)GDKA(258), (268)GF(269)
fcbC gene		
Nucleotide number	432 bp	429 bp
Different nucleotides	(320)CGGTCAGCT(328)	(320)CTCAGT(325)
Amino acid residue	143 aa	142 aa
Different amino acids	(106)PVSL(109)	(106)PQL(108)
Degradation/Dechlorination		
4-chlorobenzoate	+/+	+/+
4-chlorobiphenyl	+/+	+/+
4-chlorocatechol	–/–	+/+

DJ-12 were transposed from the *Comamonas* sp. P08 plasmid.

Acknowledgments

This work was supported by a research grant (R01-2002-000-00022-0) from the Korea Science and Engineering Foundation. We gratefully acknowledge Marco Bazzicalupo for helpful discussion which was provided by a visiting program (01H020000500) of the International Cooperative Research Project between Korea and Italy.

References

Arensdorf, J.J. and D.D. Focht. 1995. A *meta* cleavage pathway for 4chlorobenzoate, an intermediate in the metabolism of 4-chlorobiphenyl by *Pseudomonas cepacia* P166. *Appl. Environ. Micriobiol.* 61, 443-447.

Babbitt, P.C., G.L. Kenyon, B.M. Martin, H. Charest, M. Sylvestre, J.D. Schoten, K.H. Chang., P.H. Liang, and D. Dunaway-Mariano. 1992. Ancestry of the 4-chlorobenzoate dehalogenase: analysis of amino acid sequence identities among families of acyl : adenylligase, enoyl-CoA hydratases/isomerases, and acyl-CoA thioesterases. *Biochemistry* 31, 5594-5604.

Bergmann, J.G. and J. Sanik, 1957. Determination of trace amounts of chlorine in naphtha. *Anal. Chem.* 29, 241-243.

Chae, J.C. and C.K. Kim. 1997, Dechlorination of 4-chlorobenzoate by *Pseudomonas* sp. DJ-12. *J. Microbiol.* 35, 290-295.

Chae, J.C., Y.S. Kim, Y.C. Kim, G.J. Zylstra, and C.K. Kim. 2000. Genetic structure and functional implication of the *fcb* gene cluster for hydrolytic dechlorination of 4-chlorobenzoate from *Pseudomonas* sp. DJ-12. *Gene* 258, 109-116.

Chang, K.H., P.H. Liang, W. Beck, J.D. Scholten, and D. Dunaway-Mariano. 1992. Isolation and characterization of the three polypeptide components of 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. strain CBS-3, *Biochemistry* 31, 5606-5601

Cork., D.J. and J.P. Krueger. 1991. Microbial transformations of herbicides and pesticides. *Adv. Appl. Microbiol.* 36, 1-66.

Dunaway-Mariano, D. and P.C. Babbitt. 1994. On the origins and functions of the enzymes of the 4-chlorobenzoate to 4-hydroxybenzoate converting pathway. *Biodegaradation* 5, 259-276.

Eden, P.A, T.M. Schmidt, R.P. Blakemore, and N.R. Pace. 1991. Phylogenetic analysis of *Aquaspirillum magnetotacticum* using polymerase chain reaction-amplified 16S rDNA-specific DNA. *Int. J. Syst. Bacteriol.* 41, 324-325.

Furukawa, K. 1994. Molecular genetics and evolutionary relationship of PCB-degrading bacteria. *Biodegradation* 5:289-300

Gartemann, K.H. and R. Eichenlaub. 2001. Isolation and characterization of IS*1409*, an insertion element of 4-chlorobenzoate-degrading *Arthrobacter* sp. strain TM1, and development of a system fro transposon mutagenesis. *J. Bacteriol.* 183, 3729-3736.

Häggblom, M.M. 1992. Microbial breakdown of halogenated aromatic pesticides and related compounds. *FEMS Micriobiol. Rev.* 103, 29-72.

Higson, F.K. 1992. Microbial degradation of biphenyl and its derivatives. *Adv. Appl. Microbiol.* 37, 135-164.

Johnsen, K., S. Andersen, and C.S. Jacobsen. 1996. Phenotypic and genotypic characterization of phenanthrene-degrading fluorescent *Pseudomonas biovars*. *Appl. Environ. Microbiol.* 62,

3818-3825.

Kikuchi, Y., Y. Yasukochi, Y. Nagata, and M. Fukuda. 1994. Nucleotide sequence and functional analysis of the *meta*-cleavage pathway involved in biphenyl and polychlorinated biphenyl degradation in *Pseudomonas* sp. strain KKS102. *J. Bacteriol.* 176, 4269-4276.

Koetsier, P.A., J. Schorr, and W. Doerfler. 1993. A rapid optimized protocol for downward alkaline southern blotting of DNA. *Biotechniques* 15, 260-262.

Layton, A.C., J. Sanseverino, W. Wallace, C. Corcoran, and G.S. Sayler. 1992. Evidence for 4-chlorobenzoic acid dehalogenation mediated by plasmids related to pSS50. *Appl. Environ. Microbiol.* 58, 399-402.

Loffler, F., R. Muller, and F. Lingens. 1991. Dehalogenation of 4-chlorobenzoate by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3: an ATP/coenzyme A dependent reaction. *Biochem. Biophy. Res. Commun.* 176, 1106-1111.

Miller, J.H. 1992. Experiments in molecular genetics, Cold Spring Harbor Laboratoty, Cold Spring Harbor, New York.

Reineke, W., 1988. Microbial degradation of haloaromatics. *Ann. Rev. Microbiol.* 42, 263-287.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning, A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. Sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 74, 5463-5467.

Savard, P., L. Peloquin, and M. Sylvestre. 1986. Cloning of *Pseudomonas* sp. strain CBS3 genes specifying dehalogenation of 4-chlorobenzote. *J. Bacteriol.* 168, 81-85.

Schmitz, A., K.H. Gartemann, J. Fiedler, E. Grund, and R. Eichenlaub. 1992. Cloning and sequence analysis of genes for dehalogenation of 4-chlorobenzoate from *Arthrobacter* sp. strain SU. *Appl. Environ. Microbiol.* 58, 4068-4071.

Scholten, J.D., K.H. Chang, P.C. Babbitt, H. Charest, M. Sylvestre, and D. Dunaway-Mariano. 1991. Novel enzymic hydrolytic dehalogenation of a chlorinated aromatic. *Science* 253, 182-185.

Thiele, J., R. Muller, and F. Lingens. 1987. Initial characterization of 4-chlorobenzoatedehalogenase from *Pseudomonas* sp. CBS2. *FEMS Microbiol. Lett.* 41, 115-119.

Yi, H.R., K.H. Min, C.K. Kim, and J.O. Ka. 2000. Phylogenetic and phenotypic dirversity of 4-chlorobenzoate-degrading bacteria isolated from soils, *FEMS Microbiol. Ecol.* 31, 53-60.

Youn, D.J., J.J. Han, C.K. Kim, and Y.S. Kim. 1992. Divergence of the *cbp* genes in 4CB catabolizing bacteria. *Kor. J. Microbiol.* 30, 53-59.