PcpA, which is involved in the degradation of pentachlorophenol in *Sphingomonas chlorophenolica* ATCC39723, is a novel type of ring-cleavage dioxygenase

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Abstract The pentachlorophenol (PCP) mineralizing bacterium *Sphingomonas chlorophenolica* ATCC39723 degrades PCP via 2,6-dichlorohydroquinone (2,6-DCHQ). The pathway converting PCP to 2,6-DCHQ has been established previously; however, the pathway beyond 2,6-DCHQ is not clear, although it has been suggested that a PcpA plays a role in 2,6-DCHQ conversion. In this study, PcpA expressed in *Escherichia coli* was purified to homogeneity and shown to have novel ring-cleavage dioxygenase activity in conjunction with hydroquinone derivatives, and converting 2,6-DCHQ to 2-chloromaleylacetate.

Key words: Pentachlorophenol; Ring-cleavage dioxygenase; Biodegradation; *Sphingomonas*

1. Introduction

Pentachlorophenol (PCP) is one of the world's most serious pollutants, and its degradation by microorganisms has been studied [1]. *Sphingomonas* (formerly *Flavobacterium*) *chlorophenolica* ATCC39723, which was isolated from a PCP-contaminated soil in Minnesota, exhibits PCP degrading activity [2]. The initial steps of the degradation pathway of PCP in this strain have been identified. PCP is converted to tetrachlorohydroquinone (TeCHQ) by PCP 4-monooxygenase encoded by the *pcpB* gene [3,4], and subsequently TeCHQ is reductively dechlorinated to trichlorohydroquinone (TrCHQ) and subsequently to 2,6-dichlorohydroquinone (2,6-DCHQ) by reductive dehalogenase, which is encoded by the *pcpC* gene [5–7]. The pathway beyond 2,6-DCHQ is unknown, although PcpA has been proposed to be involved in the conversion of 2,6-DCHQ, based on the accumulation of 2,6-DCHQ by a *pcpA* mutant [8].

In this study, using purified PcpA protein, we investigated the products of the 2,6-DCHQ cleavage formula converted by PcpA. We show that PcpA has novel ring-cleavage dioxygenase activity that cleaves aromatic rings with two hydroxyl groups *para* to each other, and that PcpA converts 2,6-DCHQ to 2-chloromaleylacetate.

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Abbreviations: HQ, hydroquinone; CHQ, chlorohydroquinone; 2,6-DCHQ, 2,6-dichlorohydroquinone; PCP, pentachlorophenol; TeCHQ, tetrachlorohydroquinone; TrCHQ, trichlorohydroquinone

2. Materials and methods

2.1. Culture conditions

Escherichia coli was grown on Luria broth (LB) at 37°C. Ampicillin (Ap) was used at a final concentration of 50 μg/ml. To induce the expression of PcpA, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM.

2.2. Purification of PcpA

In the purification steps, PcpA activity was routinely assayed with hydroquinone (HQ) as a substrate. A suitable amount of enzyme was incubated with HQ, and the increase in OD_{320} was monitored. The amount of protein was determined by a protein assay kit (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as a standard. To overproduce PcpA, plasmid pLDP26 was used. pLDP26 was isolated as a clone, which is known to confer 2,5-dichlorohydroquinone degradation activity on *E. coli* MV1190 (unpublished data). In this plasmid, pcpA was placed under the control of the *lac* promoter. *E. coli* MV1190 containing pLDP26 was cultured in 1 liter of Luria broth at 37°C. Cells were harvested after induction with 1 mM IPTG, washed with 25 mM potassium phosphate buffer, pH 8.0, and resuspended in the same buffer. The cells were disrupted by sonication (Sonifier 250; Branson, Danbury, CT, USA). After centrifugation at $100\,000 \times g$ for 1 h, the supernatant was used as the crude extract. PcpA was purified on a hydroxy apatite column (Bio-Gel HT Gel; Bio-Rad Laboratories, Richmond, CA, USA), a DEAE column (DEAE-650M; Tosho, Tokyo, Japan), and a MonoQ column (Pharmacia, Uppsala, Sweden). Only one protein band was observed on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis after three steps of purification. After purification, the activity of PcpA was low, but it was reactivated by the addition of $FeSO_4$ and ascorbic acid.

2.3. Enzymes and reagents

The enzymes necessary for the DNA manipulations were purchased from Takara Shuzo Co., Kyoto, Japan. Authentic HQ and chlorohydroquinone (CHQ) were purchased from Nacalai Tesque (Kyoto, Japan) and Aldrich (Milwaukee, WI, USA), respectively. 2,6-DCHQ was prepared as follows. A solution of 2,6-dichloro-1,4-benzoquinone (purchased from Tokyo Chemical Industry Co., Tokyo, Japan) (5% w/v in ethanol) was mixed with the same amount of ascorbic acid solution (5% w/v in 100 mM phosphate buffer, pH 7.0) and was stored as a 2,6-DCHQ stock solution.

2.4. GC-mass spectrometry analysis

The GC-MS analyses were performed under the same conditions as described previously [9].

2.5. Analysis of the metabolites

Purified PcpA protein was diluted in 20 mM potassium phosphate buffer, pH 7.3, containing 1 mM $FeSO_4$ and 2 mM ascorbic acid. HQ, CHQ, or 2,6-DCHQ was added at 25 ppm, and the solution was incubated at 30°C with shaking for 3 h. After incubation, the metabolites were extracted by ethyl acetate after acidification by HCl. Following the evaporation of the ethyl acetate layer, the substances were trimethylsilylated by *N*-methyl-*N*-trimethylsilyl-trifluoroacet-

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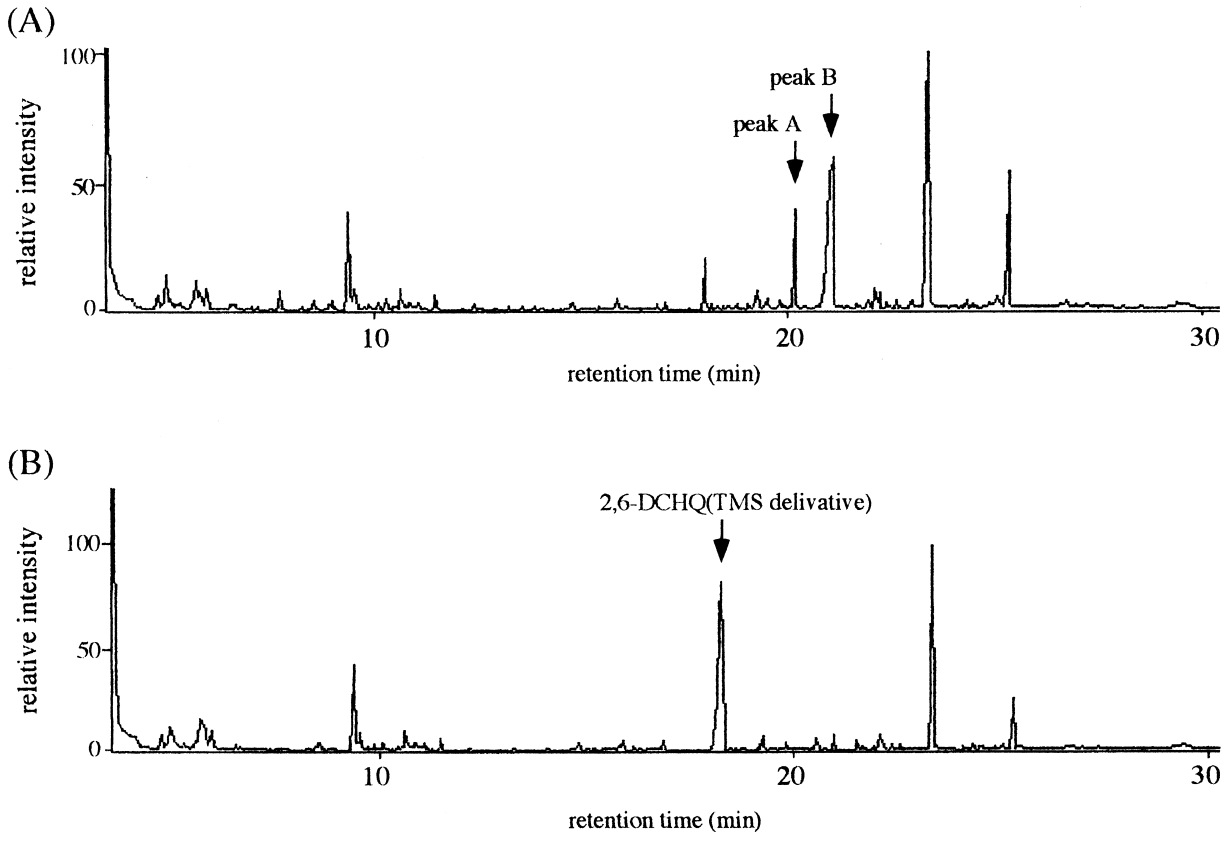

Fig. 1. GC-MS analysis of the metabolite of 2,6-DCHQ by PcpA. As described in Section 2, the metabolite of 2,6-DCHQ by PcpA was trimethylsilylated and analyzed by GC-MS. A: Two specific peaks are indicated by arrows. B: The results of the control experiment (without PcpA) are shown. A TMS derivative of 2,6-DCHQ is indicated.

amide (Nacalai Tesque, Kyoto, Japan) at 65°C. The resultant samples were analyzed by GC-MS.

3. Results

3.1. Identification of metabolites of 2,6-DCHQ by PcpA

To identify the degradation product of 2,6-DCHQ by PcpA, 2,6-DCHQ (25 ppm) was incubated with purified PcpA protein with vigorous shaking for 3 h. After incubation, the samples were acidified by HCl, extracted with ethyl acetate, treated with the reagents for trimethylsilylation, and analyzed by GC-MS. Two specific peaks that were not observed in the control experiment (incubated without PcpA protein) appeared (Fig. 1, compare panels A and B). The mass spectrogram of the specific peak A in Fig. 1 is shown in Fig. 2. This mass spectrogram contains some indicative peaks (a molecular ion at *m/z* 408 accompanied by a half-size peak at *m/z* 410 indicating the presence of a chlorine atom, a base peak at *m/z* 373 (M^+-Cl), and major fragments at *m/z* 291 (M^+-TMS-O-CO-) and *m/z* 393 (M^+-CH3)) that suggest that the degradation product of 2,6-DCHQ is 2-chloromaleylacetate.

Peak B in Fig. 1 appeared reproducibly, but we were not able to identify it. It may be a TMS derivative of the lactol form of *cis*-2-chloro-4-keto-pent-2-enoic acid, which could possibly have been produced from 2-chloromaleylacetate by thermal decarboxylation and lactonization on acidification

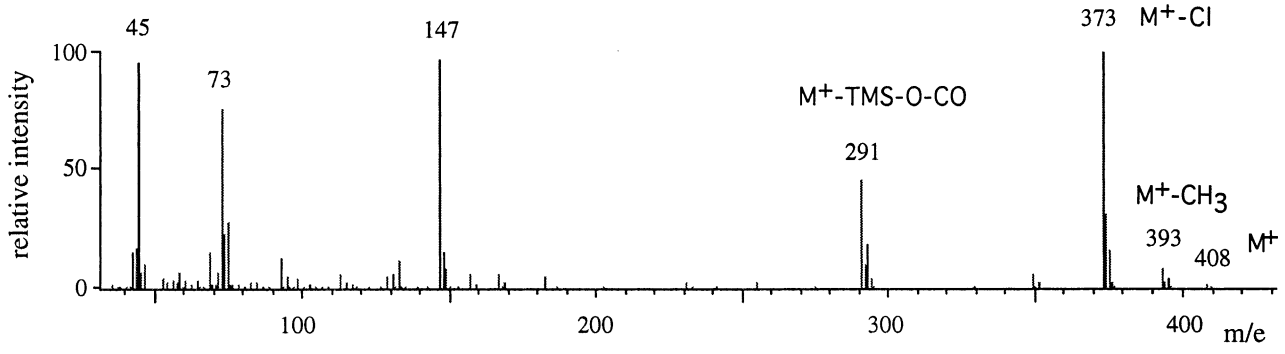

Fig. 2. Mass spectrogram of peak A in Fig. 1.

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PcpA    1    --VETNHITSLHHITICTGTAQGDIDFFVKVMGQRFVKRTLFYDGSIPIYHLYFADELGT
LinE    1    MMQLPERVEGLHHITVATGSAQGDVDLLVKTLGQRLVKKTMFYDGARPVYHLYFGNELGE
                  ..  *****..**.****.*  ** .*** **.*.****. *.*****  ***

PcpA   59    PGTVMTTFPTRRTGQKGRKGSNQFTVCTYAIPKGSLEWWIGHLNAHGIATGEPGTRFGQR
LinE   61    PGTLYTTFPVRQAGYTGKRGAGQISAVSYNAPVGTLSWWQEHLIKRAVTVSEVRERFGQK
             ***. ****.*..* .*..*..*... .*  * *.*.** **  ......*   ****.

PcpA  119    YVGFQHPDCGIDFEVLEDEND-TRQPYDSPYVPIEHAQRGFHSWTASVRELEDMDFFMEN
LinE  121    YLSFEHPDCGVGFEIIEQDTDGQFEPWDSPYVPKEVALRGFHSWTATLNRNEEMDSFMRN
             *. *.*****. **..* ..*   .* ****** * * ********..   *.** ** *

PcpA  178    CWNFEKIGEEGNRHRYRVKGTTESGTIIDLLHEPDRRQGSWTIAEGIIHHGAFAVPDMDI
LinE  181    AWNLKPQGRDGNYQRY-AFGNGGAAKVLDVYIDEDERPGTWALGEGQVHHAAFEVADLDV
             .**    * .** .**   *.  .  ..*.  . * * *.*.. ** .** ** * *.*.

PcpA  238    QARIKFETEGVGFTDFSDRKNRGYFESTYVRTPGGVMFEATHSLGFTHDEDERSLGMDLK
LinE  241    QAALKFDVEGLGYTDFSDRKHRGYFESIYVRTPGGVLFEASVTLGFTHDESPEKLGSEVK
             ** .**. **.*.*******.****** ********.***. .*******    ** ..*

PcpA  298    VSPQFDDKKHLIEQAMEDDPIVV
LinE  301    VAPQLEGVKDELLRTMN-DPIVI
             *.** .  *  . ..*  ****.
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Fig. 3. Alignment between the deduced amino acid sequences of PcpA and LinE. Identical and similar residues between PcpA and LinE are indicated by asterisks and dots, respectively.

[10]. The formation of chlorolactol was further confirmed by GC-MS analysis, in which samples were injected without trimethylsilylation. This procedure resulted in a peak with a fragmentation pattern consistent with the structure assigned (data not shown).

3.2. PcpA shares homology with LinE of ring-cleavage dioxygenase

We found that the deduced amino acid sequence of PcpA is very similar to that of LinE from *Sphingomonas pauchimobilis* UT26 (51.3% identity, 74.7% similarity in 321 amino acid residues). The alignment between PcpA and LinE is shown in Fig. 3. We have recently shown that LinE is a ring-cleavage dioxygenase that converts HQ and CHQ to γ-hydroxymuconicsemialdehyde and maleylacetate, respectively [11]. This reaction is a dioxygenation of the aromatic ring between the carbon atoms at positions 1 and 2. As inferred from the reaction of LinE with CHQ, PcpA should dioxygenize 2,6-DCHQ to yield 2-chloromaleylacetate (Fig. 4A).

Table 1
Mass spectra of the TMS derivatives of the metabolites

Substrate used	Mass spectrum *m/z* (relative intensity)
HQ	286 (1.49), 271 (52.79), 257 (5.39), 243 (9.41), 169 (48.59), 153 (26.36), 147 (36.85), 143 (41.09), 73 (100)
CHQ	374 (0.34), 359 (8.08), 315 (6.19), 257 (100), 241 (3.30), 197 (3.46), 147 (51.49), 123 (9.21), 95 (17.61), 73 (51.98)
2,6-DCHQ	410 (0.86), 408 (1.84), 393 (8.56), 373 (100), 291 (45.44), 147 (96.56), 133 (11.92), 93 (13.04), 73 (75.49)

3.3. Activities of PcpA for HQ and CHQ

Because PcpA has homology with the ring-cleavage dioxygenase LinE, we tested the ring-cleavage dioxygenase activity of PcpA for HQ and CHQ, which are the substrates for LinE. The degradation products of HQ and CHQ by resting cells of *E. coli* overexpressing PcpA were trimethylsilylated and analyzed by GC-MS. As expected, the peaks of the TMS derivatives of HQ and CHQ disappeared, and new peaks that were not observed in the control experiment (resting cells of *E. coli* not expressing PcpA were used) appeared. When CHQ was used, three specific peaks appeared, all of which had the same mass spectrogram profile that has been reported to be indicative of a TMS derivative of maleylacetate (Table 1) [12]. These peaks were also observed when purified PcpA and CHQ were incubated. When HQ was used, a specific peak with a mass spectrogram indicative of a TMS derivative of γ-hydroxymuconicsemialdehyde was observed (Table 1). The retention time and mass spectrogram perfectly matched those of the degradation product of HQ catalyzed by LinE [11]. These results

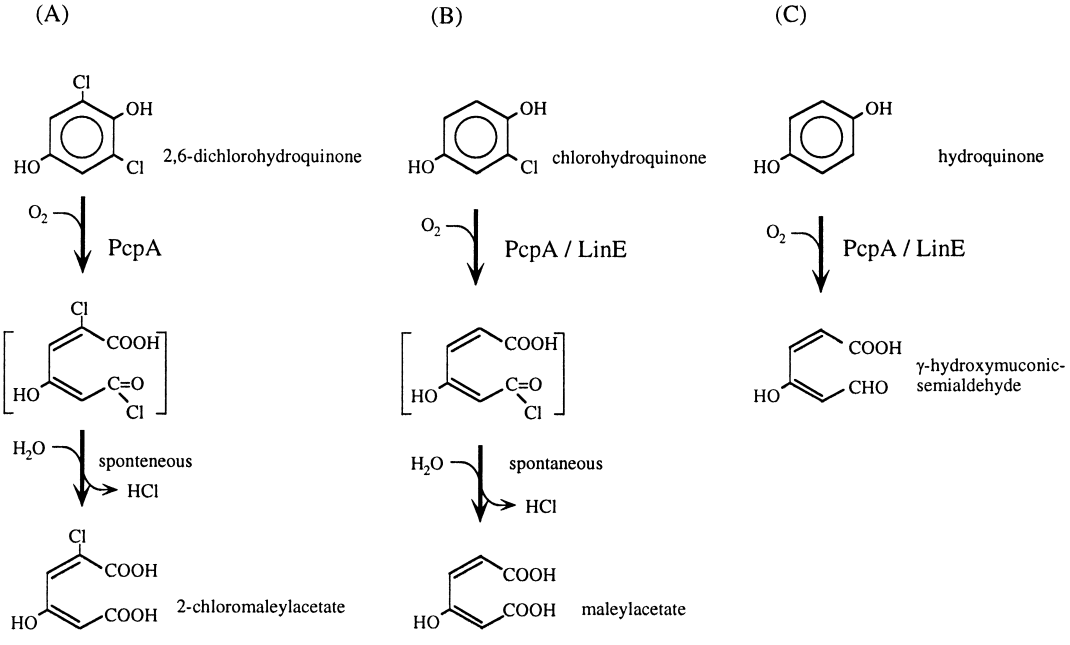

Fig. 4. Dioxygenation of HQs by PcpA. A: PcpA converts 2,6-DCHQ to 2-chloromaleylacetate. B and C: Ring-cleavage dioxygenation by PcpA and LinE of chlorohydroquinone and hydroquinone, respectively. PcpA cleaves the benzene ring between the carbon atoms at positions 1 and 2.

also suggest that PcpA is a ring-cleavage dioxygenase in its activity toward HQ derivatives (Fig. 4).

4. Discussion

PcpA has been suggested to play an essential role in PCP degradation, especially in the conversion of 2,6-DCHQ [8] in ATCC39723. However, there has been no direct evidence that PcpA converts 2,6-DCHQ. In this study, our results allow us to conclude that PcpA converts 2,6-DCHQ to 2-chloromaleylacetate.

Several lines of evidences indicate that PcpA converts 2,6-DCHQ to 2-chloromaleylacetate. First, PcpA is highly homologous to the ring-cleavage dioxygenase LinE that dioxygenizes CHQ to maleylacetate. By analogy, PcpA dioxygenizes 2,6-DCHQ to 2-chloromaleylacetate. Second, PcpA converts HQ and CHQ to γ-hydroxymuconicsemialdehyde and maleylacetate, respectively, by dioxygenation. By analogy with the reaction for CHQ conversion, 2,6-DCHQ is converted to 2-chloromaleylacetate. The dioxygenation activity of PcpA is confirmed by the finding that PcpA is inactive in the absence of oxygen (data not shown). Third, and most important, in the GC-MS analysis, a specific peak, whose mass spectrogram contained indicative peaks for a TMS derivative of 2-chloromaleylacetate, was detected when 2,6-DCHQ was incubated with PcpA. Fourth, the lactol form of *cis*-2-chloro-4-ketopent-2-enoic acid, which has been shown to arise from 2-chloromaleylacetate [10], was detected by the GC-MS analysis. These findings are all consistent with the conversion of 2,6-DCHQ to 2-chloromaleylacetate by PcpA.

Lee et al. [13] have purified and characterized 2,6-DCHQ chlorohydrolase, which converts 2,6-DCHQ to chlorohydroxyquinol from ATCC39723. This conversion suggests that ATCC39723 possesses at least two degradation pathways for 2,6-DCHQ. One is the pathway through hydroxyquinol, and the other is a direct cleavage of 2,6-DCHQ. The pathway converting 2,6-DCHQ to 2-chloromaleylacetate seems to be a primary pathway in PCP mineralization since the *pcpA* mutant has been shown to accumulate 2,6-DCHQ. The pathway beyond 2-chloromaleylacetate remains to be elucidated.

Two types of pathways for the aerobic degradation of (chloro)phenols have been described thus far; one is via (chloro)catechols, and the other is via (chloro)hydroquinones. In the pathway via (chloro)catechols, these chlorophenols are further metabolized via *ortho* or modified *ortho* cleavage pathways [14]. In the (chloro)hydroquinone pathway, the introduction of a third hydroxyl group leads to the formation of (chloro)hydroxyquinol [13,15–19], which is subsequently cleaved by (chloro)hydroxyquinol 1,2-dioxygenase [16,18,20] or by a *meta*-cleavage enzyme [15,19].

On the other hand, pathways that involve the direct cleavage of hydroquinone have been suggested in the *p*-nitrophenol degrading bacterium *Moraxella* [21] and the 4-ethylphenol degrading bacterium *Pseudomonas putida* JD1 [22]. However, no information is available regarding the gene and enzyme involved in this reaction. Enzymes homologous to PcpA and LinE may be involved in this reaction.

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References

[1] McAllister, K.A., Lee, H. and Trevors, J.T. (1996) Biodegradation 7, 1–40.
[2] Saber, D.L. and Crawford, R.L. (1985) Appl. Environ. Microbiol. 50, 1512–1518.
[3] Orser, C.S., Lange, C.C., Xun, L., Zahrt, T.C. and Schneider, B.J. (1993) J. Bacteriol. 175, 411–416.
[4] Xun, L. and Orser, C.S. (1991) J. Bacteriol. 173, 4447–4453.
[5] Orser, C.S., Dutton, J., Lange, C., Jablonski, P., Xun, L. and Hargis, M. (1993) J. Bacteriol. 175, 2640–2644.
[6] Xun, L., Topp, E. and Orser, C.S. (1992) Biochem. Biophys. Res. Commun. 182, 361–366.
[7] Xun, L., Topp, E. and Orser, C.S. (1992) J. Bacteriol. 174, 8003–8007.
[8] Chanama, S. and Crawford, R.L. (1997) Appl. Environ. Microbiol. 63, 4833–4838.
[9] Miyauchi, K., Suh, S.-K., Nagata, Y. and Takagi, M. (1998) J. Bacteriol. 180, 1354–1359.
[10] Tiedje, J.M., Duxbury, J.M., Alexander, M. and Dawson, J.E. (1969) J. Agric. Food Chem. 17, 1021–1026.
[11] Miyauchi, K., Adachi, Y., Nagata, Y. and Takagi, M. (1999) J. Bacteriol. 181, in press.
[12] Rieble, S., Joshi, D.K. and Gold, M.H. (1994) J. Bacteriol. 176, 4838–4844.
[13] Lee, J.Y. and Xun, L. (1997) J. Bacteriol. 179, 1521–1524.
[14] van der Meer, J.R., de Vos, W.M., Harayama, S. and Zehnder, A.J.B. (1992) Microbiol. Rev. 56, 677–694.
[15] Uotila, J. (1993) Ph.D. Thesis, University of Helsinki.
[16] Zaborina, O., Daubaras, D.L., Zago, A., Xun, L., Sido, K., Klem, T., Nikolic, D. and Chakrabarty, A.M. (1998) J. Bacteriol. 180, 4667–4675.
[17] Golovleva, L.A., Zaborina, O., Pertsova, R., Baskunov, B., Schurukhin, Y. and Kuzmin, S. (1992) Biodegradation 2, 201–208.
[18] Latus, M., Eberspcher, H.-J. and Lingens, F. (1995) Appl. Environ. Microbiol. 61, 2453–2460.
[19] Uotila, J.S., Kitunen, V.H., Coote, T., Saastamoinen, T., Salkinoja-Salonen, M. and Apajalahti, J.H. (1995) Biodegradation 6, 119–126.
[20] Zaborina, O., Latus, M., Eberspcher, J., Golovleva, L.A. and Lingens, F. (1995) J. Bacteriol. 177, 229–234.
[21] Spain, J.C. and Gibson, D.T. (1991) Appl. Environ. Microbiol. 57, 812–819.
[22] Darby, J.M., Taylor, D.G. and Hopper, D.J. (1987) J. Gen. Microbiol. 133, 2137–2146.