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Cloning and Sequencing of a Novel *meta*-Cleavage Dioxygenase Gene Whose Product Is Involved in Degradation of γ-Hexachlorocyclohexane in *Sphingomonas paucimobilis*

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***Sphingomonas* (formerly *Pseudomonas*) *paucimobilis* UT26 utilizes γ-hexachlorocyclohexane (γ-HCH), a halogenated organic insecticide, as a sole source of carbon and energy. In a previous study, we showed that γ-HCH is degraded to chlorohydroquinone (CHQ) and then to hydroquinone (HQ), although the rate of reaction from CHQ to HQ was slow (K. Miyauchi, S. K. Suh, Y. Nagata, and M. Takagi, J. Bacteriol. 180: 1354–1359, 1998). In this study, we cloned and characterized a gene, designated *linE*, which is located upstream of *linD* and is directly involved in the degradation of CHQ. The LinE protein consists of 321 amino acids, and all of the amino acids which are reported to be essential for the activity of *meta*-cleavage dioxygenases are conserved in LinE. *Escherichia coli* overproducing LinE could convert both CHQ and HQ, producing γ-hydroxymuconic semialdehyde and maleylacetate, respectively, with consumption of O_2 but could not convert catechol, which is one of the major substrates for *meta*-cleavage dioxygenases. LinE seems to be resistant to the acylchloride, which is the ring cleavage product of CHQ and which seems to react with water to be converted to maleylacetate. These results indicated that LinE is a novel type of *meta*-cleavage dioxygenase, designated (chloro)hydroquinone 1,2-dioxygenase, which cleaves aromatic rings with two hydroxyl groups at *para* positions preferably. This study represents a direct demonstration of a new type of ring cleavage pathway for aromatic compounds, the hydroquinone pathway.**

γ-Hexachlorocyclohexane (γ-HCH; also called γ-BHC and 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. Moreover, some countries are presently using γ-HCH for economic reasons, and new sites are continually being contaminated.

Sphingomonas (formerly *Pseudomonas*) *paucimobilis* UT26 utilizes γ-HCH as a sole source of carbon and energy (12). UT26 degrades γ-HCH through the pathway shown in Fig. 1 (24, 27, 28). γ-HCH is likely converted by two steps of dehydrochlorination via γ-pentachlorocyclohexene (γ-PCCH) to 1,3,4,6-tetrachloro-1,4-cyclohexadiene (1,4-TCDN). This is productively metabolized to 2,5-dichloro-2,5-cyclohexadiene-1,4-diol (2,5-DDOL) by two steps of hydrolytic dehalogenation. 2,5-DDOL is further degraded to 2,5-dichlorohydroquinone (2,5-DCHQ) and 2,5-DCHQ is dechlorinated to CHQ or HQ, then to be mineralized. Two dead-end products, 1,2,4-trichlorobenzene (1,2,4-TCB) and 2,5-dichlorophenol (2,5-DCP), are also produced in this pathway.

In previous studies, we cloned and sequenced four genes involved in the γ-HCH degradation in UT26 (11, 23, 27, 28). The *linA* gene encodes γ-HCH dehydrochlorinase (LinA), which converts γ-HCH to 1,2,4-TCB via γ-PCCH. LinA shows no homology to known proteins (11). The *linB* gene encodes 1,4-TCDN chlorohydrolase (LinB), which converts 1,4-TCDN to 2,5-DDOL via 2,4,5-trichloro-2,5-cyclohexadiene-1-ol (2,4,5-DNOL). LinB shows significant similarity to hydrolytic dehalogenase (DhlA) from *Xanthobacter autotrophicus* GJ10 (13). The *linC* gene encodes 2,5-DDOL dehydrogenase, which converts 2,5-DDOL to 2,5-DCHQ (28). LinC shows homology to the members of the short-chain alcohol dehydrogenase family (29). The *linD* gene encodes 2,5-DCHQ dechlorinase, which converts 2,5-DCHQ to HQ via CHQ, and its activity rises in the presence of glutathione. LinD shows similarity to some members of class theta glutathione *S*-transferase family (23). We also showed that *linA*, *linB*, and *linC* were constitutively expressed (11, 27, 28), whereas *linD* was inducibly expressed in the presence of its substrate (23).

In this study, we describe the isolation and characterization of *linE* gene which is directly involved in the degradation of CHQ, one of the intermediates of γ-HCH degradation pathway in *S. paucimobilis* UT26. We show that the protein product of *linE* is a novel type of *meta*-cleavage dioxygenase, (chloro) hydroquinone 1,2-dioxygenase, which cleaves the aromatic ring with two hydroxyl groups at *para* positions preferably.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Sphingomonas* strains, *Pseudomonas* strains, and *Escherichia coli* were grown in Luria broth (21) or on W minimal medium (12). Cultures were incubated at 30°C for *Sphingomonas* and *Pseudomonas* strains and at 37°C for *E. coli* strains. Antibiotics were used at final concentrations of 50 μg/ml for ampicillin and kanamycin, 25 μg/ml for nalidixic acid, and 20 μg/ml for tetracycline.

Isolation of DNA. Plasmid DNA of *E. coli* was isolated by the alkaline lysis method of Maniatis et al. (21) and, if needed, purified by a cesium chloride-ethidium bromide density gradient centrifugation. Total DNAs from *Sphingomonas* and *Pseudomonas* strains were isolated as described previously (25).

Assay for CHQ degradation activity. Cosmid clones with LinD activity were assayed for CHQ degradation activity. A small quantity of each colony was picked and suspended in 100 μl of the assay solution (20 mM phosphate buffer [pH 7.0] containing CHQ at 1 μg/ml). The solution was incubated for 12 to 18 h at 30°C for *Pseudomonas putida* and at 37°C for *E. coli*, 500 μl of ethyl acetate was added, and the mixture was vortexed for 1 min. After centrifugation, the ethyl acetate layer was recovered. Five microliters of this extract was used for gas chromatography-mass spectroscopy (GC-MS) analysis. CHQ degradation activity was detected as the disappearance of the peak for CHQ.

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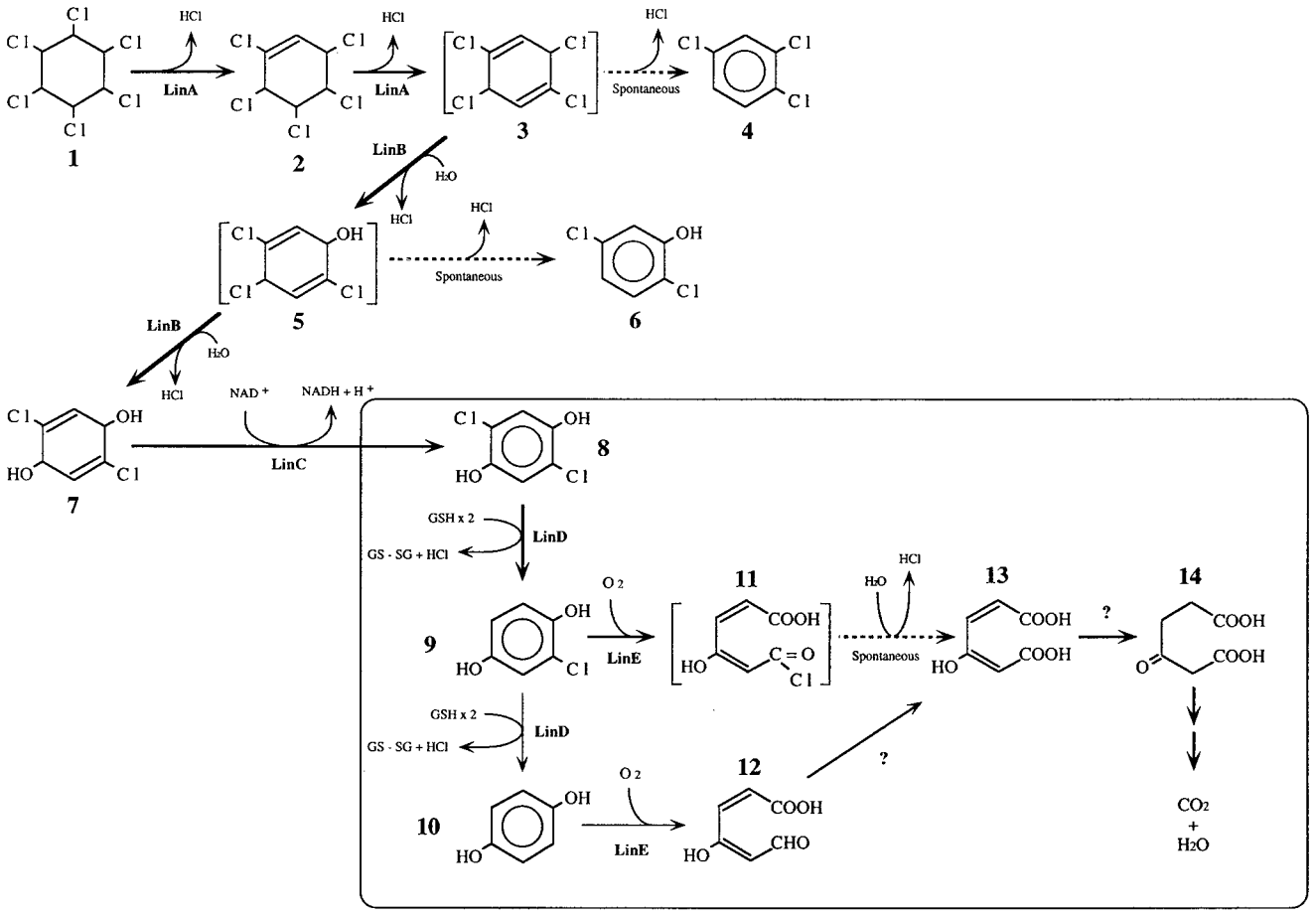

FIG. 1. Proposed assimilation pathway of γ-HCH in *S. paucimobilis* UT26. Compounds: 1, γ-HCH; 2, γ-PCCH; 3, 1,4-TCDN; 4, 1,2,4-TCB; 5, 2,4,5-DNOL; 6, 2,5-DCP; 7, 2,5-DDOL; 8, 2,5-DCHQ; 9, CHQ, 10, HQ; 11, acylchloride; 12, γ-HMSA; 13, maleylacetate; 14, β-ketoadipate.

To measure CHQ degradation activity of *Sphingomonas* strains, CHQ solution (20 mM potassium-phosphate buffer [pH 7.0] containing CHQ and ascorbic acid (each at a final concentration of 100 μM) was added to each whole cell (100 mg [wet weight]/ml). The mixture was extracted with ethyl acetate at specified times and analyzed by GC-MS. CHQ degradation activity was detected as the disappearance of the peak for CHQ.

CHQ was purchased from Aldrich (Milwaukee, Wis.), and a stock solution was made by dissolving the compound in ethanol.

GC-MS analyses. GC-MS analysis was performed as described previously (23). The column temperature was increased from 80 to 160°C at a rate of 5°C/min and then from 160 to 260°C at a rate of 10°C/min. The carrier gas flow rate was 20 ml/min.

Nucleotide sequence determination. Nucleotide sequences were determined by the dideoxy-chain termination method with a LI-COR model 4000L DNA sequencing system (LI-COR, Lincoln, Neb.).

Southern blot analysis. Southern blot analysis was performed with the ECL (enhanced chemiluminescence) gene detection system (Amersham, Arlington Heights, Ill.) according to the protocol provided by the manufacturer.

Northern blot analysis. Northern blot analysis was performed as described previously (23). 2,5-DCHQ, CHQ, and HQ (10 μM each) were used as inducers.

Analysis of the *linE* gene product. For overexpression of the *linE* gene product, plasmid pMYLE2 was constructed from pAQN (38). Plasmid pAQN was digested with *Eco*RI and *Hin*dIII to replace the 1.8-kb aqualysin coding fragment with the 1.3-kb *Eco*RI-*Hin*dIII fragment including the *linE* gene from pLE1. In pMYLE2, the *linE* gene is expressed under the control of the *tac* promoter. Expression is repressed tightly by the *lacI*^{q} gene product which is produced from the same plasmid, without IPTG (isopropyl-β-D-thiogalactopyranoside). Overexpression of *linE* was achieved as described previously (11) by using *E. coli* MV1190 containing pMYLE2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described previously (27).

Analysis of the metabolites. *E. coli* overproducing LinE was suspended in 20 mM potassium-phosphate buffer (pH 7.0) containing 100 ppm of (C)HQ and was incubated at 37°C with shaking for 12 h. Cells were removed by centrifugation, and the supernatant was extracted with ethyl acetate after acidification by HCl. Following evaporation of the ethylacetate layer, the substances were trimethylsilylated by *N*-methyl-*N*-trimetylsilyl-trifluoroacetamide (Nacalai Tesque, Kyoto, Japan) at 65°C. The resultant samples were analyzed by GC-MS. *E. coli* MV1190 was used as a negative control.

O_2 consumption assays using crude cell extract. Cells overproducing LinE or XylE were suspended in 20 mM Tris-HCl buffer (pH 7.5) and were disrupted by sonication (Sonifier; Branson, Danbury, Conn.). After centrifugation (12,000 × *g*) at 4°C for 10 min, the supernatant was used as the crude cell extract. Three hundred microliters of crude extract was diluted in 2.7 ml of O_2-saturated 20 mM potassium-phosphate buffer (pH 7.0). Substrates were diluted to 100 mM with ethanol. The assay was started by injecting 1 μl of substrate solution to the diluted crude extract. The consumption of O_2 was measured with an O_2 electrode system (MD-1000; Iijima Electronics Co., Aichi, Japan). The protein concentration of the crude extract was measured with a protein assay kit (Bio-Rad, Hercules, Calif.). One unit of activity was defined as the amount which consumes 1 μmol of O_2 in 1 min. Values reported are those from which the value of the endogeneous oxygen consumption was subtracted.

Site-directed mutagenesis. Site-directed mutagenesis was performed by using an LA PCR in vitro mutagenesis kit (Takara, Kyoto, Japan). Primers used for the mutants were 5′-GTCCAGCTGGCAAAGCCC-3′ for H162A, 5′-CGCGGCG GCATGAACTTGG-3′ for H229A, and 5′-GACCGAGGCCGCGAACAAC-3′ for E278A.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper are registered with the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession no. AB021867.

RESULTS

CHQ degradation activity of the LinD-less mutant. We previously cloned the *linD* gene, whose product (LinD) is responsible for the conversion of 2,5-DCHQ to CHQ and CHQ to

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Reference
S. paucimobilis		
UT26	HCH^+ Nal^r	12
UT103	HCH^- Nal^r Δ*linD* genome::Tn*5*	26
UT116	HCH^- Nal^r Δ*linD* Δ*linE* genome::Tn*5*	26
P. putida PpY101	Met Nal^r	19
E. coli		
MV1190	Δ*lac-proAB thi supE* Δ*srl-recA306*::Tn*10* F′ *traD36 proAB lacI*^{q}ZΔM15	39
HB101	F^- *hsdS recA ara proA lacY galK rpsL xyl mtl supE*	21
Plasmids		
pRK2013	ColE1::RK2 Tra^+ Km^r	7
pKTY320::Tn*5*	Ap^r Cm::Tn*5* (Km^r Ble^r Str^r)	25
pKS13	RK2 replicon, *cos* Mob^+ Tc^r	19
pUC18	pMB9 replicon, Ap^r	39
pUC118	pMB9 replicon, Ap^r	39
pHSG399	pMB9 replicon, Cm^r	39
pAQN	pMB9 replicon, *lacI*^{q} *aqn* Ap^r	38
pKSM1920	pKS13 with about 20 kb of UT26 DNA containing *linD*	This study
pKSM208	pKS13 with about 20 kb of UT26 DNA containing *linD*	This study
pSS1	pUC18 containing a 1.3-kb *Sac*I-*Sac*I fragment upstream of *linD*	This study
pSS2	pUC18 containing a 7-kb *Sac*I-*Sac*I fragment upstream of *linD*	This study
pLE1	pUC18 carrying a 1.3-kb blunted *Pst*I-*Nae*I fragment including *linE* at its *Sma*I site. *linE* is the same direction as the *lac* promoter	This study
pLE11	pHSG399 carrying a 5.5-kb *Pst*I-*Pst*I fragment from which *Nae*I-*Nae*I fragment is removed. *linE* is the same direction as the *lac* promoter	This study
pPP55	pUC18 carrying a 5.5-kb *Pst*I-*Pst*I fragment including *linE* at its *Pst*I site. *linE* is the same direction as the *lac* promoter.	This study
pMYLE2	pAQN carrying the *Eco*RI-*Nae*I fragment of pLE11	This study
pCY385	pUC18 carrying *xylE*	20
pLEH162A	pUC18 containing *linE* mutant (H162A)	This study
pMYH162A	pAQN containing *linE* mutant (H162A)	This study
pLEH229A	pUC18 containing *linE* mutant (H229A)	This study
pMYH229A	pAQN containing *linE* mutant (H229A)	This study
pLEE278A	pUC18 containing *linE* mutant (E278A)	This study
pMYE278A	pAQN containing *linE* mutant (E278A)	This study

[a] HCH^+, grown on γ-HCH. *aqn*, aqualysin I gene of *Thermus aquaticus*.

HQ (23). However, the conversion of CHQ to HQ by LinD seems not to be essential for the degradation pathway of γ-HCH in UT26, because the conversion rate of CHQ to HQ is much lower than that of 2,5-DCHQ to CHQ (23). Therefore, we investigated whether UT26 has another gene involved in CHQ degradation. The CHQ degradation activity of UT103, the LinD-less mutant (22, 23, 26), was tested. UT103 had the same extent of CHQ degradation activity as UT26 (100 mg [wet weight] of both strains degraded 100 nmol of CHQ within 1 h), indicating that UT26 has another gene (designated *linE*) for CHQ degradation. We also tested the CHQ degradation activity of UT116, carrying a deletion of the *linD* gene and its flanking region (23, 26). UT116 had no LinE activity, suggesting that the putative *linE* gene resides near the *linD* gene.

Screening of a cosmid clone which has CHQ degradation activity. In a previous study, we obtained six clones of *P. putida* PpY101, each of which holds a 20- to 30-kbp insert containing the *linD* gene (23). We tested each clone for CHQ degradation activity (LinE activity) and found that two of the six clones (one carrying pKSM1920 and one carrying pKSM208) had LinE activity. This activity was not detected when we used *E. coli* HB101 carrying pKSM1920 and pKSM208.

Subcloning and sequence analysis of the *linE* gene. Subcloning analysis revealed that the 5.5-kb *Pst*I-*Pst*I fragment containing the *linD* gene and 1.3-kb *Pst*I-*Nae*I fragment were responsible for LinE activity (Fig. 2). One open reading frame (ORF) of reasonable size (963 bp) was found in the 1.3-kb *Pst*I-*Nae*I fragment (Fig. 2). As this ORF is preceded by a putative Shine-Dalgarno sequence, we designated it *linE*. The *linE* gene encodes a polypeptide of 321 amino acids, and its deduced molecular mass is 36.0 kDa. Neither a sequence which shows a high level of similarity to the promoter sequence in *E. coli* nor one which is expected to form a stem-loop structure to function as a terminator was found around the *linE* gene. The G+C content of the *linE* gene is 60.1%, which is close to the total G+C content of a type strain of *S. paucimobilis* (65%) (31); the G+C contents of *linB*, *linC*, and *linD* are 64.3% (27), 62.5% (28) and 60.8% (23), respectively.

Southern blot analysis of the above-mentioned six cosmids containing the *linD* gene by using the 1.3-kb *Pst*I-*Nae*I fragment containing *linE* as a probe revealed that only two cosmids which expressed LinE activity in *P. putida* (pKSM1920 and pKSM208) contained the whole *linE* gene (data not shown). Northern blot analysis revealed that *linE* is inducibly expressed in the presence of CHQ, HQ, and 2,5-DCHQ (data not shown).

Overexpression of the *linE* gene in *E. coli* and identification of its protein product. To identify the protein product of the *linE* gene, we constructed plasmid pMYLE2, in which the *linE* gene was under the control of the *tac* promoter. *E. coli*

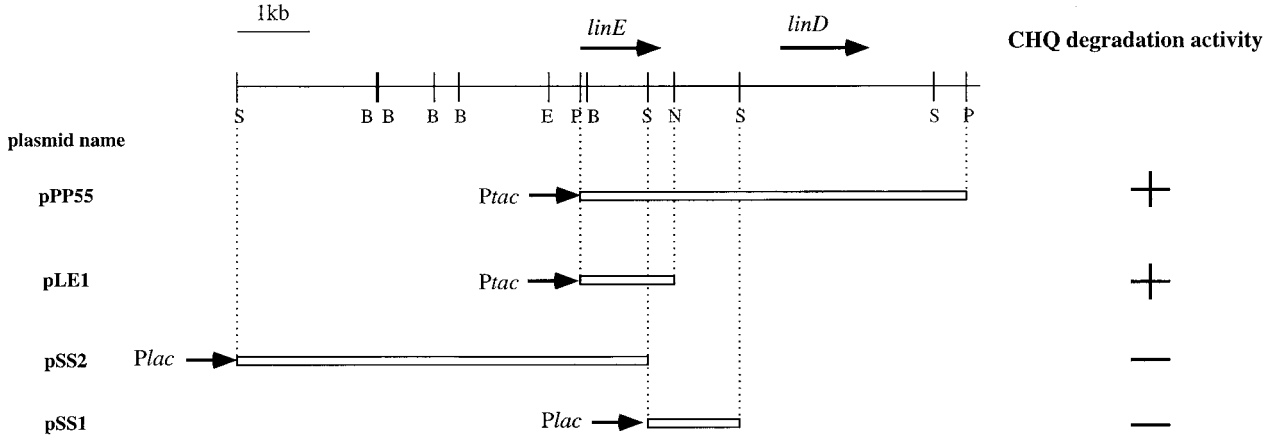

FIG. 2. Restriction map and deletion analysis of the region around the *linD* gene. Directions of transcription by the *lac* promoter are indicated by arrows. The procedure for the measurement of the LinE activity is described in Materials and Methods. The deduced location and direction of transcription of the *linE* gene are indicated by the arrow above the restriction map. B, *Bam*HI, E, *Eco*RI; N, *Nae*I; P, *Pst*I; S, *Sac*I.

MV1190 transformed with this plasmid was incubated with or without IPTG, and total proteins were analyzed by SDS-PAGE. An overproduced protein band corresponding to about 36 kDa was observed in the IPTG-treated cells (Fig. 3, lanes 2 and 3). The molecular mass of this protein was almost equal to that deduced from the nucleotide sequence of the *linE* gene, thus confirming that the product of *linE* is a protein with a molecular mass of about 36 kDa.

Homology search analysis of the LinE protein. The computer search revealed that two genes and their protein products, *pcpA* of *Sphingomonas chlorophenolica* (41) (recently the sequence of PcpA was revised) and *orf88′* of *Methylobacterium extorquens* AM1 (37), showed significant similarity to *linE* and LinE (46 and 37% amino acid identity, respectively) (Fig. 4). LinE also showed high levels of similarity to ORFs of *Bacillus subtilis* (YkcA, YodE, and YdfO [28, 29, and 23% amino acid identity, respectively]), but their functions are unknown. PcpA is involved in the degradation of pentachlorophenol (PCP) in *S. chlorophenolica* (41). It is known that PcpA is a periplasmic protein induced by PCP, although its function is unknown. Orf88′ of *M. extorquens* AM1 shares homology with only the N terminus of the LinE protein, because it is the product of a partial ORF which is located at the 3′ end of the published sequence, and consists of 88 amino acids. *orf88′* resides near the *pqqEF* operon, which is responsible for the synthesis of pyrroloquinolinequinone. The putative product of *orf88′* shares some identity with some members of catechol 2,3-dioxygenases (C23Os) (37). Therefore, we aligned LinE with some *meta*-cleavage dioxygenases (Fig. 4).

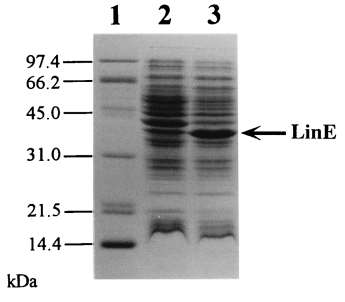

FIG. 3. Expression of *linE* under the *tac* promoter in *E. coli*. Lanes: 1, molecular mass markers; 2 and 3, total proteins of *E. coli* containing pMYLE2 not induced by IPTG and induced by IPTG, respectively. LinE protein is indicated by the arrow. The conditions for induction are described in Materials and Methods.

Three-dimensional structures of two kinds of *meta*-cleavage dioxygenases, 2,3-dihydroxybiphenyl 1,2-dioxygenases (BphC), from *Pseudomonas* sp. strain KKS102 (35) and *Burkholderia cepacia* LB400 (9), were determined. The important residues for the Fe(II) binding and its enzymatic activity were proposed from these data (Fig. 4). Eltis and Bolin have made an alignment of 23 *meta*-cleavage dioxygenases including the above two BphCs (6). Alignment of LinE with some of these dioxygenase (Fig. 4) revealed that all of the residues for Fe(II) binding (His162, His229, and Glu278 [LinE numbering]) are conserved. Although the level of similarity around the whole sequence is low, it is likely that LinE belongs to the *meta*-cleavage dioxygenase family. We also investigated the sequence similarity between LinE and gentisate 1,2-dioxygenases (8, 40), whose substrate (gentisate) has a hydroquinone structure, but there is not significant sequence similarity between LinE and known gentisate 1,2-dioxygenases.

HQ degradation activity of *E. coli* overproducing LinE. We previously observed that when HQ was incubated with UT26 with shaking, the color of the solution turned yellow, like the *meta*-cleavage product of catechol, and the accumulation of the yellow compound corresponded to the increase of the absorption peak at 320 nm (26). When *E. coli* overproducing LinE was incubated in potassium-phosphate buffer containing HQ with shaking, similar results were obtained: disappearance of HQ, increase of the absorption peak at 320 nm, and accumulation of the yellow compound. When *E. coli* not expressing *linE* was used as a control, the color of the solution turned red, apparently as a result of autooxidants of HQ. These results suggest that LinE can degrade HQ in addition to CHQ.

LinE cleaves the aromatic ring of (C)HQ with consumption of O_2. Because amino acids at the catalytic sites of *meta*-cleavage dioxygenases are conserved in LinE, we investigated the possibility that LinE functions as a ring cleavage dioxygenase.

To identify the metabolites of CHQ and HQ produced by the LinE protein, each substrate was incubated, with shaking, with resting *E. coli* cells overproducing LinE. The metabolites were extracted by ethyl acetate after acidification, then trimethylsilylated, and analyzed by GC-MS. The mass spectra of the peaks which specifically appeared when the cells were

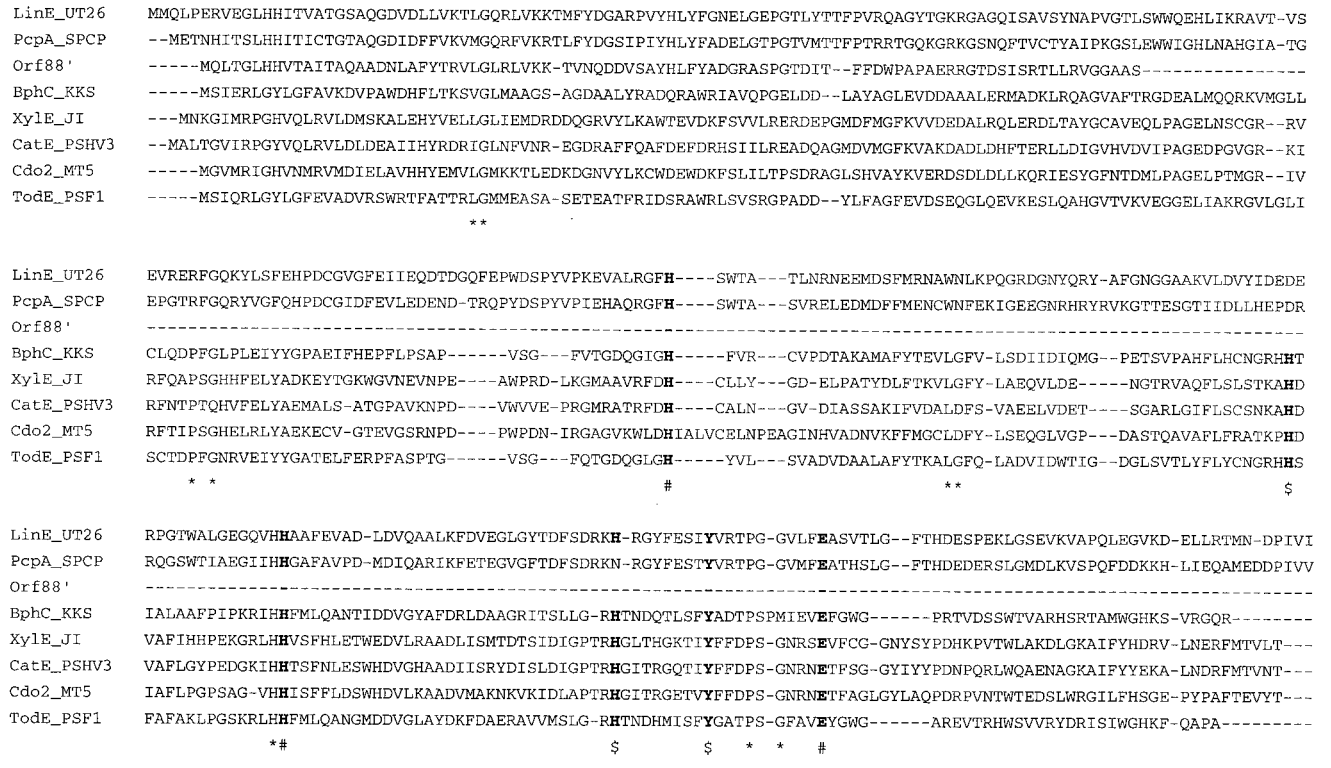

FIG. 4. Alignment between LinE, its homologs, and some *meta*-cleavage dioxygenases as obtained by CLUSTAL X and modified according to the crystal structure of BphC. The residues involved in Fe(II) binding (#) and in its activity ($) in BphC of *Pseudomonas* sp. strain KKS102 are shown, as are the residues referred to as conserved residues in reference 6) (*). The identities and similarities between LinE and each dioxygenase are shown following each sequence. The alignment was generated by using the CLUSTAL X sequence alignment program. Abbreviations: LinE_UT26, *S. paucimobilis* UT26, LinE; PcpA_SPCP, *S. chlorophenolica*, PcpA (41); Orf88′, *M. extorquens* AM1, Orf88′ (37); BphC_KKS, *Pseudomonas* sp. strain KKS102, 2,3-dihydroxybiphenyl 1,2-dioxygenase (19); XylE_JI, *P. aeruginosa* JI104, C23O (20); CatE_PSHV, *Pseudomonas* sp. strain HV3, C23O (42); Cdo2_MT5, *P. putida* MT-15, C23O (18); TodE_PSF1, *P. putida* F1, 3-methylcatechol 2,3-dioxygenase (43).

incubated with CHQ (Fig. 5a and b, peaks A through D) were identical to trimethylsilylated maleylacetate (peak A through C) and trimethylsilylated 3-oxoadipate (β-ketoadipate) (peak D) (Table 2) (33). The appearance of three peaks for maleylacetate appeared to be due to its isomers (33). When the authentic 3-oxoadipate was also trimethylsilylated and analyzed by GC-MS, the retention time and mass spectrum were identical to those of peak D in Fig. 5b (Table 2). The other peaks (for example, the peak whose retention time is 6.1 min in Fig. 5b) were not reproducible. The mass spectra of the peaks which appeared when cells were incubated with HQ (Fig. 5c and d, peaks E through I) were characteristic of those of trimethylsilylated γ-hydroxymuconic semialdehyde (γ-HMSA). The appearance of five peaks also seemed to be due to its isomers.

Next, we investigated O_2 consumption during the reaction by LinE. We used crude cell extracts of *E. coli* MV1190 overproducing LinE and of *E. coli* MV1190 overproducing XylE (C23O of *Pseudomonas aeruginosa* JI104 [20]) as a control. The results (Table 3) indicated that LinE consumed O_2 when CHQ and HQ were added but not when catechol was added. On the other hand, XylE consumed O_2 only when catechol was added. The activity of LinE against (C)HQ was much lower than that of XylE against catechol. Possibly because LinE formed inclusion bodies when overproduced in *E. coli* (data not shown) and in the resultant crude cell extract, there seemed to be very little soluble LinE.

These results indicated that LinE is a novel type of *meta*-cleavage dioxygenase, (C)HQ 1,2-dioxygenase, which cleaves aromatic rings with two hydroxyl groups at *para* rather than *ortho* positions.

Site-directed mutagenesis. For further confirmation that LinE is a member of *meta*-cleavage dioxygenases, we constructed three kinds of mutants. His162, His229, and Glu278, which correspond to the putative Fe(II) binding residues, were changed to alanine and designated H162A, H229A, and E278A, respectively. Activities of *E. coli* cell extracts producing mutant or wild-type LinE were measured by using CHQ as a substrate (Table 3). We confirmed by SDS-PAGE analysis that LinE and its mutants were synthesized in the same quantity. Activities of the mutants were not detected, indicating the importance of these amino acids for the activity of LinE, like other *meta*-cleavage dioxygenases.

DISCUSSION

We have cloned and sequenced the *linE* gene and partially characterized its protein product, LinE. Our findings suggest that LinE is a novel type of *meta*-cleavage dioxygenase which cleaves the aromatic ring with two hydroxyl groups at *para* positions. "*meta* cleavage" describes the manner of cleavage of the ring fission dioxygenases, which cleave outside two adjacent hydroxyl groups. Although the cleavage style of LinE does not fit the definition of *meta* cleavage, we categorize LinE as a member of *meta*-cleavage dioxygenases on the basis of its amino acid sequence similarity to them. In fact, catalytic amino acids of these dioxygenases are highly conserved in LinE. Some degradation pathways of aromatic compounds in which hydro-

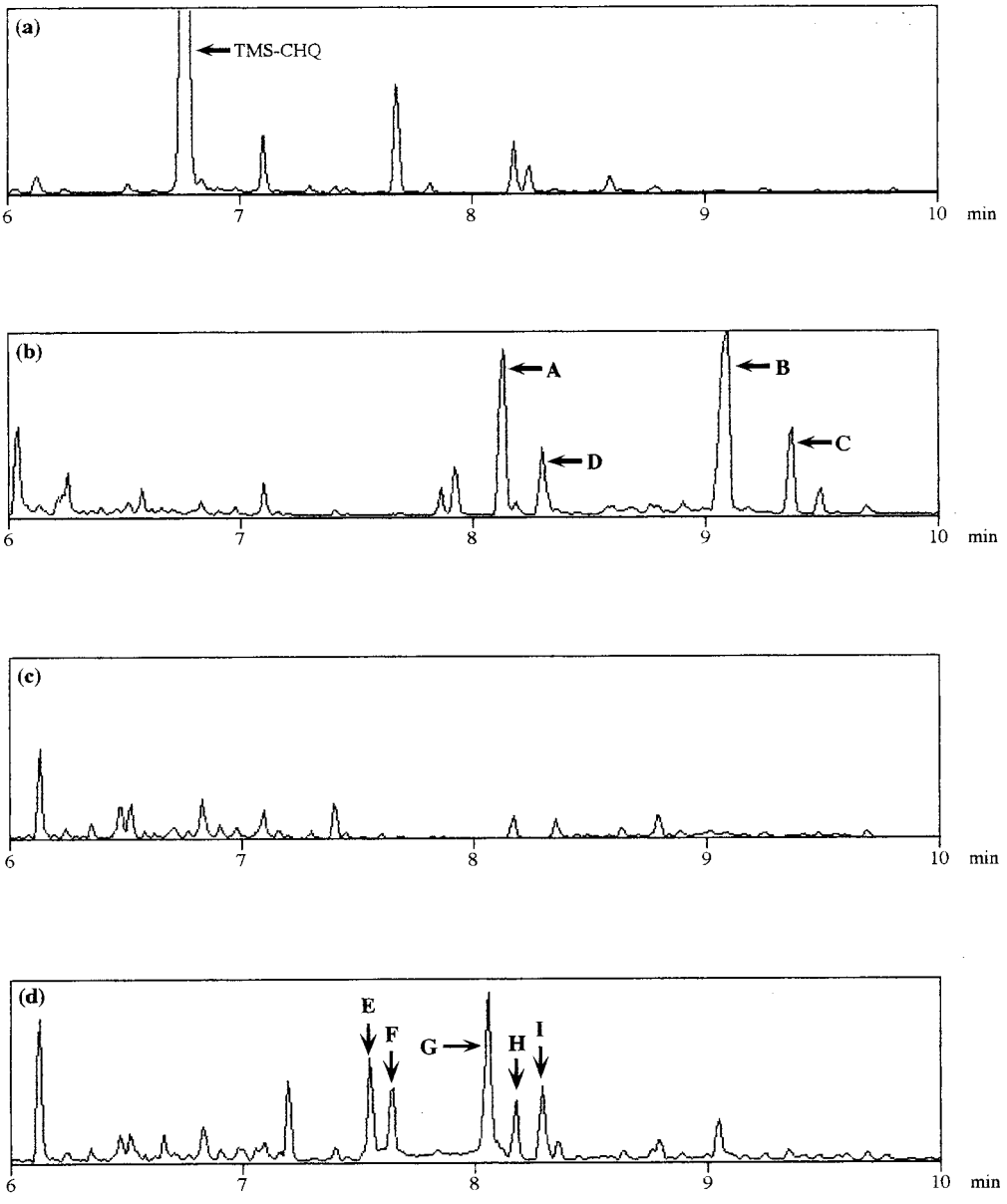

FIG. 5. GC-MS analysis of intermediates of (C)HQ by LinE reaction. (a) *E. coli* MV1190 incubated with CHQ; (b) *E. coli* MV1190(pMYLE2) incubated with CHQ; (c) *E. coli* MV1190 incubated with HQ; (d) *E. coli* MV1190(pMYLE2) incubated with HQ. After incubation, all samples were extracted by ethyl acetate followed by trimethylsilylation. The mass spectrum of each peak is indicated in Table 2.

quinones are used as a direct ring cleavage substrate have been reported (4, 36, 32), but to our knowledge there is no information about a gene or a protein responsible for cleavage of hydroquinones. LinE is the first ring cleavage enzyme which cleaves (C)HQ in preference to catechol, which is one of the general substrates for *meta*-cleavage dioxygenases. In fact, we showed that C23O (XylE) cleaves catechol rather than (C)HQ. We believe that LinE recognizes two hydroxyl groups at *para* positions. This study represents the direct demonstration of a new degradation pathway for aromatic compounds, the hydroquinone pathway. Next, we plan to purify and characterize the LinE protein.

The homology search showed that LinE does not exhibit a high level of similarity to known enzymes except for some proteins. One of them, Orf88′ of *M. extorquens* AM1, has homology to some C23Os, although *orf88′* is an incomplete ORF and its function is unknown (37). The alignment between LinE and *meta*-cleavage dioxygenases showed that LinE has nearly all residues for an active center and Fe(II) binding which are well conserved among the *meta*-cleavage dioxygenases. LinE also has a high level of similarity to PcpA of *S. chlorophenolica*, whose function is not known (41). Chanama and Crawford discussed the function of PcpA as 2,6-DCHQ chlorohydrolase (3). However, we suspect that PcpA is also a kind of (chloro)hydroquinone dioxygenase because of its sequence similarity to LinE. We had also isolated the *pcpA* gene from *S. chlorophenolica* independently as the gene which is responsible for 2,5-DCHQ degradation and showed that PcpA also had a ring cleavage dioxygenase activity (30).

The metabolites of (C)HQ were also identified. LinE cleaves CHQ between two carbon atoms (C-1 and C-2) which are substituted by hydroxyl group and chlorine group, respectively.

TABLE 2. Mass spectra of the peaks in Fig. 5

Peak[a]	Mass spectrum *m/z* (relative intensity)
A, B, C	374 (0.46), 359 (8.58), 315 (5.08), 257 (100), 241 (2.56), 197 (3.02), 147 (47.16), 123 (8.13), 95 (14.41), 73 (71.15)
D	376 (8.65), 361 (61.96), 317 (26.81), 286 (53.72), 259 (15.51), 243 (25.93), 231 (48.03), 169 (70.72), 147 (71.03), 125 (38.33), 97 (17.79), 73 (100)
Authentic β-ketoadipate	376 (7.73), 361 (59.74), 317 (27.87), 286 (53.82), 259 (16.18), 243 (27.78), 231 (46.83), 169 (63.55), 147 (64.13), 125 (47.89), 97 (19.76), 73 (100)
E, F, G, H, I	286 (2.13), 271 (62.34), 257 (7.73), 243 (10.15), 169 (70.51), 153 (34.31), 147 (50.42), 143 (60.95), 73 (100)

[a] All peaks are those of trimethylsilylated samples.

The product, acylchloride, seems to react with water to form the resultant product, maleylacetate. In this study, however, β-ketoadipate was also detected. The conversion of maleylacetate to β-ketoadipate may not to be due to LinE because when we used partially purified LinE, the peak of β-ketoadipate did not appear (22). The cause of conversion of maleylacetate to β-ketoadipate in *E. coli* is unknown. Maleylacetate reductase, which converts maleylacetate to β-ketoadipate, was found in some degradation pathways of aromatic compounds (5, 15, 17, 34). We are now trying to identify the gene encoding maleylacetate reductase from UT26. So far, we have not found a region homologous to maleylacetate reductase in the flanking regions of *linE* and *linD*. As for acylchloride converted from CHQ by LinE, a previous study reported that when 3-chlorocatechol was cleaved by C23O, the resultant metabolite, acylchloride, bound with the C23O and inactivated it (1). C23O of *P. putida* GJ31, however, can avoid the suicidal inactivation by acylchloride which was formed from 3-chlorocatechol by its own activity, and the resultant acylchloride reacts with water to form 2-hydroxymuconate (16). From our results, LinE can also avoid the inactivation by the acylchloride, and the acylchloride is likely to react with water to form maleylacetate.

From this and previous studies, *S. paucimobilis* converts γ-HCH to (C)HQ by LinA, LinB, LinC, and LinD, and then (C)HQ is cleaved by LinE in a *meta*-cleavage manner. The resultant metabolite, maleylacetate, appears to be degraded by the following β-ketoadipate pathway, which is known as part of the *ortho*-cleavage degradation pathway of catechol or 1,2,4-trihydroxybenzene (10). The pathway from CHQ to γ-HMSA via HQ may not be a major pathway because the conversion rate of CHQ to HQ by LinD is very slow (23), and the substrate specificity of LinE against HQ is lower than that for CHQ (Table 3). We are now trying to identify the gene involved in the degradation of γ-HMSA. However, the possibility that γ-HMSA is a dead-end product in the γ-HCH degradation pathway of UT26 as 1,2,4-TCB and 2,5-DCP cannot be excluded.

TABLE 3. Substrate specificities of crude cell extracts overproducing LinE or XylE against (C)HQ and catechol and activities of LinE mutants

Protein	Sp act (U/mg of protein) for[a]:		
	CHQ	HQ	Catechol
LinE	8.8×10^{-1}	2.7×10^{-1}	ND
XylE	ND	ND	99
LinE H162A	ND	NT	NT
LinE H229A	ND	NT	NT
LinE E278A	ND	NT	NT

[a] ND, not detectable; NT, not tested.

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