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Cloning and Characterization of *linR*, Involved in Regulation of the Downstream Pathway for γ-Hexachlorocyclohexane Degradation in *Sphingomonas paucimobilis* UT26

Keisuke Miyauchi,[1,2]* Haeng-Seog Lee,[1] Masao Fukuda,[2] Masamichi Takagi,[1] and Yuji Nagata[1,3]

Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657,[1] Department of Bioengineering, Kamitomioka, Nagaoka, Niigata 940-2188,[2] and Graduate School of Life Sciences, Tohoku University, Katahira, Sendai 980-8577,[3] Japan

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In *Sphingomonas paucimobilis* UT26, LinD and LinE activities, which are responsible for the degradation of γ-hexachlorocyclohexane, are inducibly expressed in the presence of their substrates, 2,5-dichlorohydroquinone (2,5-DCHQ) and chlorohydroquinone (CHQ). The nucleotide sequence of the 1-kb upstream region of the *linE* gene was determined, and an open reading frame (ORF) was found in divergent orientation from *linE*. Because the putative protein product of the ORF showed similarity to the LysR-type transcriptional regulator (LTTR) family, we named it *linR*. The fragment containing the putative LTTR recognition sequence (a palindromic $TN_{11}A$ sequence), which exists immediately upstream of *linE*, was ligated with the reporter gene *lacZ* and was inserted into the plasmid expressing LinR under the control of the *lac* promoter. When the resultant plasmid was introduced into *Escherichia coli*, the LacZ activity rose in the presence of 2,5-DCHQ and CHQ. RNA slot blot analysis for the total RNAs of UT26 and UT102, which has an insertional mutation in *linR*, revealed that the expression of the *linD* and *linE* genes was induced in the presence of 2,5-DCHQ, CHQ, and hydroquinone in UT26 but not in UT102. These results indicated that the *linR* gene is directly involved in the inducible expression of the *linD* and *linE* genes.

γ-Hexachlorocyclohexane (γ-HCH; also called γ-BHC or lindane) is a haloorganic insecticide which has been used worldwide. *Sphingomonas paucimobilis* UT26 can utilize γ-HCH as a sole source of carbon and energy (4). The degradation pathway of γ-HCH in UT26 consists of upstream and downstream pathways, which are shown in Fig. 1 (11). In the upstream pathway, γ-HCH is transformed to 2,5-dichlorohydroquinone (2,5-DCHQ), which is further degraded in the downstream pathway. In the previous studies, we isolated five genes (*linA*, *linB*, *linC*, *linD*, and *linE*) involved in these pathways and characterized their protein products (3, 7, 8, 12, 13). The *linA*, *linB*, and *linC* genes, for the upstream pathway, exist separately on the UT26 genome and are constitutively expressed (4, 12, 13). On the other hand, the *linD* and *linE* genes, for the downstream pathway, are located near each other and are inducibly expressed in the presence of their substrates (7, 8). They may constitute an operon (7). LinE is a novel *meta*-cleavage dioxygenase which cleaves aromatic rings with two hydroxy groups at *para* positions. We also demonstrated that PcpA from a pentachlorophenol-degrading bacterium, *Sphingomonas chlorophenolica* ATCC 39723, has activity similar to that of LinE (14). These results directly demonstrated a new type of ring cleavage pathway for aromatic compounds, the "hydroquinone (HQ) pathway." Although reports of the HQ pathway have been limited, we consider it one of the major degradation pathways for aromatic compounds whose regulation system for gene expression has been established. However, the regulation system for the genes of the HQ pathway in UT26 is still unknown. In this study, we cloned and characterized a regulatory gene encoding a transcriptional regulator for *linD* and *linE*.

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 and *Escherichia coli* were grown on Luria broth (6). The cultures were incubated at 30°C for *Sphingomonas* strains and at 37°C for *E. coli*. Antibiotics were used at final concentrations of 50 μg/ml for ampicillin and kanamycin and 25 μg/ml for nalidixic acid.

Isolation of DNA. Plasmid DNA of *E. coli* was isolated by the alkaline lysis method of Maniatis et al. (6). Total DNAs from *Sphingomonas* strains were isolated as described previously (9).

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.

Nucleotide sequence determination. The nucleotide sequences of a 1.0-kb *Pst*I-*Sma*I fragment of plasmid pLR1 and the fragments amplified by PCR were determined by the dideoxy chain termination method with the LI-COR (Lincoln, Neb.) model 4000L DNA-sequencing system.

Construction of plasmids for promoter activity assay. Various lengths of the region upstream of *linE* followed by the 5′ end of the *linE* gene were amplified by PCR and fused to the *lacZ* gene in the same frame on the plasmid pMC1403 (18) to form plasmids pMEU1, pMEU2, and pMEU3. The primers used to amplify the region upstream of *linE* are LEUP-1 (5′-GCCGAATTCTCGTGC AGCGGCGCTGA-3′), LEUP-2 (5′-GCGGGATCCAGTTGCATCATGATCG CTC-3′), LEUP-3 (5′-GCCGAATTCTATATTCACAATCTG-3′), and LEUP-4 (5′-GCCGAATTCTATGAAGGTCCGC-3′). To amplify the fragments for pMEU1, pMEU2, and pMEU3, primers LEUP-4, LEUP-3, and LEUP-2, respectively, were used in combination with LEUP-1. The resultant plasmids can express the LinE-LacZ fusion protein if the upstream region has promoter activity. To form pMEU1R, pMEU2R, and pMEU3R, the *Pvu*II-*Pvu*II fragment containing P_{lac}-*linR* was isolated from pLR1 and inserted into the *Bal*I site of each plasmid. To make the *Bal*I sites easily digested, each plasmid was purified from *E. coli* JM110 (*dcm* mutant).

β-Galactosidase assay. *E. coli* harboring each plasmid was incubated with isopropyl-β-D-thiogalactopyranoside (1 mM). The culture was divided, and each

* Corresponding author. Mailing address: Department of Bioengineering, Kamitomioka, Nagaoka, Niigata 940-2188, Japan. Phone: 81-258-47-9427. Fax: 81-258-47-9450. E-mail: kmiya@vos.nagaokaut.ac.jp.

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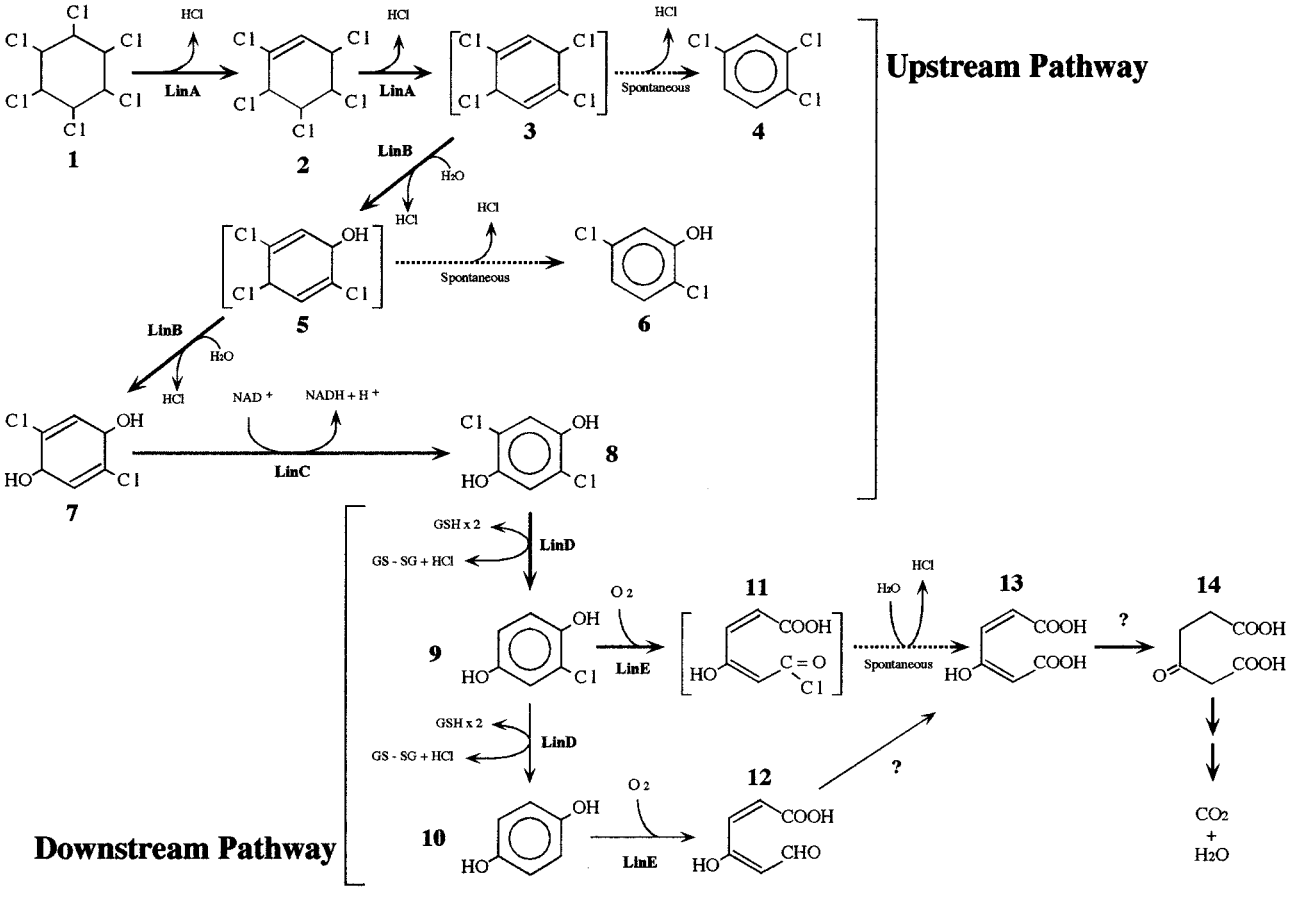

FIG. 1. Proposed assimilation pathway of γ-HCH in *S. paucimobilis* UT26. Compounds: 1, γ-HCH; 2, γ-pentachlorocyclohexene; 3, 1,3,4,6-tetrachloro-1,4-cyclohexadiene; 4, 1,2,4-trichlorobenzene; 5, 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 6, 2,5-dichlorophenol; 7, 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 8, 2,5-DCHQ; 9, CHQ; 10, HQ; 11, acylchloride; 12, γ-hydroxymuconic semialdehyde; 13, maleylacetate; 14, β-ketoadipate.

substrate was added (10 μM final concentration). After incubation for 2 h, the cells were harvested and resuspended in 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol). The cells were disrupted by sonication (Sonifier 250; Branson, Danbury, Conn.) and centrifuged at 12,000 × *g*. The supernatant was used for the measurement of β-galactosidase activity. The β-galactosidase activity of the cell extract was measured as follows. One hundred microliters of 13.3 mM *o*-nitrophenyl-β-D-galactopyranoside was added to 400 μl of cell extract. After incubation at 30°C, 250 μl of Na_2CO_3 solution (1 M) was added to stop the reaction, and the absorbance at 420 nm was measured. The protein concentration of the cell extract was determined by using a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard. One unit of β-galactosidase activity was

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
S. paucimobilis		
UT26	Nal^r	4
UT102	Nal^r Km^r; Tn*5*-induced mutant of UT26	10
E. coli		
JM110	*dam dcm supE hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr tsx* Δ(*lac-proAB*)/F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	6
MV1190	Δ*lac-proAB thi supE* Δ*srl-recA306*::Tn*10* F′ *traD36 proAB lacI* ZΔM15	6
Plasmids		
pMC1403	pMB1 replicon; Ap^r	18
pHSG399	pMB9 replicon; Cm^r	19
pUC18	pMB9 replicon; Ap^r	19
pUC19	pMB9 replicon; Ap^r	19
pLR1	pUC18/*Hin*cII + blunted *Pst*I-*Sma*I fragment containing *linR*; *linR* is the same orientation as the *lac* promoter	This study
pMEU1	pMC1403 carrying 238-bp *Eco*RI-*Bam*HI fragment containing the upstream region of *linE*	This study
pMEU2	pMC1403 carrying 163-bp *Eco*RI-*Bam*HI fragment containing the upstream region of *linE*	This study
pMEU3	pMC1403 carrying 138-bp *Eco*RI-*Bam*HI fragment containing the upstream region of *linE*	This study
pMEU1R	pMEU1 carrying *Pvu*II-*Pvu*II fragment of pLR1 at its *Bal*I site	This study
pMEU2R	pMEU2 carrying *Pvu*II-*Pvu*II fragment of pLR1 at its *Bal*I site	This study
pMEU3R	pMEU3 carrying *Pvu*II-*Pvu*II fragment of pLR1 at its *Bal*I site	This study

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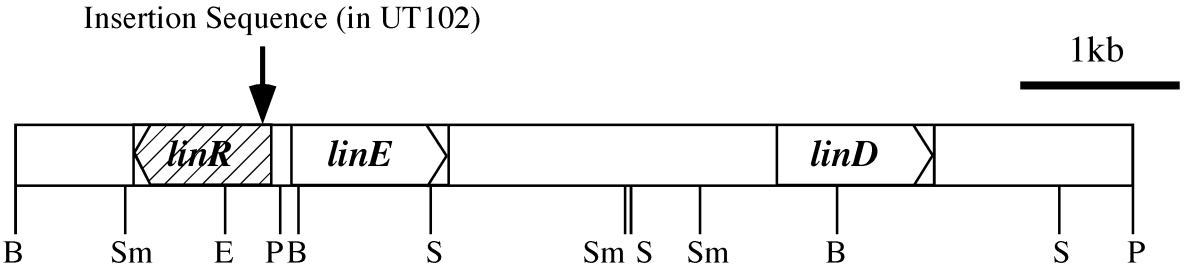

FIG. 2. Restriction map of *linR* and its flanking region. The arrow indicates the position of the insertion mutation in UT102. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sac*I; Sm, *Sma*I.

defined as the amount of enzyme that would hydrolyze 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside in 1 min at 30°C.

RNA slot blot analysis. Total RNAs of UT26 and UT102 were isolated as described previously (8). 2,5-DCHQ, chlorohydroquinone (CHQ), and HQ were used as inducers at final concentrations of 10 μM. Six micrograms of each RNA was blotted onto the nylon membrane with a PR600 slot blot manifold (Amersham, Arlington Heights, Ill.). Northern blot hybridization was performed as described previously (8).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper were registered in the DDBJ nucleotide sequence database under accession no. AB021863.

RESULTS AND DISCUSSION

Cloning and sequencing of the *linR* gene. *S. paucimobilis* UT26 inducibly expresses LinD and LinE activities in the presence of 2,5-DCHQ, substrate for LinD (8) and CHQ, substrate for both LinD and LinE (7, 8). On the other hand, UT102, which is one of the Tn*5*-induced mutants of UT26, shows faint LinD and LinE activities with or without their substrates (7, 10). It can be considered that this phenotype of UT102 is caused by a mutation in a regulatory gene for the expression of *linD* and *linE*. As UT102 has an insertional mutation other than Tn*5* in the upstream region of the *linE* gene (K. Miyauchi, Y. Nagata, and M. Takagi, unpublished data), we determined the nucleotide sequence of this region. The nucleotide sequence of a 1.0-kb *Pst*I-*Sma*I fragment was determined, and we found an open reading frame (ORF) of 909 bp in this region (Fig. 2). Because this ORF showed similarity to regulatory proteins (see below) and UT102 has an insertion sequence in the N terminus of the ORF (Fig. 2) (Miyauchi et al., unpublished), we designated the ORF *linR*. The *linR* gene appears to be divergently transcribed from *linE* and to use GTG as a start codon. The G+C content of the *linR* gene is 61.3%, which is similar to those of other *lin* genes (7, 8, 12, 13) except for *linA* (53.9%) (3). The deduced molecular mass of LinR is 33.6 kDa.

Homology search analysis of LinR. A FASTA homology search revealed that LinR shows similarity to LysR-type transcriptional regulators (LTTRs) (16). The proteins which showed high similarity to LinR are as follows: OhbR (29% identity; 69% similarity), a LysR-like protein in an operon for *ortho*-halobenzoate degradation in *Pseudomonas aeruginosa* strain JB2 (AF087482); NahR (31% identity; 68% similarity), a regulatory protein for naphthalene degradation genes in plasmid NAH7 of *Pseudomonas putida* (J04233); NagR (32% identity; 66% similarity), the regulator of the *nag* operon in *Ralstonia* sp. strain U2 plasmid pWWU2 (AF036940-2); NahR (31% identity; 68% similarity), a regulatory protein for naphthalene degradation genes in *Pseudomonas stutzeri* AN10 (AF039534-4); and SyrM1 (32% identity; 66% similarity), a LysR-like protein in *Rhizobium* sp. strain NGR234 (AE000091-2). The LTTR family is one of the ubiquitous transcriptional regulators in prokaryotes (16), and the transcription of the genes responsible for the degradation of aromatic compounds is often regulated by LTTRs (2). Like known LTTRs, LinR also contains a putative helix-turn-helix structure (S_{22}VSAAA RELDLPQPTASHGLARLRKALGDPL$_{52}$) at its N terminus, which is proposed to be responsible for DNA binding. Like many LTTRs, LinR does not have a glycine residue in the middle of the helix-turn-helix (D_{31} in LinR), which is conserved among classic helix-turn-helix structures (16), while OhbR, two NahRs, and SyrM have it. In the immediate upstream region of the *linE* gene, there is a consensus sequence for LTTR binding (ATTCACAATCTGAAT), which

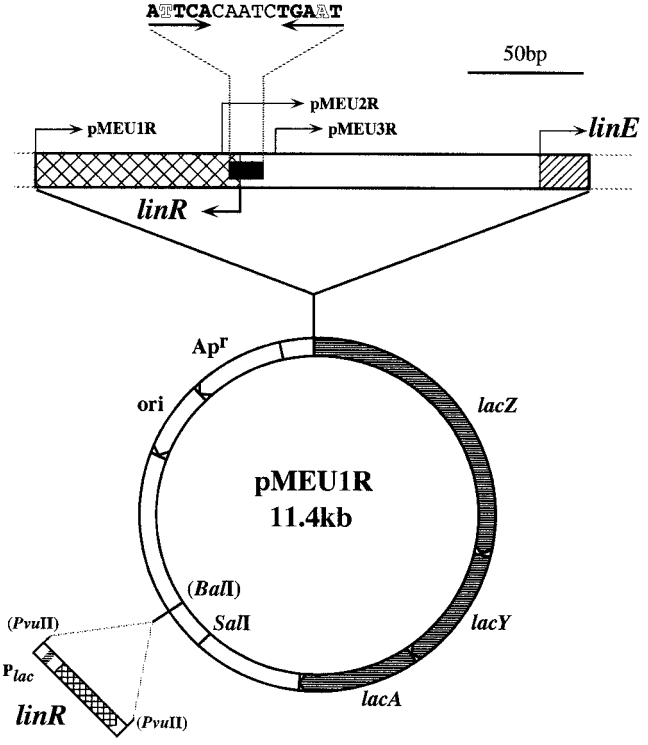

FIG. 3. Construction of plasmids pMEU1R, pMEU2R, and pMEU3R. The putative LinR-binding site and the start codons of *linR* and *linE* are indicated.

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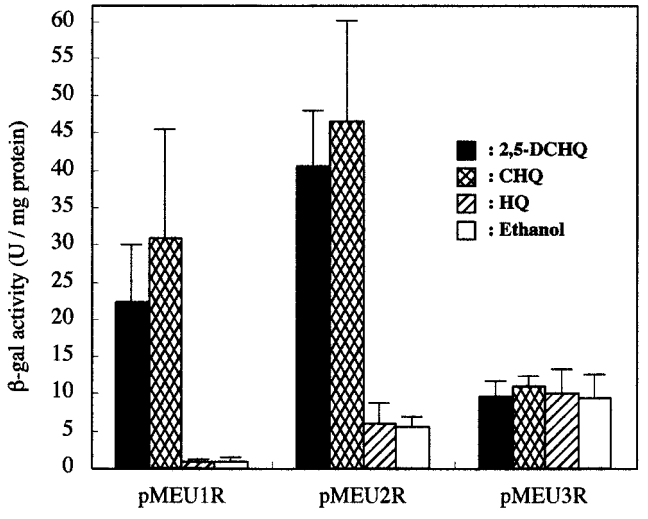

FIG. 4. β-Galactosidase (β-gal) activities of the cell extracts of *E. coli* harboring pMEU1R, pMEU2R, or pMEU3R. Standard errors calculated from three independent experiments are shown.

is a palindromic $TN_{11}A$ sequence (beginning and end underlined) (16) (Fig. 3).

In vivo analysis of the function of LinR. To characterize the function of LinR in the expression of *linE*, we constructed the plasmids pMEU1R, pMEU2R, and pMEU3R (Fig. 3). We also constructed pMEU1, pMEU2, and pMEU3, which do not have the *linR* gene, as a control for each plasmid. *E. coli* harboring pMEU1, pMEU2, or pMEU3 showed only a faint LacZ activity under every condition we tested (data not shown). *E. coli* harboring either pMEU1R or pMEU2R showed an increase in LacZ activity in the presence of 2,5-DCHQ and CHQ (23- to 36-fold), while *E. coli* harboring pMEU3R did not (Fig. 4). These results indicated that the *linR* gene and the palindromic $TN_{11}A$ sequence which is located upstream of *linE* are necessary for the upregulation of *linE* expression. It also seemed that the $TN_{11}A$ sequence is required to repress the expression of the *linE* gene when the substrates are not present.

Substrate specificity of LinR. The substrate specificity of LinR was studied with the *E. coli* in vivo system described above. In addition to 2,5-DCHQ and CHQ, only 2,6-DCHQ gave upregulation activity to *E. coli* harboring pMEU1R (Table 2). This result indicated that LinR specifically recognizes mono- or di-CHQs among the substrates we tested.

RNA slot blot analysis. To confirm the function of the *linR* gene for the expression of *linE* and *linD*, RNA slot blot analysis was performed with total RNAs of UT26 and UT102, which is a *linR* mutant. Figure 5 shows that *linD* and *linE* were inducibly expressed in the presence of 2,5-DCHQ, CHQ, and HQ in UT26, while almost no expression of *linD* and *linE* was observed in UT102 even in the presence of these substrates. The *linA* gene, which is constitutively expressed regardless of the presence of the substrates (4), was used as a control. This result indicated that HQ worked as an inducer for *linD* and *linE* expression in UT26 (Fig. 5) in addition to 2,5-DCHQ and CHQ, while it did not in the *E. coli* in vivo system (Fig. 4 and Table 2), suggesting a difference between the permeabilities of HQ in *Sphingomonas* and *E. coli*. The possibility that the intermetabolite of HQ works as an inducer in UT26 could not be excluded. However, we can conclude that CHQs are direct substrates of LinR at least, because *E. coli* does not have activity for conversion of CHQs. Like LinR, the inducer of

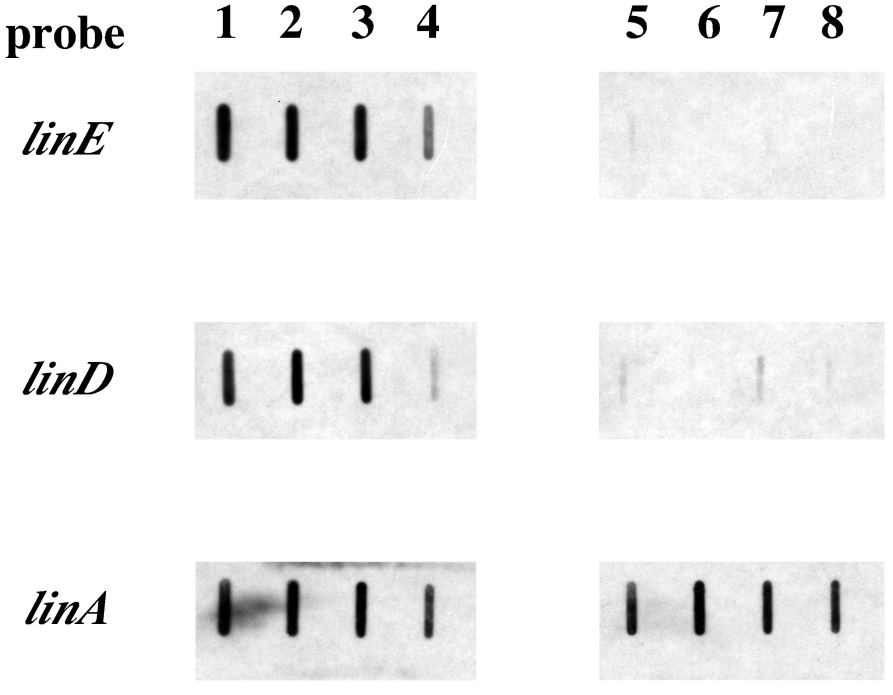

FIG. 5. RNA slot blot analysis of total RNAs of UT26 (lanes 1 to 4) and UT102 (lanes 5 to 8) incubated with 2,5-DCHQ (lanes 1 and 5), CHQ (lanes 2 and 6), HQ (lanes 3 and 7), and ethanol (lanes 4 and 8). The probes used are also shown.

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TABLE 2. Substrate specificity of LinR

Substrate	β-Galactosidase activity (U/min/mg of protein)[a]
2,5-DCHQ	22 ± 7.5
CHQ	31 ± 14
HQ	0.8 ± 0.1
2,6-DCHQ	3.9 ± 1.0
TetrachloroHQ	1.3 ± 0.1
Catechol	1.1 ± 0.2
3-Cl catechol	1.1 ± 0.1
4-Cl catechol	1.1 ± 0.2
Phenol	1.2 ± 0.2
m-Chlorophenol	1.2 ± 0.1
o-Chlorophenol	1.2 ± 0.3
Protocatechuate	1.1 ± 0.1
Gentisate	1.2 ± 0.1
Salicylate	1.2 ± 0.2
5-Cl salicylate	1.2 ± 0.2

[a] *E. coli* MV1190(pMEU1R) was used for the assay.

NahR for the expression of naphthalene-degradative genes is an aromatic compound, salicylate (17), while that of CatR for the expression of catechol-degradative genes is *cis*,*cis*-muconate, which is the ring cleavage product of catechol (15). The BenM protein, which regulates the expression of the *ben* operon in *Acinetobacter* sp. strain ADP1, responds to both aromatic compounds and ring cleavage products, i.e., benzoate and *cis*,*cis*-muconate (1). It would be very interesting to know why these substrates are selected as the inducers for the regulation system of each degradation pathway.

HQ pathway. The results described above indicate that the *linR* gene is necessary for the inducible expression of *linE* and *linD*. Because (C)HQs are specific substrates not only for LinD and LinE but also for LinR (Fig. 4 and 5 and Table 2), the genes encoding these three proteins seem to constitute a specific system for the degradation of (C)HQs, while the gene expression of some dehalogenases for xenobiotic haloalkanes is constitutive (5). The existence of a well-established degradation system strongly suggests that the HQ pathway is one of the major pathways for the degradation of aromatic compounds. *linD* and *linE* are considered to form an operon for the following reasons, although we could not detect the band corresponding to the predicted length for the putative *linE-linD* operon by Northern blot analysis (data not shown). First, the *linD* and *linE* genes are located close to each other. Second, the expression of *linD* is induced simultaneously with that of *linE* (Fig. 5). Third, we could not identify an obvious terminator sequence between the *linE* and *linD* genes. We are trying to identify other genes which belong to this operon.

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