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Organization and Transcriptional Characterization of the cat_1 Gene Cluster in *Acinetobacter lwoffii* K24

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Previously, we have reported that two clustered *cat* genes from *Acenitobacter lwoffii* K24 had different arrangements, $catB_1C_1A_1$ and $catB_2A_2C_2$ (Kim, S. I., S.-H. Leem, J.-S. Choi, Y. H. Chung, S. Kim, Y.-M. Park, Y. K. Park, Y. N. Lee, and K.-S. Ha. 1997, *J. Bacteriol.* 179, 5226–5231). By further analysis of the organization of the cat_1 gene cluster, we obtained a complete sequence of the $catB_1$ gene, which encoded 40.8-kDa polypeptide containing 379 amino acids, and found a open reading frame (ORF) coding a putative regulatory protein in upstream region of $catB_1$ on plasmid pCD1-1. This ORF encoded 34.2-kDa polypeptide containing 379 amino acids and had more than 40% identity with *catR, LysR* family regulatory protein of *Pseudomonas putida.* RT-PCR, Northern blot analysis and primer extension assay for transcriptional analysis of the cat_1 gene cluster revealed that the $catB_1C_1$ genes were cotranscribed and the $catA_1$ gene was independently transcribed.
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The β–ketoadiphate pathway is widely used as a central reaction in microbial dissimilation of aromatic compounds [1]. In this pathway, catechol is converted into succinate and the *catABC* genes are involved in the first three steps. The *cat* genes are extensively studied in *Pseudomonas putida* and *Acenitobacter calcoaceticus* [2-7]. Two bacteria have different gene organization but similar transcriptional regulation. In *P. putida,* three *cat* genes are compactly clustered with the transcriptional activator *catR* in order of *catR, catBCA. CatR* can activate *catBC* and *catA* in the presence of intermediate metabolite *cis,cis*-muconate, which functions as a inducer [3, 8]. In *A. calcoaceticus,* the *catA* gene is separated 3.8 kbp away from *catBCEFD* and the regulator gene *catM* is present in upstream of *catB* [2, 9, 10]. All of the genes of catechol degradation in *A. calcoaceticus* are activated by *catM,* which previously was thought to act as negative regulator [2], in concert with *cis,cis*-muconate [7]. Both *catR* and *catM* are *LysR*-type transcriptional regulators. The diversity in gene organization and regulation mechanism of the *cat* genes suggests the presence of different gene organization and transcriptional control in other bacteria. Recently *Rhodococcus erythropolis* 1CP was reported to have the *cat* genes in the order of *catA catB catC* [11].

Previously we have cloned two *catA* genes ($catA_1$ and $catA_2$) from chromosomal DNA of *Acinetobacter lwoffii* K24 [12]. Two *catA* genes had different gene organization with other *cat* genes; $catB_1C_1A_1$ and $catB_2A_2C_2$. The $catB_2A_2C_2$ gene cluster has not been reported yet in other bacteria. In this study, we completely sequenced the cat_1 gene cluster containing putative regulatory gene and analyzed transcriptional characterization of the cat_1 gene cluster.

MATERIALS AND METHODS

Bacterial strains and plasmid. *Acinetobacter lwoffii* K24 is a wild type strain capable of growing on media containing aniline as a sole carbon and nitrogen source [12]. *A. lwoffii* K24 was cultured in aniline medium at 27°C as previously described by Kim et al [12]. *E. coli* DH5α (pCD1-1) and *E. coli* DH5α (pCD1) were selected in colony hybridization for having the $catA_1$ gene of *A. lwoffii* K24. Plasmid pCD11, pCD12 and pCD13 were subcloned from pCD1 and pCD1-1 (Fig. 1).

Induction and enzyme activity assay. *E. coli* DH5α containing the *cat* genes were grown to an optical density of 0.3-0.4 at 600 nm in LB containing Ap at a concentration of 100 μg/ml and then incubated for overnight at 30°C in the final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested, resuspended in 2 ml of 50 mM Tris-HCl (pH 8.0) and disrupted by sonication (VCX 400 Sonics & Materials, Danbury, Conn.) and were centrifuged at 15,000 $\times$ g for 10 min. The supernatant was used for enzyme assays. Catechol 1,2-dioxygenase (*catA*) activity was assayed as previously described [13]. Muconate lactonizing enzyme (*catB*) activity was assayed by the method of Ornston [14]. The substrate of muconate lactonizing enzyme, *cis,cis*-muconate was produced enzymati-

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cally from catechol by purified catechol 1,2-dioxygenase and purified by proRPC column (5 by 200 mm, Pharmacia).

DNA manipulations. Plasmid DNA was isolated using QIAGEN plasmid kit (Chatsworth, Calf.). DNA fragments were isolated from 1% agarose gels by the prep-A clean kit (Bio-Rad, Hercules, Calf.) according to the recommendations of the supplier. DNA digestion with restriction enzymes, ligation and transformation were performed by the procedures of Sambrook et al [15].

Nucleotide sequence analysis. The nucleotide sequences of the upstream region of the $catB_1$ gene in pCD1-1 were determined by a modification of the dideoxy-chain termination methods [16], using Prism DyeDeoxy Terminator Cycles Sequencing Kit (Perkin-Elmer, Norwalk, Conn.). Sequencing reactions were prepared according to the supplier's instructions and analyzed by electrophoresis using Applied Biosystem Model 373A DNA sequencer on 6% polyacrylamide gel. The oligonucleotides used as primers in sequencing reaction were supplied by Bioneer Corp (Taejon, Korea). Interpretation of the DNA sequence and the deduced amino acid sequence was done by MacDNASIS DNA and Protein Sequence Analysis System of Hitach (San-Bruno, Calif.). The nucleotide sequence reported here has been submitted to the GenBank database under accession number U77658.

RT-PCR. RT-PCR was performed with Promega Access RT-PCR system (Promega, Madison, Wisc). The used primers are listed in the Fig. 5C. PCR with 10 ng pCD1 as templates was carried out as a positive control in the same condition of RT-PCR except for reverse transcription. RNA was extracted from a log phase culture of *A. lwoffii* K24 in aniline media by Qiagen Rneasy kit (Chatsworth, Calif.) and was treated with Rnase-free Dnase I (Takara, Japan) to remove DNA contamination according to the supplier's protocol. To ensure prepared RNA has no DNA contaminant, PCR was performed with 1 μg prepared RNA as a template and founded to have no PCR products.

Northern blotting. RNA was extracted as described by Simpson et al. [17]. RNA (10μg) was separated in 1% agarose-formaldehyde gel and transferred to a Zeta-probe blotting membrane in 10× SSC (1.5M NaCl plus 0.15 M sodium citrate) and immobilized on the membrane by UV irradiation (UV cross linker, Bio-Rad). DNA probes were prepared from the PCR product of 450-bp for $catA_1$ [12] and radiolabelled by [^{32}P]dCTP (NEN, Beverly, Mass.) using Exo-free Klenow fragment (Takara). The filters were hybridized, washed at 65°C and then exposed to X-ray film overnight at 70°C.

Primer extension. A oligonucleotide primer for primer extension was 5′-end labeled with [γ-32P]ATP (NEN) by using T4 polynucleotide kinase (promega). The labeled oligonucleotide was hybridized with total RNA (10μg) and used as primer by avian myeloblastosis virus reverse transcriptase (promega) for reverse transcription. The cDNA product was analyzed on 8% denaturing polyacrylamide gel with dideoxy sequencing standards prepared by using the same primer and T7 DNA polymerase (USB, Cleveland, Ohio). The primer was 5′CCCTTGCCTTCGAGATTGGTCGCCGCCTTC3′ (30mer) at position 2827 to 2798.

RESULTS

Cloning of the cat_1 gene cluster. We have selected pCD1 containing the $catA_1$ gene in colony hybridization and found the cat_1 gene order as $catB_1$–$catC_1$–$catA_1$ in subcloned vector pCD11. In this study, other positive clone, which have the upstream region of $catB_1$ and shows *catA* and *catB* activity by IPTG, was selected and named as pCD1-1 (Fig. 1). *E. coli* containing subcloned vectors pCD12 and pCD13 also showed *catB* activity under IPTG induction.

DNA sequence analysis of $catB_1$ and ORF_{R1} in the cat_1 gene cluster. Nucleotide sequence analysis of the upstream of the $catB_1$ gene in plasmid pCD1-1 was performed and 2009 bp nucleotides were sequenced (Fig. 2). The open reading frame (ORF) of the $catB_1$ gene initiates at ATG (bp 1196) and terminates at TAA (bp 2335). $CatB_1$ have 379 amino acids and deduced molecular weight is 40788 dalton. Multi-alignment analysis of the deduced amino acid sequence of the $catB_1$ gene with other homologous genes showed that the gene has more than 50% identity with *A. calcoaceticus* (M76991) and *P. putida* PRS2000 [8]. The $catB_1$ gene also have more than 40% identity with *clcB* [18], *tcbD* [19] and *tfdD* [20] (Data not shown). These results suggest that the *catB* and *catC* genes are more conserved than the *catA* genes in soil bacteria [11, 12]. Several amino acid residues (Asp, Glu and Lys) of the *catB* genes, which are assumed to be involved in magnesium coordination and the enzyme mechanism, are conserved in the $catB_1$ gene [11]. By computer analysis,

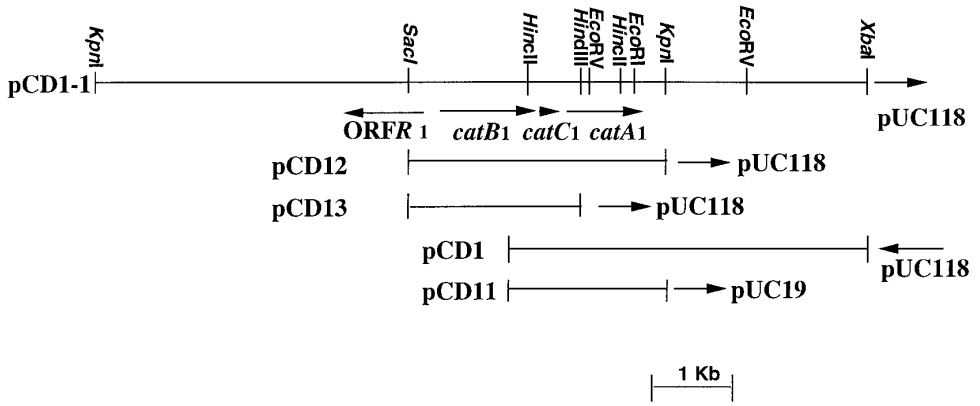

FIG. 1. Restriction endonuclease map of the 10-kbp *Kpn*I-*Xba*I fragment of pCD1-1 containing the cat_1 genes. Arrows indicate the direction of transcription.

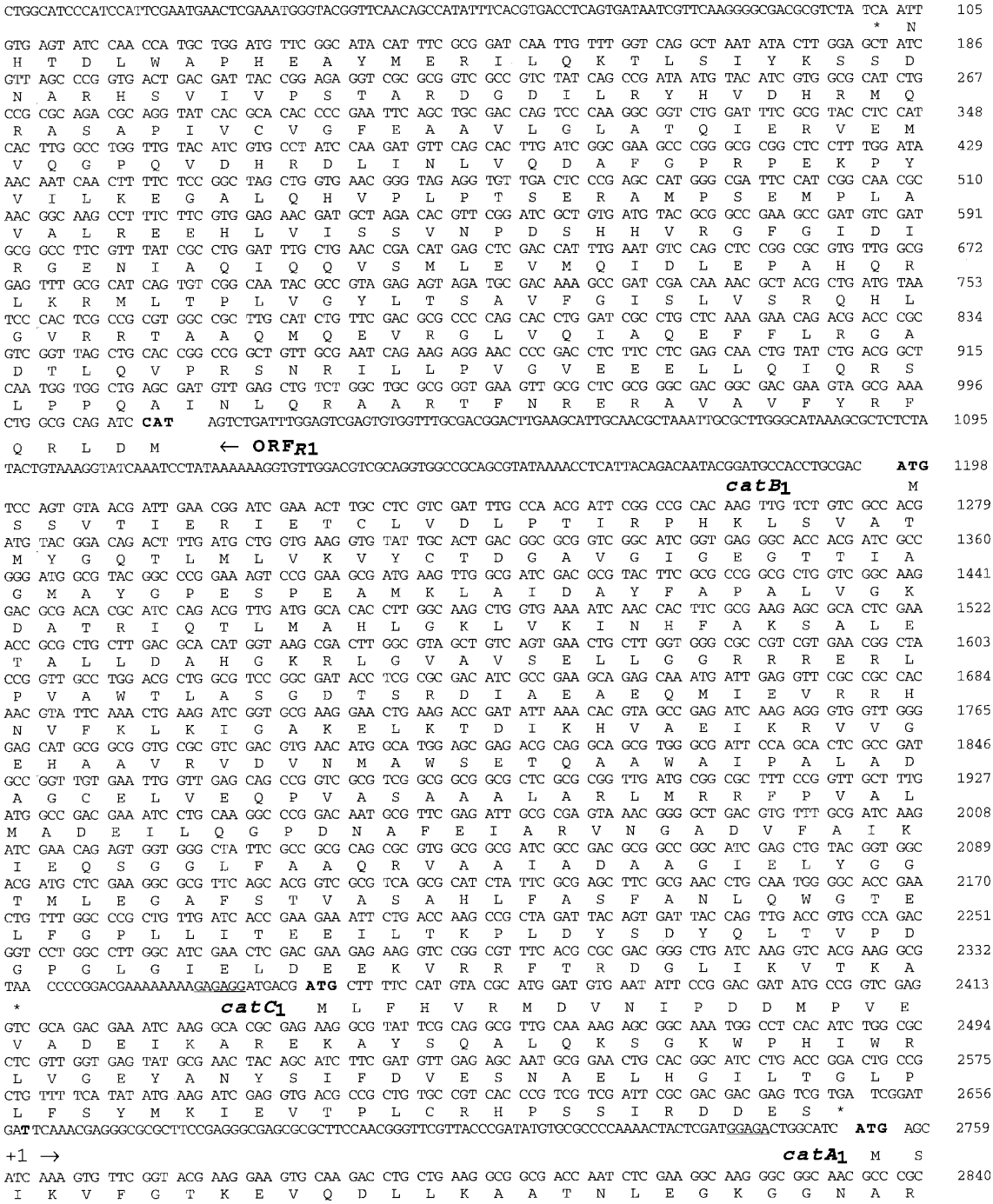

FIG. 2. Nucleotide sequences and deduced amino acid sequences of ORF$_{R1}$ and the *cat*$_1$ genes in *Acinetobacter lwoffii* K24. The putative ribosome-binding sites are underlined. Asterisks indicate the translational stop codons. The arrow of ORF$_{R1}$ indicated the direction of transcription. The transcription start sites of *catA*$_1$ was defined by +1.

another open reading frame was found in 224 bp upstream of *catB*$_1$. This ORF has reverse direction and significant identity with *catR*s (43.9% and 44.1%), *catM* (38.2%) and *clcR* (31.4%) (Fig. 3). *CatR*, *catM* and *clcR* are known as *LysR* family regulator proteins in *P. putida* and *A. calcoaceticus* [2, 5, 8, 18]. This ORF has also common characteristics of *LysR* family in molecular weight, highly homology in the N-terminal region containing helix-turn-helix motif and lower homology in the C-terminal region [21]. We designated this ORF as

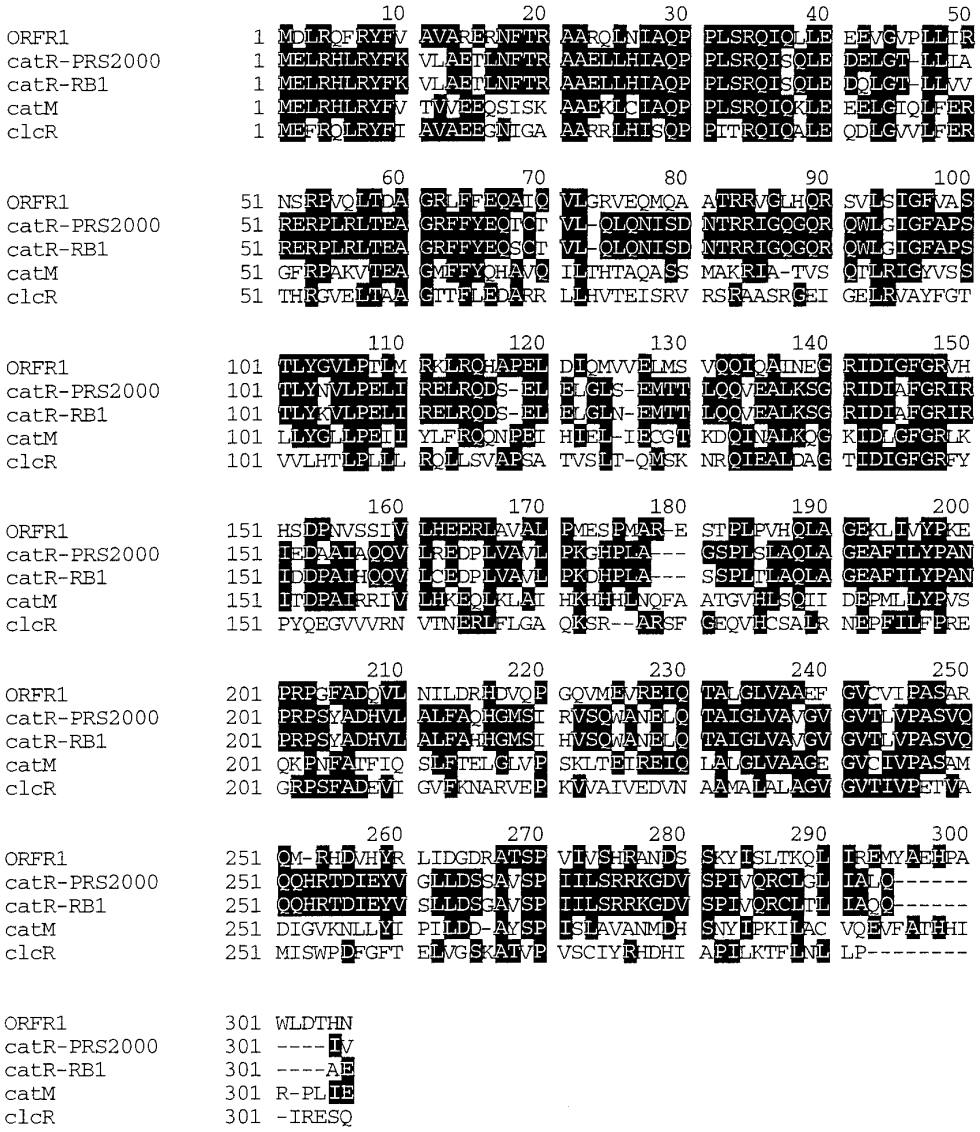

FIG. 3. Multiple alignment of the deduced amino acids of ORF_{R1} of *Acinetobacter lowffii* K24 with homologous proteins of various species. ORFR1, ORF_{R1} of cat_1 of *A. lwoffii* K24; catR-PRS2000, *catR* of *P. putida* PRS2000 [8]; catR-RB1, *catR* of *P. putida* RB1 [5]; catM, *catM* of *A. calcoaceticus* [2]; clcR, *clcR* of *P. putida* [27].

ORF_{R1}. ORF_{R1} has 303 amino acids and its deduced molecular weight is 34245. The ORF_{R1}-$catB_1$ intergenic region has highly homologous sequence with the sequence of repression binding site (RBS) and activation binding site (ABS) in the *catR-catB* intergenic regions of *P. putida* (Fig. 4). Interestingly, suggested consensus binding motif of *LysR* family regulatory protein, T-N_{11}-A is founded in this region (T_{1096}-A_{1108} of cat_1 genes). Codon usage analysis showed that the cat_1 gene cluster has higher biased G or C (71.4% for cat_1 genes) in the wobble position than total genes of *E. coli* (55.9%) and have similar ratio to that of *P. putida* (72.4 %) [22].

Transcriptional analysis of the cat_1 gene cluster. To analyze the transcriptional characterization of the $catB_1C_1A_1$ gene cluster of A. lwoffii K24, RT-PCR was carried out using the oligonucleotides synthesized on the base of nucleotide sequence of the cat_1 genes. Five oligonucleotides used in PCR and RT-PCR were indicated in Fig. 5 C. By this method mRNA coding $catB_1$, $catB_1C_1$ and $catA_1$ were detected but not $catB_1C_1A_1$ (Fig. 5 A). This results show that $catB_1$ and $catC_1$ may be cotranscribed but $catA_1$ may be independently transcribed (Fig. 5 B). Northern blotting and primer extension analysis confirmed this proposal. Northern blotting with 450 bp PCR product of $catA_1$ [12] revealed that the transcript coding $catA_1$ was about 1.6 kb, which is enough size for covering the $catA_1$ gene but not the $catB_1$ gene (Fig. 6 A). To identify the 5′ termini

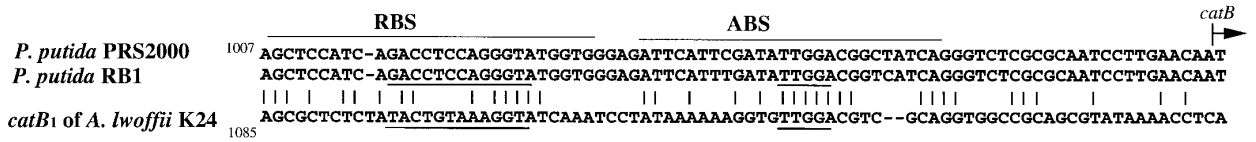

FIG. 4. Alignment of promoter regions of the $catB_1$ gene and the *catB* genes of *P. putida* PRS2000 [8] and *P. putida* RB1 [3]. Homologous nucleotides are indicated by bars. The sequences of RBS and ABS in *P. putida* RB1 were indicated and were from the studies by Parsek et al.[3]. The -35 hexamer sequences, G-N_{11}-A sequence in *P. putida* and T-N_{11}-A sequence in *A. lwoffii* K24 were underlined.

of the $catA_1$ gene, primer extension analysis was performed with RNA purified from A. lwoffii K24 cultured in aniline media. The extension product of the $catA_1$ gene was ended at T_{2659} on the coding strand suggesting the transcription start of $catA_1$ at T_{2659} (Fig. 6 B).

DISCUSSION

The cat_1 gene cluster of *A. lwoffii* K24 was sequenced and the transcriptional pattern was characterized. The cat_1 gene cluster has the gene order of $ORFR_1$-$catB_1$-$catC_1$-$catA_1$, which is similar to the *cat* gene cluster of *P. putida* PRS2000 [8]. The cat_1 genes have other common properties with those of *P. putida* PRS2000. 1) Independent transcription of $catA_1$ and cotranscription of $catB_1C_1$. 2) Significant homology in the promoter region of the $catB_1$ gene and the *catB* gene of *P. putida* PRS2000 (Fig. 4). 3) Putative regulatory protein coding gene, ORF_{R1}, which has 43.9% identity with *catR* of *P. putida* PRS2000. But the cat_1 genes have different characteristics from *cat* genes of *P. putida* PRS 2000. 1) Difference in the length and DNA sequence of intergenic space between the *cat* genes. The 85-bp palindromic sequence, which cause genetic difference in *P. putida* PRS2000 and *P. putida* RB1, was not found in the cat_1 genes in *A. lwoffii* K24. 2) In spite of different placement of an 85-bp palindromic sequence, the *cat* genes of *P. putida* PRS2000 and *P. putida* RB1 have about 90% homology showing their close relation, but the cat_1 genes of *A. lowffii* K24 have less than 60% homology with the *cat* genes of these bacteria. These results suggest that though the *cat* genes of two soil bacteria have the same gene arrangement, there is considerable gene diversity between the cat_1 genes of *A. lowffii* K24 and the *cat* genes of *P. putida* PRS2000. Primer extension analysis of $catA_1$ suggests the possibility of regulatory protein binding on the $catC_1$ region for transcription of $catA_1$. *CatR* of *P. putida* PRS 2000 was reported to bind to the *catC* region as well as the upstream region of the *catB* gene and *catM* of *A. calcoaceticus* also was known to bind to the upstream region of *catA* [7, 8]. However, we could not found common DNA sequences for binding of *LysR* family regulatory proteins in this region. It is necessary to study the

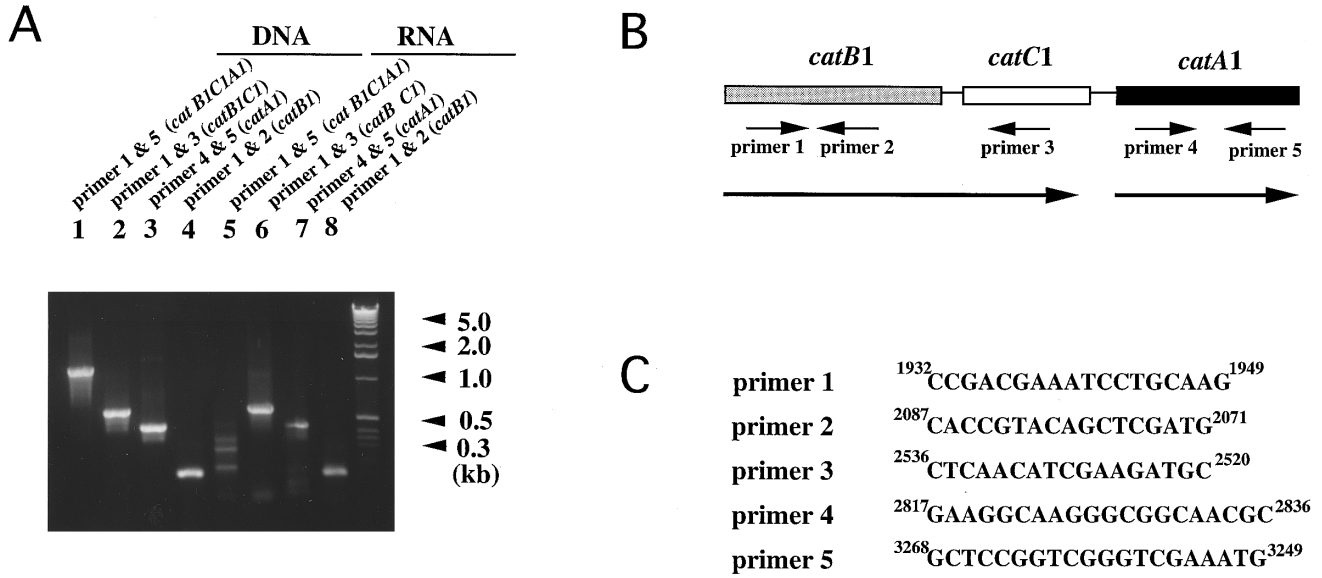

FIG. 5. Analysis of transcriptional pattern of the $catB_1C_1A_1$ gene cluster by RT-PCR. (A) Lane 1-4; control PCR. pCD1-1 was used as a template. Lane 5-8; RT-PCR was performed according to the Material and Methods. (B) Transcription pattern of the cat_1 genes was depicted as bold line. (C) The used oligonucleotides.

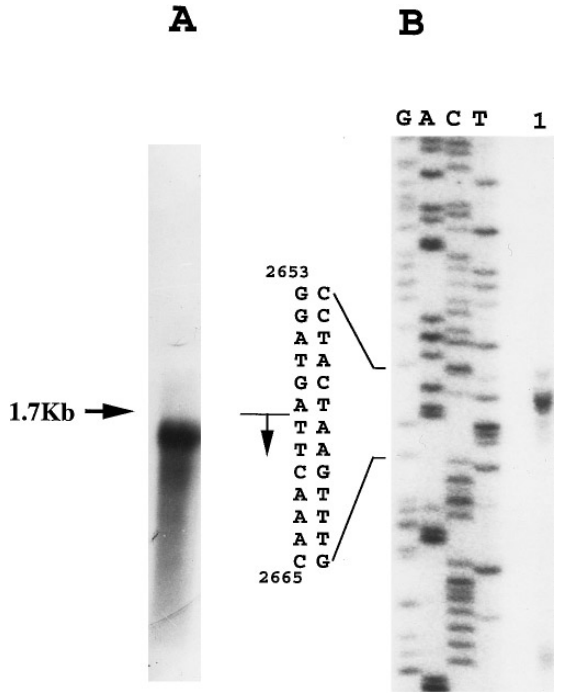

FIG. 6. (A) Northern blot analysis with 450bp PCR product of $catA_1$ as probe. (B) Determination of $catA_1$ transcription start site by primer extension. The sequence in the region of transcription initiation is shown at the left side, and the arrow indicates the sequence positions of the major extension products.

function of the putative regulatory protein-coding gene, ORF_{R1} as regulator for the cat_1 and cat_2 cluster. *CatR*, the regulator of the *catBC* and *catA* gene of *P. putida* RB1, was known to be able to control the transcription of the *pheBA* operon in *P. putida* PaW85 as well as the *clcABD* gene clusters in *P. putida* PRS2000 [23, 24]. The cross regulation of *catR* is common characteristics found in other Lys-R family member such as *benR* and *TcbR* [25, 26]. High homology of ORF_{R1} with *catR* of *P. putida* (44.1%) shows the possibility of cross talking of ORF_{R1} and *catR* as regulators.

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