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Nucleotide Sequence and Initial Functional Characterization of the *clcR* Gene Encoding a LysR Family Activator of the *clcABD* Chlorocatechol Operon in *Pseudomonas putida*

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The 3-chlorocatechol operon *clcABD* is central to the biodegradative pathway of 3-chlorobenzoate. The *clcR* regulatory gene, which activates the *clcABD* operon, was cloned from the region immediately upstream of the operon and was shown to complement an insertion mutation for growth on 3-chlorobenzoate. ClcR activated the *clcA* promoter, which controls expression of the *clcABD* operon, in *trans* by 14-fold in an in vivo promoter probe assay in *Pseudomonas putida* when cells were incubated with 15 mM 3-chlorobenzoic acid. Specific binding of ClcR to the *clcR-clcA* intergenic promoter region was observed in a gel shift assay. Nucleotide sequence analysis of the *clcR* gene predicts a polypeptide of 32.5 kDa, which was confirmed by using specific in vivo ^{35}S labeling of the protein from a T7 promoter-controlled ATG fusion construct. ClcR shares high sequence identity with the LysR family of bacterial regulator proteins and has especially high homology to a subgroup of the family consisting of TcbR (57% amino acid sequence identity), TfdS, CatR, and CatM. ClcR was shown to autoregulate its own production in *trans* to 35% of unrepressed levels but partially relieved this autorepression under conditions that induced transcription at the *clcA* promoter. Several considerations indicate that the *clcR-clcABD* locus is most similar to the *tcbR-tcbCDEF* regulon.

Modified *ortho* cleavage pathways are responsible for the biodegradation of a large array of chloroaromatic compounds and are widespread in soil bacterial communities. A well-characterized group of modified *ortho* cleavage pathway operons that encode functions for the dissimilation of catechol and chlorocatechols provides a model system for studying the regulation of evolutionarily related pathways. These include operons in degradative pathways for 3-chlorobenzoic acid (3Cba) from *Pseudomonas putida*, 1,2,4-trichlorobenzene from a *Pseudomonas* sp., 2,4-dichlorophenoxyacetic acid from *Alcaligenes eutrophus*, and benzoic acid from *P. putida* and from *Acinetobacter calcoaceticus*. In the above order, the relevant operons of the first three pathways include *clcABD* (15), *tcbCDEF* (50), and *tfdCDEF* (12), which convert 3-chlorocatechol, 3,4,6-trichlorocatechol, and 3,5-dichlorocatechol to maleylacetic acid, 5-chloromaleylacetic acid, and 2-chloromaleylacetic acid, respectively (12, 14, 44, 45, 52). The catechol-degradative genes in the benzoate pathways of *P. putida* and *A. calcoaceticus* are clustered in dissimilar operons. In *P. putida* (after the conversion of catechol to *cis,cis*-muconate by the *catA* gene product), the *catBC* operon encodes the conversion of the latter to β-ketoadipate enol lactone, which is then transformed to β-ketoadipate through the action of other loci (36). Catechol degradation in *A. calcoaceticus* is directed by the *catA* gene and the *catBCEFD* operon, which convert catechol through β-ketoadipate to succinyl coenzyme A and acetyl coenzyme A (36). In nearly every case, the analogous reactions of the above five operons are catalyzed by homologous enzymes.

Transcription of the *catBCEFD*, *catBC*, and *tcbCDEF* operons is known to be controlled by the respective regulatory proteins CatM, CatR, and TcbR (35, 41, 51), each of which is a member of the LysR family of bacterial regulators (24). While the regulation of the *tfdCDEF* operon is not yet clear, the operon may be regulated by the LysR family member TfdS (see Discussion).

The chlorocatechol (*clc*) pathway genes (Fig. 1) were subcloned from the biodegradative plasmid pAC27 as an *Eco*RI fragment (9), localized to a 4.2-kb *Bgl*II fragment (19), sequenced, and expressed (15). The conversions mediated by the pathway enzymes have been well characterized (14, 44, 45).

The potential for inducible regulation of the pAC27-borne pseudomonal 3-chlorocatechol-biodegradative pathway was first indicated by Dorn et al. (13) by using a highly similar (8) but not identical (53) plasmid, pWR1. When cells containing pWR1 were incubated with 3Cba, they consumed oxygen at high rates only when pregrown on 3Cba. Cells not pregrown on 3Cba were not induced by 3Cba during the 2-h time course of the experiment (13). More recently, a computer homology search (39) of the previously published *clcABD* operon revealed a partial open reading frame upstream of *clcA* that was similar to the N terminus of LysR family regulatory proteins (24). The predicted gene would be transcribed divergently from the *clcABD* operon.

In order to better understand the regulatory strategies employed by the above similar group of catechol/chlorocatechol operons, and to better understand the molecular evolution of the related regulatory protein-promoter systems, the nature of the activator protein of the *clcABD* operon was investigated. This study focuses on the cloning and sequencing of the *clcR* regulatory gene and the function of the ClcR gene product.

MATERIALS AND METHODS

Bacteria, plasmids, bacteriophages, and media. Strains, plasmids, and bacteriophages used in this study are listed in

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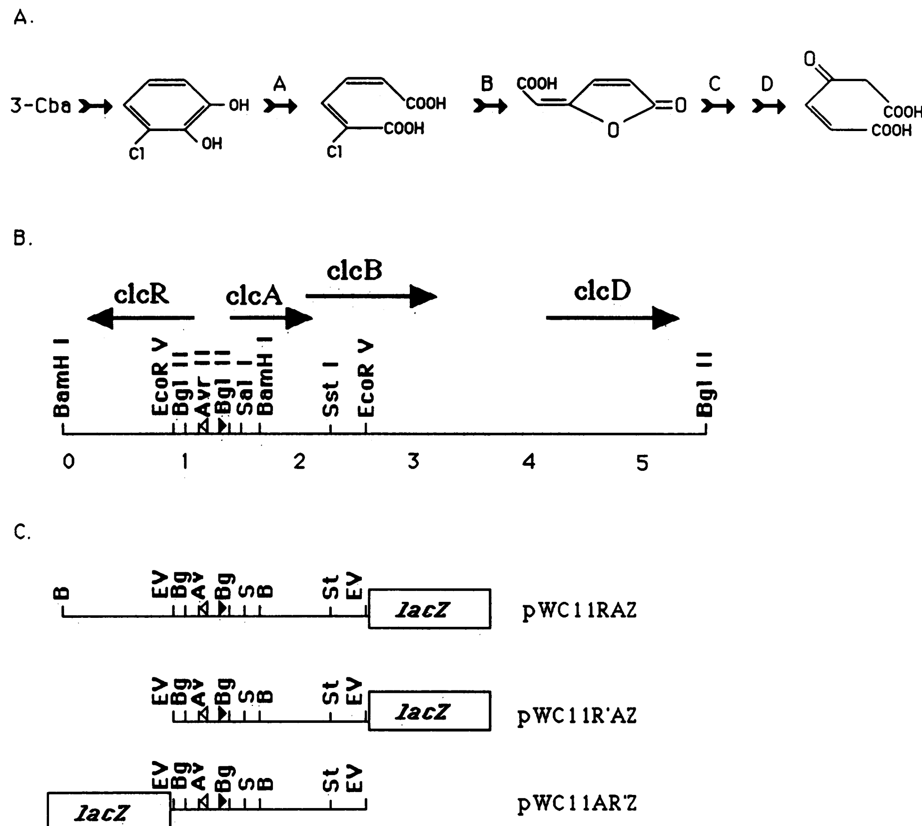

FIG. 1. (A) The chlorocatechol-biodegradative operon is central to the 3Cba pathway. Steps A, B, and D are mediated by *clcA*, *clcB*, and *clcD*, respectively. *clcA* encodes a chlorocatechol-1,2-dioxygenase, *clcB* encodes a cycloisomerase, and *clcD* encodes a hydrolase. Step C involves a muconolactone isomerization which requires no enzyme catalysis. (B) Genetic arrangement of the *clcR-clcABD* locus with relevant restriction endonuclease cleavage sites. The *clcR* and *clcA* promoters are symbolized by open and solid triangles, respectively. (C) Diagram of the inserts of the three broad-host-range promoter probe constructs used to study transcription from the *clcR* and *clcA* promoters. Abbreviated restriction endonuclease cleavage sites correspond to those in panel B.

Table 1. Bacterial cultures were grown on either Luria broth or basal salts medium (BSM) supplemented with 20 mM glucose, 5 mM benzoate, or 5 mM 3Cba as described previously (1). *Escherichia coli* and *P. putida* strains were grown at 37 and 30°C, respectively. Media and agar plates were supplemented with antibiotics for selection as follows (in milligrams per liter): for *E. coli* in Luria broth and Luria agar plates, streptomycin (100), ampicillin (50), kanamycin (50), and tetracycline (30); for *P. putida* in Luria broth and BSM, streptomycin (100), carbenicillin (1,000), kanamycin (1,000), and tetracycline (30); for *P. putida* on *Pseudomonas* isolation agar, streptomycin, carbenicillin, and kanamycin (1,000 each) and tetracycline (300).

DNA preparations and sequencing. Plasmid DNA was isolated by the alkaline lysis (31) or the Holmes and Quigley rapid boiling method (25). Single-stranded M13 sequencing templates were generated from M13mp18 and M13mp19 cloning vehicles as previously described (54). The sequence of *clcR* was determined by the dideoxy chain termination method of Sanger et al. (43).

Molecular cloning and mobilization of genetic constructs. Restriction endonucleases were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and were used according to the manufacturer's recommendations. Before ligations, vector DNA was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim GmbH, Mannheim, Germany). Ligation reactions were performed at 16°C for 2 to 16 h and then the mixtures were brought to 30 mM KCl at room temperature at least 2 h before transformation into JM109. Broad-host-range plasmids were mated into *P. putida* strains by pelleting, washing in Luria broth, and mixing 1.0 ml each of 12-h cultures of HB101/pRK2013, PRS2000, or PRS3026 and JM109 containing the plasmid to be transferred. After 2 min of microcentrifugation, the cell pellet was deposited on a Luria agar plate and incubated at 30°C for 24 h. Transconjugates were selected on *Pseudomonas* isolation agar plates with the appropriate antibiotics.

Fusion of the φ-10 Shine-Dalgarno sequence to the *clcR* open reading frame by recombination PCR. The recombination polymerase chain reaction (PCR) method of Jones and Howard (26) for site-specific mutagenesis and fragment insertion was modified in order to create an ATG fusion of the *clcR* open reading frame with the pT7-7 vector ATG as shown in Fig. 2. One primer (P1; 5′ **AATTCCAT**ATGTAT ATCTCCTTCTTA) was made to direct synthesis in the upstream direction from the ATG start of the supercoiled vector. The free 5′ end, or tail, of P1 (in boldface), however, was made complementary to the first 8 bases of the *clcR* open reading frame (without duplicating the ATG). Conversely, the second primer (P2; 5′**ATATACAT**ATGGAATT TGGGCAGCTT) was made to direct synthesis from the *clcR* ATG start codon in the downstream direction. Its 5′ tail (in

TABLE 1. Bacterial strains, plasmids, and phages

Strain, plasmid, or phage	Relevant properties	Reference or source
Strains		
E. coli		
JM109	Δ(*pro-lac*) *recA1 thi-1 supE endA gyrA96 hsdR relA1* (F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*)	54
K38/pGP1-2	Includes heat-inducible T7 polymerase gene on plasmid pGP1-2	49
P. putida		
PRS2000	Wild type; CatR$^+$ Ben$^+$	M. Wheelis
PRS3026	Mutant of PRS2000; CatR$^-$ Ben$^-$ Km^r	E. Hughes
Plasmids		
pDC15	17-kb *Bgl*II insert with *clcR-ABD* in pLAFR1; 3Cba$^+$	19
pWC15R′	pDC15 with Km^r cassette mutation in *clcR*; 3Cba$^-$	This study
pJRD215	Broad-host-range vector; Km^r Sm^r	11
pWC212	1.8-kb *Bam*HI insert with *clcR* in pJRD215	This study
pOT153	Vector; Ap^r Km^r trimethoprim resistant	I. Lamont
pWC1513a	1.6-kb *Eco*RV-*Sst*I insert with *clcR′-AB′* in pOT153	This study
pKRZ1	Broad-host-range *lacZ* promoter probe vector; Ap^r Km^r	42
pWC11AR′Z	2-kb *Eco*RV *clcB′A-R′* insert with *clcRp-lacZ* in pKRZ1	This study
pWC11R′AZ	2-kb *Eco*RV *clcR′-AB′* insert with *clcAp-lacZ* in pKRZ1	This study
pWC11RAZ	2.7-kb *Bam*HI-*Eco*RV *clcR-AB′* insert; *clcAp-lacZ* in pKRZ1	This study
pT7-5	T7 promoter (T7p) expression vector; Ap^r	48
pT7-6	pT7-5, reversed multicloning bank	48
pT7-7	pT7-5, with ϕ-10 ribosomal binding site and ATG	48
pWC52	T7p-(*clcR* reversed orientation); 1.2-kb *Avr*II-*Bam*HI insert in pT7-5 *Xba*I-*Bam*HI sites	This study
pWC62	T7p-*clcR*; 1.2-kb *Avr*II-*Bam*HI insert in pT7-6 *Xba*I-*Bam*HI sites	This study
pWC72	T7p-*clcR*; 1.2-kb *Hin*cII-*Bam*HI insert from pWC62 in pT7-7 *Nde*I (blunted)-*Bam*HI sites	This study
pWC72f	T7p-ATG fusion with *clcR* in pT7-7 (see text)	This study
pUC1021	Vector containing Km^r cassette; Km^r Ap^r	3
pWC102R	1.8-kb *Bam*HI *clcR* insert replaces the Km^r cassette in pUC1021 *Bam*HI sites	This study
pWC102R′	*Hin*cII Km^r cassette insertion mutation in *Eco*RV site of *clcR* in pWC102R	This study
pWC322R′	2.8-kb *Sal*I insert from pWC102R′ in pBR322 *Sal*I site	This study
Phages		
M13mp18	Sequencing cloning vehicle; Ap^r	54
M13mp19	As M13mp18, with cloning bank in opposite orientation	54

boldface) corresponded to the 8 bases immediately upstream of the vector ATG start (vector upstream sequence). PCR amplification was performed as recommended for site-directed mutagenesis, except the Mg^{2+} concentration was 1.1 mM and the template concentration was 10^5 supercoiled molecules per 100 μl of reaction mixture. After PCR amplification, the resultant linear molecule possessed a 16-bp direct terminal repeat including the vector upstream sequence with the ATG fused to the *clcR* open reading frame. This linear product was transformed into *E. coli* JM109. In vivo recombinants were screened on ampicillin plates, and their genotypes were confirmed. The final ATG fusion construct was named pWC72f.

In vivo labeling of the ClcR protein. T7 promoter expression and in vivo labeling studies were carried out by the method of Tabor and Richardson (49).

Preparation of ClcR protein extracts and promoter binding studies. The construct pWC212, in which *clcR* is transcribed from its own promoter, was used to show specific binding of ClcR to the *clcR-clcABD* intergenic promoter region. Plasmid pJRD215 was used as a vector control throughout. Crude extracts of the ClcR protein were produced from pWC212 in the *catR* mutant *P. putida* strain PRS3026 as follows. One milliliter of an overnight culture of PRS3026/pWC212 (or PRS3026/pJRD215) was used to inoculate 50 ml of Luria broth containing 1 g of streptomycin per liter. The cultures were grown to an optical density at 540 nm of 0.8, harvested, washed in buffer 1 (50 mM Tris-Cl [pH 8.0], 10 mM $MgCl_2$, 10% glycerol, 5 mM β-mercaptoethanol), sonicated, and centrifuged at 80,000 × *g* for 30 min at 4°C. The supernatants were then brought to 50% glycerol and frozen at −70°C. All procedures after the initial harvesting were at 4°C except where otherwise noted. The enrichment of ClcR from the crude extract proceeded as follows. Two-liter cultures of PRS3026/pWC212 (and the PRS3026/pJRD215 control) were grown, washed, and sonicated as described above. The cell extract was then centrifuged at 39,000 × *g* for 10 min, and the resultant supernatant was centrifuged at 150,000 × *g* for 1 h. The final supernatant (approximately 25 ml) was pooled and loaded onto a heparin-agarose column which had been preequilibrated with the mentioned buffer 1. The column was washed with buffer 1 until nonbound proteins were eluted as monitored at 280 nm. The remaining bound proteins were eluted from the column in 50 0.5-ml fractions by using buffer 1 supplemented with 1 M NaCl. The protein peak (in 15 0.5-ml fractions) was pooled, dialyzed against three changes of 1.5 liters of buffer 1, and brought to 50% glycerol before storage at −70°C.

Gel shift assays were carried out by the method of Fried and Crothers (16).

Promoter activity assays. Transcriptional activity from the *clcA* and *clcR* promoters in the *lacZ* transcriptional fusion vectors described below was determined by the method of Miller (34). Log-phase cells (optical density at 620 nm, 0.8) were harvested, washed with 1/3 volume of BSM, and resuspended in the same volume of BSM containing 20 mM

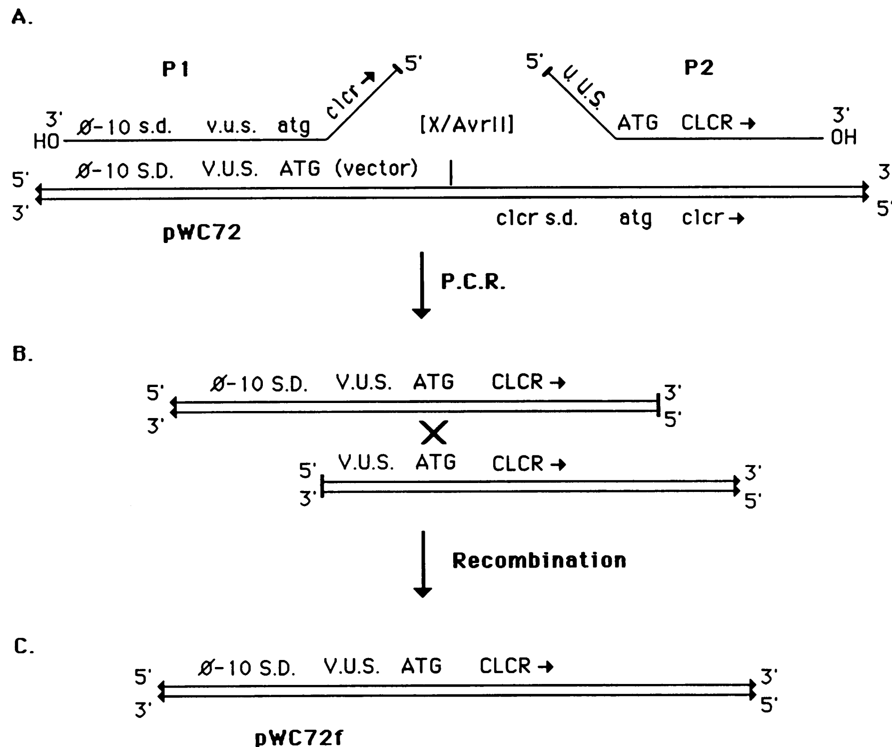

FIG. 2. Method used to delete the *clcR* Shine-Dalgarno (S.D.) sequence and fuse the vector and *clcR* ATG start codons. For clarity, elements of the sense strand are capitalized and those of the anti-sense strand are in lowercase letters. (A) Upstream of the vector ATG is vector upstream sequence (V.U.S.), 8 bases of which were incorporated into the 5′ tail of the primer (P2) used to elongate from the *clcR* ATG rightward around the supercoiled template, pWC72. Conversely, the P1 primer, used to elongate leftward, included the first 8 bases of the *clcR* reading frame in its 5′ tail. Both primers were designed to include an *Nde*I site at the ATG start codon. (B) PCR amplification results in a linear double-stranded DNA molecule, the ends of which are identical over 16 bp. The unpurified PCR mixture was transformed into *E. coli*, and recombination occurred in vivo to yield the desired ATG fusion, pWC72f (C). This strategy is a modification of the recombination PCR method developed by Jones and Howard (26) as described in the text.

glucose. This final cell suspension was supplemented with 15 mM 3Cba and/or incubated (shaken at 30°C) where noted. After sonication for one 30-s pulse at 4°C, the lysate was centrifuged for 5 min at 10,000 × *g* at room temperature. The colorimetric β-galactosidase assay was performed as recommended (34).

Protein determinations. Protein concentrations were determined by the method of Bradford (5).

Nucleotide sequence accession number. The DNA sequence reported here has been assigned the GenBank/EMBL accession number L06464.

RESULTS

Localization of a *clcABD* regulatory region by *trans* complementation for growth on 3Cba. *P. putida* PRS2000 and PRS3026 containing plasmid pDC15 are wild type for growth on 3Cba. Plasmid pDC15 was derived as a 17-kb subclone of the 3Cba-biodegradative plasmid isolate pAC27 (19) and includes the chlorocatechol-degradative genes and approximately 13 kb upstream of the *clcABD* operon. A kanamycin cassette was inserted into pDC15 via in vivo recombination in order to interrupt the region of the potential regulatory gene at the *Eco*RV site (Fig. 1B) as follows. A 1.8-kb *Bam*HI fragment (Fig. 1B) subclone of pDC15 including 0.4 kb of the 5′ end of *clcA* and 1.4 kb immediately upstream of *clcA* was cloned into the *Bam*HI sites of the nonmobilizable vector pUC1021 (3) (thus replacing the vector kanamycin gene) to yield pWC102R. The kanamycin resistance gene was then isolated from pUC1021 as a *Hin*cII fragment and was cloned into the *Eco*RV site (Fig. 1B) centered in the insert of pWC102R to yield pWC102R′. The potential regulatory gene with the kanamycin cassette insertion was excised from pWC102R′ as a 2.8-kb *Sal*I fragment and cloned into the *Sal*I site of the mobilizable vector pBR322. The resultant construct, pWC322R′, was mated into wild-type *P. putida* PRS2000/pDC15, and recombinants were screened for the acquisition of kanamycin resistance. A recombinant plasmid, which was confirmed by restriction endonuclease mapping to differ from pDC15 only in having the expected cassette insertion in the potential regulatory region, was named pWC15R′.

In contrast to pDC15, pWC15R′ did not allow growth of *P. putida* PRS2000 on BSM supplemented with 3Cba. Growth on 3Cba was restored by *trans* complementation of the mutation in pWC15R′ with the compatible plasmid pWC212, which contained the 1.8-kb *Bam*HI insert from pWC102R in vector pJRD215.

DNA sequence of *clcR*. DNA (1,372 bases) from the initiation codon of the divergently transcribed *clcA* gene to the *Bam*HI site distal to *clcR* was sequenced (Fig. 3). Open reading frames were analyzed in all six frames, and the reading frame from bases 173 to 1057 was designated *clcR*. The predicted gene displays a reasonable ribosomal binding site (AGAGGT) at −11 bases and encodes a polypeptide of 294 residues with a predicted molecular mass of 32,507 Da.

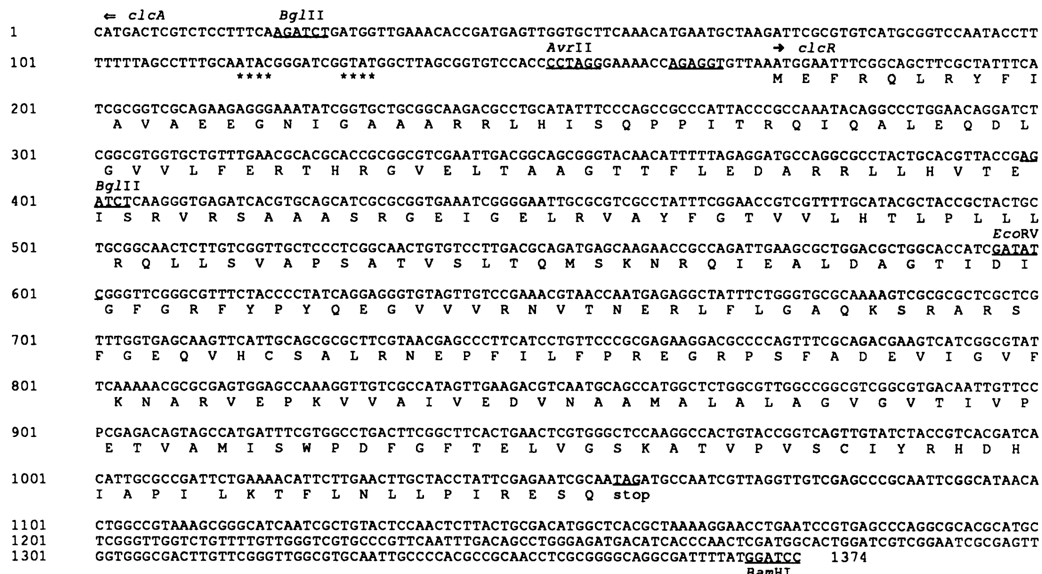

FIG. 3. Nucleotide sequence of *clcR*. The deduced amino acid sequence of the ClcR gene product is shown, with the solid arrow indicating the start of translation. The putative ribosomal binding sequence is underlined. Sequences bearing similarity to a proposed LysR family consensus binding site (20) are indicated by asterisks. Also shown are the *Avr*II, *Bam*HI, *Bgl*II, and *Eco*RV sites. The ATG translational start codon (open arrow) of the divergently transcribed *clcA* gene is located at nucleotides 1 to 3 (i.e., CAT).

The reading frame of *clcR* has an ATG start codon 171 bp upstream of the ATG start of the *clcABD* operon and is transcribed divergently from *clcABD*. The second largest reading frame in the region sequenced corresponded to a polypeptide of only 66 residues and had no clear Shine-Dalgarno consensus sequence, and no product was observed in in vivo labeling studies. Alternative ATG start codons were ruled out as follows. First, no alternative in-frame ATG start was possible upstream of the assigned translational start site since the three nucleotides immediately preceding would constitute a TAA stop codon. Second, the next downstream ATG codon corresponds to the methionine residue at position 127. There is no reasonable ribosomal binding site upstream of this ATG. If this downstream ATG were used as a start codon, it would result in the production of a polypeptide of only 29.8 kDa that would not include the conserved LysR family helix-turn-helix motif region. Further analysis shows that the *clcR* gene contains 58% G+C, which is consistent with the *P. putida* genome (30) as well as with the genes of the *clcABD* operon (15). Although not marked, the *clcR* reading frame codons do show a preference for guanine and cytosine in wobble base positions, in several cases to the exclusion or near exclusion of codons frequently used in typical *E. coli* open reading frames (22).

Positive activation of the *clcA* promoter by ClcR. Promoter probe constructs were based on the broad-host-range promoter probe vector pKRZ1 (42). Two broad-host-range *clcA* promoter probe fusions were employed to assess the effect of ClcR on *clcA* transcription. The first, pWC11R'AZ, included a truncated *clcR*, the complete *clcA* gene, and a *clcB'-lacZ* fusion so that *lacZ* transcriptional levels reflect *clcA* promoter activity (solid triangle, Fig. 1C). This plasmid was constructed by cloning the 1,957-bp *Eco*RV fragment from the 3Cba-degrading subclone pDC15 (see above) into the *Sma*I site of pKRZ1. To engineer the second construct, the *clcR* gene was reconstituted in pWC11R'AZ by cloning the correct orientation of the pDC15 1,767-bp *Bam*HI fragment into *Bam*HI-digested pWC11R'AZ to yield pWC11RAZ (Fig. 1C).

All activation studies were carried out with the *catR* mutant strain PRS3026 to avoid potential cross-activation by CatR. Figure 4 shows that *clcA* promoter activity from pWC11R'AZ (31.7 ± 0.64 U) does not respond to the presence or absence of 3Cba. The presence of *clcR* in construct pWC11RAZ, however, allowed an increase in

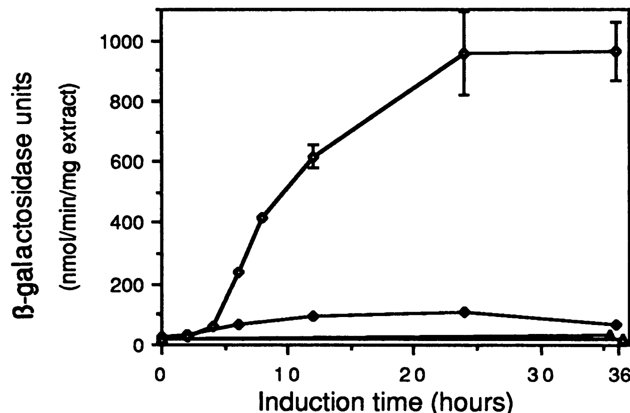

FIG. 4. The effect of 3Cba and the presence of *clcR* on transcriptional activation at the *clcA* promoter. β-Galactosidase activity directed from the *clcA* promoter in constructs that have a full-length copy of *clcR* (pWC11RAZ) and were (◇) or were not (◆) incubated with 15 mM 3Cba or in constructs that have a truncated *clcR* gene copy (pWC11R'AZ) and were (△) or were not (▲) incubated with 3Cba is shown. Induction time represents the time after resuspension of log-phase cultures in BSM-glucose or BSM-glucose plus 15 mM 3Cba. All determinations at 0 and 36 h were done at least in triplicate; others were single to triplicate determinations to indicate the kinetics of induction.

TABLE 2. *trans* autorepression of the *clcR* promoter

Time after resuspension (h)	β-Galactosidase activity[a] in PRS3026 with:		% Activity[b]
	pJRD215 + pWC11AR'Z	pWC212 + pWC11AR'Z	
0	12.8 ± 0.2	8.1 ± 0.9	63.3
36	30.9 ± 0.1	11.0 ± 0.0	35.6

[a] Each assay performed in triplicate.
[b] Values show the amount of repressed activity divided by the amount of unrepressed activity.

β-galactosidase activity from the *clcA* promoter to 66.4 U in the absence of 3Cba. When cells containing pWC11RAZ were incubated with 15 mM 3Cba, the β-galactosidase activity was induced to 960 U (Fig. 4) in 36 h. A similar, albeit lower, response was seen with pWC11R'AZ when *clcR* was provided in *trans* on the compatible plasmid pDC15 (data not shown).

Autorepression of *clcR* transcription. Potential autoregulation at the *clcR* promoter by the ClcR protein product was studied by using a *lacZ* transcriptional fusion to the truncated *clcR* gene in the broad-host-range construct pWC11AR'Z. Plasmid pWC11AR'Z contained the 1,957-bp *Eco*RV fragment in the *Sma*I site of pKRZ1 oriented so that β-galactosidase activity reflected the level of transcription from the *clcR* promoter (open triangle, Fig. 1C). This plasmid was mated into PRS3026, as was a compatible vector with or without a cloned copy of *clcR* (i.e., pWC212 or pJRD215, respectively). With ClcR provided by pWC212, and in the absence of 3Cba, activity from the *clcR* promoter was observed to be autorepressed to 63.3% of the vector control immediately after resuspension of the Luria broth-grown, washed cells in BSM-glucose (Table 2). Although for as-yet-undefined reasons, the baseline of transcriptional activity in both cases increased over the 36-h time course of the experiment, a clear trend was evident. After 36 h of incubation on BSM-glucose, the level of autorepression in the presence of ClcR tightened until 35.6% of the vector control activity remained (Table 2).

After 36 h of induction (i.e., incubation in the presence of 15 mM 3Cba), the PRS3026/pJRD215 + pWC11AR'Z vector control cells showed a decrease to 81.2% activity compared with the same cells without induction (Table 3). In contrast, the presence of ClcR provided by pWC212 allowed an increase to 173% of uninduced levels upon induction with 3Cba (Table 3). That is, under conditions which result in the activation of the *clcA* promoter, autorepression at the *clcR* promoter can be partially relieved.

In vivo labeling of ClcR. Overexpression of the *clcR* gene was accomplished by using S. Tabor's T7 promoter expression vectors pT7-5, pT7-6, and pT7-7 (48, 49). A 1,220-bp *Avr*II-*Bam*HI fragment containing the complete *clcR* open reading frame and 12 bp upstream of the putative Shine-Dalgarno sequence was cloned into the *Xba*I-*Bam*HI sites of pT7-5 and pT7-6 to yield pWC52 (*clcR* in opposite orientation) and pWC62 (Table 1). The construct pWC72 is similar to pWC62 but contained a vector ϕ-10 Shine-Dalgarno sequence 25 bp upstream of the *clcR* Shine-Dalgarno sequence. Plasmid pWC72 was constructed by removing the *clcR* insert from pWC62 as a *Hin*cII-*Bam*HI fragment and cloning it into the pT7-7 *Nde*I (blunted)-*Bam*HI sites.

TABLE 3. Relief of autorepression at the *clcR* promoter during induction of the *clcABD* operon with 3Cba

Strain PRS3026 with plasmids	β-Galactosidase activity[a] 36 h after resuspension		% Activity[b]
	Without 3Cba	With 3Cba	
pJRD215 + pWC11AR'Z	30.9 ± 0.1	25.1 ± 1.1	81.2
pWC212 + pWC11AR'Z	11.0 ± 0.0	19.0 ± 2.0	173

[a] Each assay performed in triplicate.
[b] Values shown are for induced versus uninduced activity.

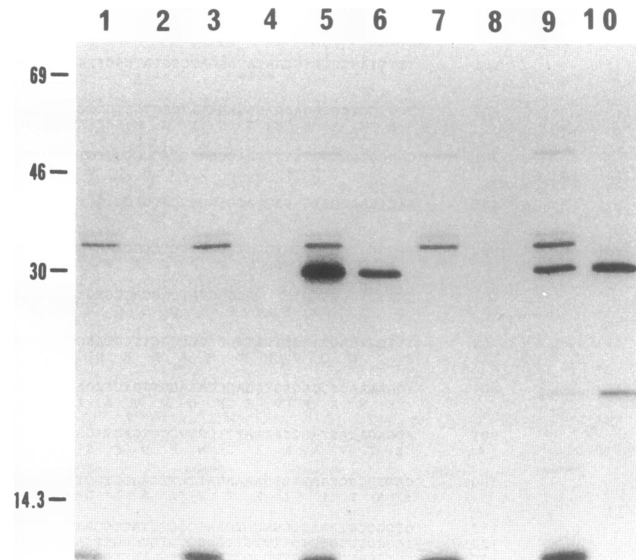

FIG. 5. In vivo labeling of ClcR from the T7 promoter. Odd- and even-numbered lanes contain uninduced and induced extracts, respectively. Lanes: 1 and 2, pT7-7 vector control; 3 and 4, pWC72 (*clcR* with its own ribosomal binding site in pT7-7); 5 and 6, pWC72f (*clcR* ATG fusion in pT7-7); 7 and 8, pWC52 (*clcR* in opposite orientation in pT7-5); 9 and 10, pKRT6-2 (CatR expressed from pT7-6 [41]) for comparison.

While no expression was noted with *clcR* cloned in the opposite orientation to the T7 promoter in pWC52 (Fig. 5, lanes 7 and 8), at best inconsistent and low expression was observed when *clcR* production was directed from the T7 promoter and its own ribosomal binding site in pWC62 (data not shown). In Fig. 3, a center of dyad symmetry (see Discussion) is evident coincident with the *clcR* ribosomal binding site. To avoid possible interference by the inverted repeat and to maximize translation, pWC72 was used as a PCR template to create the ATG fusion pWC72f as diagrammed in Fig. 2. As expected from the spacing of the vector ribosomal binding site to the start of *clcR*, no observable expression from pWC72 was noted (Fig. 5, lanes 3 and 4; compare pT7-7 controls, lanes 1 and 2). Construct pWC72f, however, showed high levels of expression, as seen in Fig. 5, lanes 5 and 6. The presence of labeled host proteins in the uninduced lanes that are not observed upon induction is most likely due to decreased permeability of the expressing cells to rifampin at the lower control temperature. Finally, the technique used is at best semiquantitative; minor lane-to-lane variations in band intensity cannot be meaningfully interpreted.

Specific binding of ClcR to the *clcA*-*clcR* promoter region. The DNA binding properties of the ClcR protein were

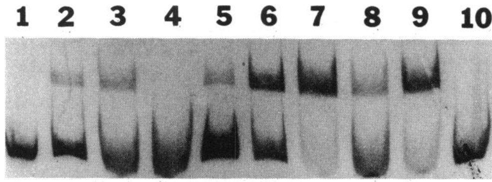

FIG. 6. Electrophoretic mobility shift assays of the 386-bp *clcA-clcR* promoter region with cell extracts containing ClcR. Lanes: 1, no extract; 2 and 3, crude ClcR extract at 2.0 and 9.5 μg, respectively; 4, pJRD215 vector control crude extract at 9.5 μg; 5 to 7, heparin-agarose-enriched extracts at 0.25, 2.0, and 10 μg, respectively; 8, same as lane 7 but with unlabeled competitor DNA at 0.5 μg in vector pOT153 (i.e., pWC1513a); 9, same as lane 7 but with unlabeled noncompeting DNA at 0.5 μg (pOT153); 10, heparin-agarose-enriched pJRD215 control extract at 10 μg.

determined in a gel shift assay. The 386-bp *Bgl*II intergenic promoter fragment (Fig. 1B and 3), which included the divergent *clcR-clcA* promoter region, was used as the retardation probe. The fragment was [α-^{32}P]dCTP labeled by filling the 3′ recessed ends with the Klenow fragment and was gel purified by polyacrylamide gel electrophoresis (PAGE). The optimized binding reaction mixture contained 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) (pH 7.5), 3% glycerol, 0.1 mM EDTA, 0.25 mM DL-dithiothreitol, 2.0 mM $MgCl_2$, 1.5 μg of poly(dI-dC); 0.1 μg of bovine serum albumin per μl, 8 mM spermidine, 50 mM KCl, 50 mM NaCl, 0.25 to 10.0 μg of ClcR-containing protein extract, and approximately 2,300 cpm of labeled probe. Competitor (pWC1513a) or noncompetitor (pOT153) DNA was added at 0.5 μg where noted. All binding reactions were carried out in a total volume of 35 μl at 22°C for 15 min. As shown in Fig. 6, the binding of crude extracts containing ClcR expressed from its own promoter (from PRS3026/pWC212) resulted in the retardation of a limited amount of the probe (lanes 2 and 3). Under equivalent conditions, the crude extract from vector control cultures (PRS3026/pJRD215) showed no observable binding to the probe (lane 4). However, when increasing amounts of heparin-agarose-enriched extracts (see Materials and Methods) were employed, near complete retardation of the probe was observed (lanes 5 to 7). Similarly treated vector control extracts again showed no retardation (lane 10). Further, the addition of unlabeled DNA (pWC1513a) containing a copy of the promoter region showed significant competition for ClcR away from the labeled probe (lane 8), while the same amount of the vector (pOT153) DNA did not decrease the binding of ClcR (lane 9). DNA competition control experiments were also performed with crude extracts, with analogous results.

Homologies with other members of the LysR family of bacterial regulators. The TFASTA or FASTA protein sequence comparison program (D. Lipman and W. R. Pearson, National Institutes of Health, Bethesda, Md.) was used to scan the computer-translated GenBank (version 68) sequence library (Intelligenetics, Inc., Mountain View, Calif.) in all six frames as well as the Swis-Prot (release 18), EMBL (release 27), and PIR (release 29) protein sequence libraries (located at, respectively, the Departement de Biochemie Medical, Centre Medical Universitaire, Geneva, Switzerland; the European Molecular Biology Laboratory, Heidelberg, Germany; and the Protein Identification Resource, National Biomedical Research Foundation, Washington, D.C.). A sequence identity comparison with the four best matches found is depicted in Fig. 7 and includes TcbR (51) (57% identity and no gaps over 294 residues), TfdS (28) (62% identity and no gaps over the available 177 residues of this as-yet-incomplete sequence), CatR (41) (32.5% identity and five gaps over 289 residues), and CatM (35) (31.4% identity and three gaps over 262 residues), all of which are members of the LysR family of bacterial regulator proteins (24). Also included in Fig. 7 is a consensus sequence composed of nine proteins from the LysR regulatory family for comparison (24).

DISCUSSION

Confirmation of the *clcR* open reading frame. In addition to the arguments made by analysis of the sequence data, the assertion that the proposed open reading frame encodes the regulatory protein ClcR was confirmed by the 3Cba$^-$ phenotype of the *clcR* insertion mutant pWC15R′ and by genetic deletion of the 3′ end of the reading frame, which resulted in the abolition of activation of the *clcA* promoter (Fig. 4). Also, only extracts from cells containing a plasmid carrying the *clcR* open reading frame were observed to retard the *clcR-clcA* promoter region (Fig. 6). Finally, the polypeptide size predicted from this open reading frame was confirmed by in vivo labeling experiments. ClcR, with a sequence-predicted molecular mass of 32.5 kDa, migrated with an M_r of 30×10^3 (Fig. 5). For comparison, CatR (41) and TcbR (51) have sequence-calculated molecular masses of 32.2 and 32.0 kDa, respectively, and they too migrated with an M_r of 30×10^3.

ClcR is a member of a subgroup of the LysR family. The ClcR polypeptide shows high homology to members of the LysR family of bacterial regulatory proteins (24). The members of the LysR family typically exhibit several properties in common. These properties include a well-conserved N terminus with a helix-turn-helix motif, selected conserved residues throughout the protein, and a size generally ranging, with few exceptions (e.g., CatM has 251 residues [35]), from 285 to 315 residues (24). The majority of LysR family member genes are transcribed divergently from a gene or operon they control. ClcR possesses all of the above attributes and is more than 20% identical to more than 10 members of the LysR family. Within the LysR family, however, ClcR shows highest homology to a select group of regulators of biodegradative operons. This group includes the transcriptional activators TfdS (62% identity, 70% similarity over the 177 residues published), CatR (32.5% identity, 43% similarity over 289 residues), and TcbR (57% sequence identity, 68% similarity over 294 residues) and the repressor CatM (34.6% identity, 46% similarity over 251 residues). Percent sequence similarity above was defined conservatively as including identities and L = I = V = M, D = E, K = R, and S = T. In manually aligning the amino acid sequence of ClcR with those of CatM and CatR, three and five gaps were introduced, respectively, while no gaps are required for the alignment of ClcR with TcbR or with the partial TfdS sequence (Fig. 7). Kyte and Doolittle hydrophobicity profile calculations further indicate that ClcR displays a higher structural similarity to the latter two regulators. By comparison, ClcR shares 21% sequence identity with the original published nine-member LysR family consensus sequence, which includes the partial TfdS sequence (called TfdO) but which excludes ClcR, CatR, CatM, and TcbR (24). The *Bacillus subtilis* glutamate biosynthesis activator, GltC (4), with 27.2% amino acid sequence identity, was the only non-*cat* subgroup LysR protein to exceed 25% identity with ClcR. The hydrophobicity plots of the above consensus

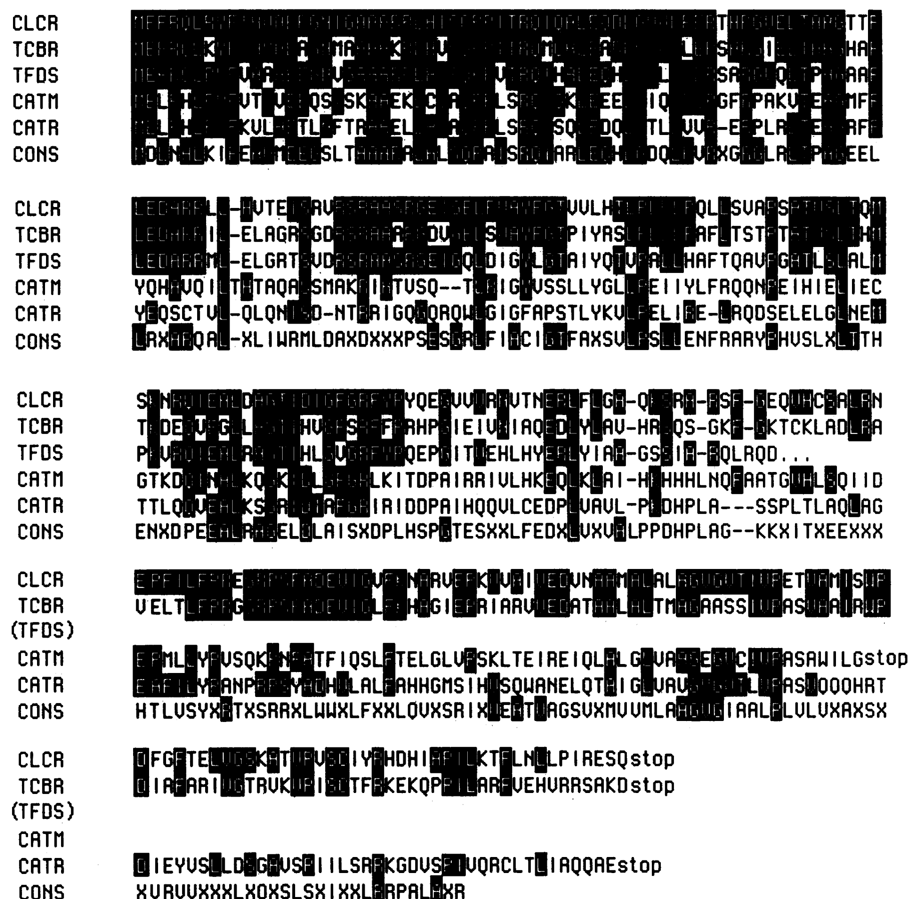

FIG. 7. Identities between the deduced amino acid sequence of ClcR and those of four members of the LysR family as well as a consensus sequence generated from nine members of the LysR family (24). Black boxes with white letters indicate residues common to ClcR and at least one other protein at a given position. Dashes indicate the introduction of gaps in the alignments.

sequence, the LysR protein, and GltC also differed significantly from that of ClcR.

For each member of the subgroup, the respective pathways encode homologous functions for the degradation of catechol and/or chlorocatechols. In each pathway, analogous reactions are catalyzed by enzymes with high sequence identity. Exceptions include the hydrolases, which are similar in the pathways for chlorocatechol assimilation but differ from those in the catechol-degradative pathways. Also, the *catC* gene in the *cat* pathway has no analog in the chlorocatechol pathways in which the analogous reactions are rapid enough without enzyme catalysis to allow growth. Although *clcR*, *tcbR*, *catR*, and *catM* are immediately upstream of and are divergently transcribed from the genes they control, there exists some controversy as to the regulation of the *tfd* pathway. The known regulatory genes of the *tfd* pathway, *tfdS* and *tfdR*, are located upstream of the genes *tfdA* and a second, apparently nonoperonic copy of *tfdC* (*tfdC1*), respectively (28). There is evidence that TfdR and TfdS are encoded by identical open reading frames and are the result of a gene duplication (55). However, other reports suggest differing repressing and/or activating functions and differing sites of actions for TfdR and TfdS (23, 27, 28). Potentially adding to the complexity of *tfd* regulation, Perkins et al. (39) observed the presence of an open reading frame transcribed divergently from the *tfdCDEF* operon with a spacing of 194 bp between their respective start codons. The 44 residues predicted in this incompletely sequenced *tfdCDEF* upstream region show 72.7% sequence identity to the first 44 residues of ClcR. It is not known whether this potential *tfdT* gene encodes a full-length protein or is expressed. No known function has been assigned to this region. Further observations (40) indicate that the *tfdCDEF* promoter region bears strong similarity to the known binding site for CatR at the *catBC* promoter and to the promoter region of *clcABD* (39), suggesting that a LysR family member, possibly TfdS, does regulate *tfdCDEF*.

The *clc* regulator/operon shares highest overall similarity to the *tcb* regulator/operon. The evolutionary advantages of divergently transcribed regulatory-structural gene units ("divergons") has been described previously (2). It may prove fruitful to consider the evolutionary relatedness of those LysR family regulatory genes, which are organized in divergons, together with the relatedness of their corresponding structural genes. In assessing the systems most similar to the *clc* system, it is noted that the lack of a published C-terminal sequence for TfdS spuriously skews the mentioned sequence identity between TfdS and ClcR toward their more conserved N termini. When TcbR and ClcR are also compared over their first 177 residues, the sequence identity observed (65.3%) is slightly higher than that found between ClcR and the 177 residues available in the TfdS partial sequence (62%). However, when the operonic arrangements are examined, one finds that *clcR* and *tcbR*, like *catR* and *catM* but

unlike *tfdS*, are transcribed divergently from the operons they regulate. As noted above, the presence of a putative *tfdT* gene has the potential to offer a LysR family member divergently transcribed from the *tfdCDEF* operon. Turning now to the operonic genes, the first two structural genes in the *clc* pathway, *clcA* and *clcB*, are more identical to *tcbC* and *tcbD*, respectively (50), than are any of these to the nevertheless highly similar *tfdC* and *tfdD* (17, 18, 40). Additionally, the *clc* and *tcb* pathways but not the *tfd* pathway possess highly similar open reading frames, designated ORF3, the function of which has not been assigned (50). Finally, although no functional analog of the *tcbF* or *tfdF* gene is apparently necessary in the *clc* system, the putative vestiges of such a gene have been noted downstream of *clcD* (40). Thus, the higher sequence identities of both the corresponding regulatory and operonic genes as well as the similar regions of unknown function indicate that the *clc* and *tcb* systems are the most similar within the subgroup of pathways analyzed here.

ClcR binds the *clcR-clcABD* promoter region. Figure 6 shows the specific binding of ClcR-containing extracts to the *clcR-clcABD* intergenic control region. This binding is consonant with the occurrence of a potential LysR family-type binding site in the *clcA* upstream region. The LysR family-type binding consensus as proposed by Goethals et al. includes a T separated from a downstream A by 11 bases and at the core of an inverted repeat (20). The *clcA* upstream region has the motif ATAC-7 bp-GTAT, which fits both the above criteria (Fig. 3). This site will be examined as a potential ClcR binding site in future studies.

Hyperproduction of ClcR. Efforts to hyperexpress *clcR* from the strong T7 promoter in pT7-6 or from the strong *lac* promoter on pUC119 were inconsistent at best when translational signals were provided by the *clcR* transcript (data not shown). Upon inspection of the *clcR* upstream region, a potential hairpin structure was noted coincident with an otherwise plausible Shine-Dalgarno sequence (AGAGGU). This center of dyad symmetry (AAAACCAGAGGTGTT) includes a stem of six nucleotides with five potentially pairing bases and a three-nucleotide loop. The ability of such structures to affect translation is well documented (21). While the role of the potential secondary structure in the *clcR* ribosomal binding site was not determined, difficulty in elongating a mutagenesis primer from single-stranded DNA at this region was encountered.

Since mutagenesis to disrupt the potential hairpin proved difficult, an alternative approach was taken in order to hyperproduce ClcR. The modified recombination PCR technique (Fig. 2) employed proved quite efficient. Of the 10 independent in vivo recombinants screened, all had generated the expected *Nde*I restriction site within the region of recombination and all expressed a polypeptide of the expected size. The resultant ATG fusion was also cloned from pWC72f along with the vector ϕ-10 Shine-Dalgarno sequence into expression vector pUC119, and a ClcR band was observed in the crude lysate when visualized on Coomassie-stained PAGE gels (data not shown).

Induction kinetics and autoregulation. Figure 4 shows a 36-h induction time course for full activity at the *clcABD* promoter. It is likely that two factors contribute to the long time course of this induction. First, by analogy with the *cat* system (33), the probable effector molecule for ClcR is the pathway intermediate 2-chloro-*cis*,*cis*-muconate. Thus, the product of the *clcA* gene would be required for production of the effector molecule and activation at the *clcA* promoter would be dependent on the low-level read-through at the *clcABD* promoter. Second, the *clcA* gene product, catechol oxygenase II, was reported to have a K_{cat} of 5.5 min^{-1}, 10-fold less than that of the analogous *catA* gene product (37). The low turnover rate would render the read-through necessary for effector molecule production that much less effective.

Transcriptional autoregulation among LysR family members is widespread (4, 10, 29, 32, 46, 47). That ClcR is no exception is thus not surprising. Although ClcR is the only member of the catechol subgroup for which autorepression has been shown by using promoter probe constructs, there is evidence that *catR* autorepresses (41), and this is likely the case with the others. More interesting is the apparent relief of autoregulation at the *clcR* promoter under conditions that induce transcription from the *clcA* promoter. Preliminary results have indicated the potential for similar regulation of the *catR* promoter (41). Although the possibilities exist that the DNA-dependent RNA polymerase interacts with ClcR and that binding of an effector molecule actively induces transcription at the *clcR* promoter, two observations argue for a simpler explanation. First, the ability of RNA polymerase to initiate transcription at the *clcR* promoter in the absence of ClcR was demonstrated in this study. Second, analyses of the binding of LysR family members (6, 7, 38) indicate a change in conformation upon binding of an effector molecule to the regulatory protein during activation of an operon. This conformational change shifts the regulatory multimer to also bind a region of the operator which lies closer to the operonic genes being activated, presumably involving or resulting in direct activator-RNA polymerase interactions which yield an open complex. If such a mechanism is used by ClcR, the conformational change and/or open complex may simply result in higher availability of the *clcR* promoter to RNA polymerase independent of the specific activator-RNA polymerase interactions thought to take place in operonic activation.

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