Purification of the LysR Family Regulator, ClcR, and Its Interaction with the *Pseudomonas putida* *clcABD* Chlorocatechol Operon Promoter

WAYNE M. COCO,† MATTHEW R. PARSEK, AND A. M. CHAKRABARTY*

Department of Microbiology and Immunology, University of Illinois
College of Medicine, Chicago, Illinois 60612

Received 19 May 1994/Accepted 27 June 1994

Previous studies have shown that the *clcABD* operon is under the transcriptional control of the LysR-type activator ClcR. In this study, the conditions leading to its aggregation were avoided and ClcR was purified and confirmed by amino-terminal sequencing. Gel filtration indicated that ClcR exists as a dimer in solution. The DNase I footprint of ClcR was determined. The binding properties of ClcR and the catechol operon regulator, CatR, were compared.

Similarities among the genes and regulatory proteins of several *ortho* cleavage pathway operons are thought to point to a shared ancestry (3, 16). Consistent with this pattern, the expression of the biodegradation pathway for 3-chlorocatechol in *Pseudomonas putida*, which is encoded by the *clcABD* operon, has been shown to require the divergently transcribed *lysR*-type regulatory gene *clcR* for activation (4). Cloning and sequencing of the *clcR* gene were reported in a previous study, along with data demonstrating that ClcR inducibly activates the *clcABD* operon and represses its own transcription (4). The extent of cross-binding and cross talk among LysR family members is as yet unclear. As a prelude to addressing this

issue, the purification of ClcR and initial characterization of the *clcABD* promoter were undertaken.

Overproduction of soluble ClcR. The *clcR* gene was expressed from the T7 promoter in construct pWC72m (Table 1), in which a vector ATG start codon was fused with the coding sequence of *clcR*. Production of ClcR from thermoinducible T7 polymerase-producing *Escherichia coli* K38/pGP1-2, with the parameters recommended for the expression system (17, 18), resulted in extensive inclusion body sequestration of ClcR. Efforts to resolubilize active ClcR were not successful. Lowering the temperature during expression did, however, result in the production of active soluble ClcR. High levels of active soluble ClcR were obtained when the duration of heat induction was restricted to 10 min at 42°C and subsequent expression was for 8 h at 30°C. The temperature dependence of

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
Strains		
<i>E. coli</i> JM 109	$\Delta(pro-lac) recA1 thi-1 supE endA$ <i>gyrA96 hsdR relA1</i> (F' <i>traD36</i> <i>proAB lacR^r lacZΔM15</i>)	20
<i>E. coli</i> K38/pGP 1-2	Heat-inducible T7 polymerase gene on plasmid pGP1-2	17
<i>P. putida</i> PRS3026	Mutant of PRS2000; CatR ⁻ Ben ⁻ Km ^r	E. Hughes
Plasmids		
pKRZ1	Broad-host-range <i>lacZ</i> promoter probe vector; Ap ^r Km ^r	13
pWC11RAZ	2.7-kb <i>Bam</i> HI- <i>Eco</i> RV <i>clcR-AB</i> insert; <i>clcAp-lacZ</i> in pKRZ-1	4
pWC102R	1.8-kb <i>Bam</i> HI <i>clcR</i> insert re- places the Km ^r cassette in vec- tor pUC1021	4
pT7-7	T7 promoter expression vector; Ap ^r	18
pWC72m	T7 promoter- <i>clcR</i> ; 1,206-bp <i>Mse</i> I- <i>Bam</i> HI insert from pWC102R in pT7-7 <i>Xba</i> I- <i>Bam</i> HI sites	This study

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of Illinois College of Medicine, 835 South Wolcott Ave., Chicago, IL 60612. Phone: (312) 996-4586. Fax: (312) 996-6415.

† Present address: Department of Biology, Yale University, New Haven, CT 06520-8103.

FIG. 1. SDS-PAGE analysis of the purification of ClcR. Lanes: 1, protein size markers; 2, clarified lysate; 3, 55% ammonium sulfate-cut; 4, heparin-agarose-purified ClcR. Total protein (30 µg) was loaded onto each lane. Bands were visualized with Coomassie brilliant blue G-250 (Bio-Rad Laboratories, Hercules, Calif.). MW, molecular weight.

FIG. 2. Start site mapping of the *clcABD* transcript. The autoradiogram shows the four DNA sequencing reactions (lanes G, A, T, and C) and the first-strand DNA synthesis reaction (lane SA). Both were generated with bottom-strand sequencing primer A (see text).

inclusion body formation in vivo in other systems has also been reported (reviewed in reference 14).

Purification of ClcR. Cultures induced as described above were ruptured in a French pressure cell. Crude lysate was centrifuged at $16,000 \times g$ for 30 min. Resulting supernatant fluid was then subjected to centrifugation at $150,000 \times g$ for 1 h to yield clarified lysate.

A fraction of clarified lysate that was precipitated by ammonium sulfate at 25% of saturation was discarded. Attempts to purify ClcR from a redissolved 55% ammonium sulfate precipitate by fast protein liquid chromatography (FPLC) with conventional NaCl gradients resulted in the rapid precipitation and inactivation of ClcR. Precipitation was avoided as follows.

The pellet produced by 55% ammonium sulfate was resolubilized and loaded onto a heparin-agarose (Sigma, St. Louis, Mo.) column. The column-bound sample was washed with buffer (50 mM Tris-Cl [pH 7.5], 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mM EDTA) and then eluted as two distinct peaks in the same buffer with 276 mM ammonium sulfate. The DNA-binding activities and purities of the resulting fractions were analyzed by gel retardation assays and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein fractions within the second elution peak were found to contain pure ClcR and were pooled. Attempts to concentrate purified ClcR preparations by ultrafiltration again led to rapid aggregation. Ammonium sulfate (60%) was therefore used to precipitate the protein before its resuspension at 2 mg/ml in 50% glycerol and storage at $-70^\circ C$.

The protein-stabilizing properties of ammonium sulfate are well known (5) and provided here a convenient and efficient means of maintaining the activity of ClcR in vitro. The total yield of purified ClcR from 12.8 g of clarified lysate protein was 25.7 mg or 0.2%. ClcR in the final preparation was estimated to be greater than 95% pure by SDS-PAGE (Fig. 1). A 2×10^2 -fold increase was observed between the activity of pure ClcR and that of the clarified lysate as estimated by gel shift assay.

Amino-terminal protein sequence analysis was performed by K.-L. Ngai at the University of Illinois at Urbana-Champaign Genetic Engineering Facility. The 11 amino-terminal residues sequenced were identical to the corresponding residues predicted by DNA sequencing (4).

Gel filtration chromatography. ClcR protein was subjected to gel filtration chromatography (FPLC) with a Superose 12 column (Pharmacia) and assessed by gel retardation (4). The elution of active fractions of ClcR corresponded to a molecular mass of 74 ± 12 kDa, corresponding to 1.9 to 2.6 times the molecular mass of the 32.5-kDa monomer. The observation that ClcR exists as a dimer in solution agrees with the findings for most other LysR-type regulators whose quaternary structures have been examined (reviewed in reference 15).

The *clcABD* transcriptional start site. Assignment of the transcriptional start site of *clcABD* was attempted by primer elongation (primer A, 5'-CGCGCCTGCGACCTCGGC). In-

duced cultures of *P. putida* PRS3026/pWC11RAZ, a *catR* mutant harboring *clcR* and *clcA*, actively transcribe the *clcABD* operon (4) and therefore were used for the isolation of total RNA. Results indicate multiple polymerase terminations (Fig. 2). The distances observed between regulator binding motifs and transcriptional start sites are fairly well conserved among operons controlled by LysR-type transcriptional activators (6). Consistent with such observations, the assignment of the largest product as the most likely start site for the *clcABD* transcript results in the expected distance to the ClcR binding motif (see below). This transcriptional start site is the A residue indicated on the top strand of the sequence in Fig. 4A. An in vitro transcription assay under development should prove useful in reconfirming the assigned site.

A 36-bp stem-loop structure is predicted for the 5' terminus of the *clcABD* transcript. Computer modeling of the *clcABD*

FIG. 3. DNase I footprint of ClcR on the *clcABD* promoter and DNase I protection of the *clcABD* antisense strand. Lane 1, the G+A sequence of the probe. Lanes 2 to 4, DNase I cleavage patterns of the probe unbound, bound by 5.5 ng of ClcR, and bound by 55 ng of ClcR, respectively. Each lane contains approximately 50 ng of promoter DNA. Regions protected from and hypersensitive to digestion are indicated with brackets and arrowheads, respectively.

FIG. 4. Summary of features for the *clcR-ABD* promoter region. (A) Top sequence, *catR-BC* promoter region with the RBS and ABS aligned under their respective boxes (11, 12). Bottom sequence, section of the *clcR-ABD* promoter region with sequences showing homology to the *catR-BC* RBS and ABS promoter regions (white lettering on black background). The DNase I footprints of ClcR on the sense and antisense strands are delimited by brackets above and below the sequence, respectively. DNase I hypersensitive cleavage sites in the footprint are indicated by vertical arrows. The -10 consensus promoter hexamer and predicted RNA polymerase binding site (RNAP) are also indicated. The RBS T and A nucleotides in the proposed LysR family consensus binding motif (T-N₁₁-A) are highlighted. (B) Putative LysR-type transcriptional activator binding consensus including surrounding core inverted repeats for *clcABD* and several promoters of *cat*-type *ortho* cleavage operons. Note the exceptional G-N₁₁-A motif in the *catBC* promoter.

untranslated leader region with the Mfold RNA folding algorithm (7, 8, 21) predicts a free energy for the stem-loop of at least -12.3 kcal/mol (1 cal = 4.184 J). Such stable structures occurring at the 5' termini of bacterial transcripts have been shown to confer resistance to RNase E cleavage and thus to extend transcript half-life (1, 2). A less extensive stem-loop structure is also predicted for the beginning of the *tcnR* transcript, and start site mapping of this transcript also resulted in multiple terminations (19). Assessment of a functional role for these structures must await future mutagenesis and mRNA half-life studies.

DNase I footprint of ClcR on the *clcABD* promoter. The promoter-binding properties of the ClcR protein were previously determined by gel shift assay (4). A more precise location of ClcR binding was determined by DNase I footprinting analysis (Fig. 3). DNase I footprinting reactions and the generation of a G+A sizing ladder were performed as previously described (10). The labeled, PCR-generated target probe (378 bp) for each strand included the entire intergenic region from *clcR* to *clcABD* (*clcR-ABD*) and part of the *clcR* and *clcA* reading frames (generated with primers B, 5'-GAGATC CTGTTCCAGGGCC, and C, 5'-AACCCTGACGCGCTTG TCC). The areas of protection and hypersensitivity are summarized graphically by the horizontal brackets and vertical arrows, respectively, in Fig. 4.

Features of the *clcABD* promoter. Included in Fig. 4 are sites on the *clcABD* promoter showing sequence identity to known sites on the *catBC* promoter (11). ClcR and CatR are members of the same subgroup of the LysR family of transcriptional activators (4). The well-characterized sequences of the CatR binding site on the *catBC* promoter offer insights into the potential roles of similar sites within the *clcABD* promoter.

Thus, in Fig. 4, the DNase I-protected regions of the *catBC* promoter have been functionally divided into the repression binding site (RBS) and activation binding site (ABS). In the *catR-BC* promoter region, the RBS and ABS are implicated in the strong binding and autorepression of CatR on the *catR* promoter (13) and in the activation of the *catBC* promoter (11), respectively. In the *clcR-ABD* promoter region, the DNase I-protected sequences can be divided into two regions with sequence identities at 12 of 26 and 13 of 27 positions compared with the *catR-BC* RBS and ABS, respectively. There is, however, a major difference in the binding of CatR and ClcR to their respective promoters. The binding of CatR to the region of the *catBC* promoter designated ABS occurs weakly and only in the presence of its effector molecule, *cis,cis*-muconate. In contrast, ClcR binds to the region of its target corresponding to both the RBS and ABS avidly and in the absence of any added effector. The reasons for this difference remain unclear.

LysR family regulators share similar binding sites that include a T-N₁₁-A nucleotide motif contained within an inverted repeat element. Like the other related promoters in Fig. 4B, the *clcABD* promoter has the proposed consensus site within the region corresponding to the *catR-BC* RBS (Fig. 4). (As previously observed [12], the CatR binding target differs from that of other LysR-type regulators in having a G-N₁₁-A within the expected inverted repeat element [Fig. 4B].)

Finally, it should be mentioned that there is a second section of the *clcR-ABD* promoter region with sequence identity to RBS-type elements. This second element is located between the mentioned *clcR-ABD* RBS region and the ATG start site of the *clcR* gene. Overall, this possible vestige RBS is as similar to the *catR-BC* RBS as the latter is to the genuine *clcR-ABD*

RBS. However, the pseudo-RBS site has no T-N₁₁-A motif in its nearly intact inverted repeat element and has a spacing of 9 bases rather than the 11 bases expected for functional LysR-type binding motifs. Analysis of the *Acinetobacter calcoaceticus catBCEFD* promoter region (9) also reveals a pseudo-RBS in a similar location. No binding of ClcR to its pseudo-RBS site was observed in DNase I footprinting studies, and no function has yet been attributed to either site.

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