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The *Escherichia coli* K-12 *cyn* Operon Is Positively Regulated by a Member of the *lysR* Family

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A regulatory gene, *cynR*, was found to be located next to the *cyn* operon but transcribed in the opposite direction. *cynR* encodes a positive regulatory protein that controls the *cyn* operon as well as its own synthesis. Positive regulation of the *cyn* operon requires cyanate and the *cynR* protein, but the negative autoregulation of the *cynR* gene appears to be independent of cyanate. The predicted amino acid sequence of the *cynR* protein derived from the DNA sequence was found to have significant homology to the predicted amino acid sequence of the *lysR* family of regulatory proteins.

The *cyn* operon includes three structural genes that are transcribed in the following order: *cynT*, encoding carbonic anhydrase (10); *cynS*, encoding cyanase; and *cynX*, encoding a protein of unknown function (28). The *cyn* operon is physically next to the *lac* operon but is transcribed in the opposite direction. The *cyn* operon produces two transcripts. A 1,500-nucleotide transcript terminates within the coding region of *cynX*, while a 2,500-nucleotide transcript terminates after the *cynX* gene. The 5′ ends of both the *cyn* mRNAs are identical. The longer message appears to overlap the *lac* mRNA by approximately 98 nucleotides at the 3′ end (28). The *cyn* operon is induced by cyanate and appears to be involved in detoxifying exogenous cyanate in *Escherichia coli* K-12 (9, 29, 30). Cyanate occurs in nature as a spontaneous dissociation of urea in aqueous solution (11) and by decomposition of carbamyl phosphate. Cyanate can react with a number of functional groups in proteins. The carbamoylation of amino groups by cyanate is irreversible, while reactions of cyanate with thiols, imidazoles, phenolic groups, and carboxylates are reversible (25). The reaction catalyzed by cyanase requires bicarbonate as well as cyanate and produces two molecules of carbon dioxide. Carbonic anhydrase is required to regenerate bicarbonate from the carbon dioxide that is a product of the reaction catalyzed by cyanase (10).

To investigate the regulation of the *cyn* operon, various fragments of DNA upstream of the *cyn* structural genes were fused to the promoterless *galK* gene (19) to generate a series of *cyn-galK* transcriptional fusions. These constructs were assayed for promoter strength and response to cyanate induction by measuring galactokinase activity in a *galK* mutant strain deleted for the chromosomal *cyn* operon (28). This strain, with plasmids containing an insert with the *cyn* promoter and 1.12 kb or more of upstream sequence, expressed *galK* activity when cyanate was present. The strain with plasmids containing a DNA fragment of 0.85 kb or shorter did not exhibit inducible *galK* expression. No significant expression was detected in any cell containing a fusion unless the cells were induced by cyanate. The 1.12-kb DNA fragment was found to contain an open reading frame (ORF) by DNA sequencing, and this ORF was found to function in *trans* in regulating the *cyn* operon. The ORF was found to have significant homology to the members of the *lysR* family of positive regulatory proteins (12). The *lysR* protein regulates the expression of the *lysA* gene by positive regulation and regulates its own synthesis by negative autoregulation. The *lysR* regulatory protein is part of a family of positive regulatory proteins that have significant protein homology as well as functional similarity.

MATERIALS AND METHODS

Bacterial strains and growth medium. SJ99 is a derivative of N99 (19), which is an *E. coli* K-12 F⁻ *galK pro thi* strain. N99 was made Δ(*pro-lac-mhp*) by mating with S10 [HfrH Δ(*pro-lac-mhp*) Δ(*pgl-bio*) *galE thi*], selecting recombinants that were fluorocytosine resistant, and checking for *lac* phenotype. The Δ(*lac-pro*) deletion removes *cod* and thus makes cells resistant to fluorocytosine. This derivative was transduced to *recA56 srlC300*::Tn*10* by P1 grown on JC10240, and transductants were selected for resistance to tetracycline (15 μg/ml) and screened for sensitivity to UV (6), yielding SJ99. Cultures were grown in Luria broth (16) for routine manipulations and in Davis minimal medium (8) for GalK assays. Cultures were grown in a New Brunswick model G76 gyratory water bath shaker, and growth was monitored by measuring optical density at 660 nm (OD_{660}) with a Beckman DB spectrophotometer.

Galactokinase assays. Galactokinase assays were carried out essentially as described by McKenny et al. (19) except that D-[1-^{3}H]galactose at 1 μCi/μmol was used instead of [^{14}C]galactose. To improve counting efficiency, the [^{3}H]galactose was eluted from DE-81 filters after the final wash with 1.5 ml of 15 mM Tris-HCl (pH 7.2)–40 mM $MgCl_2$–1.67 M $LiCl_2$–0.5 mM EDTA. After 10 min of shaking, 15 ml of Aquasol II-xylene (2.5:1) was added, and the radioactivity in the samples was counted.

DNA sequencing analysis. Sequences were determined by the dideoxy chain-termination method (24) with modification for the use of [^{35}S]dATP (3) and a synthetic 17-base universal primer. Clones for sequencing were generated by ligation of fragments by restriction enzyme digestion and by sequential deletion with T4 DNA polymerase (7). Templates for sequencing were prepared as described previously (3).

Plasmid constructs. The plasmid pKOTW1 was obtained from Tim Warner (31a) and is a derivative of pKO11 (1), obtained by partial restriction with *Eco*RI and filling in of the sticky ends to remove the upstream *Eco*RI site of pKO11. All constructs used to analyze expression of the *cyn* operon

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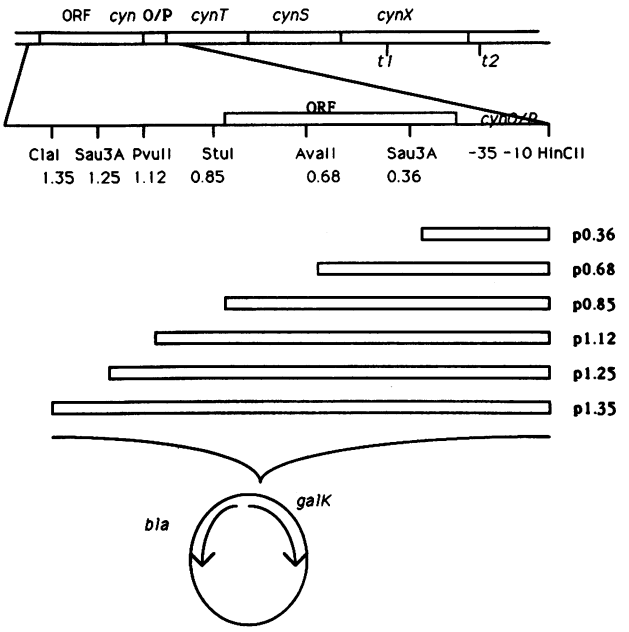

FIG. 1. Plasmid constructs used to investigate the regulation of the *cyn* operon. The upper line represents the *cyn* operon, while the second line indicates the restriction sites in the region of the promoter and upstream of the promoter that were used to construct fusion vectors. t1 and t2 represent the two transcription termination sites identified previously. All fusions were made with a downstream *Hin*cII site that is located between the ribosome-binding site and the start codon of *cynT*. The indicated fragments were blunt ended and ligated into the *Sma*I site of the vector pKOTW1 except for the fragment generated by *Sau* and *Hin*cII digestion, which was ligated into the *Sma*I and *Bam*HI sites. Correct orientation of fragments was determined by restriction digestion. Plasmid pKOTW1 contains the *galK* gene without a promoter as well as a translational stop codon in all three reading frames. O/P, operator-promoter region.

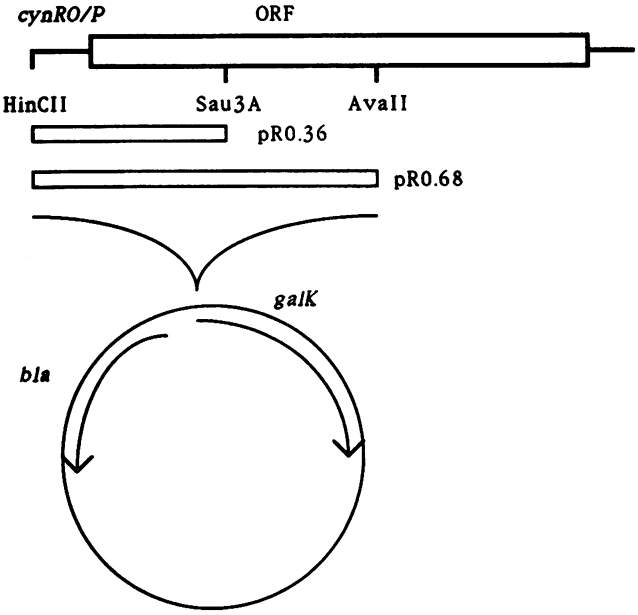

FIG. 2. Plasmid constructs used to investigate the regulation of the *cynR* transcript. The *Hin*cII site is the same site used in Fig. 1 and is located between the ribosome-binding site and the start codon of *cynT*. The indicated fragments were ligated into the promoterless *galK* vector pKOTW1. The ORF was identified as *cynR* by DNA sequencing.

promoter had the *Hin*cII site downstream of the *cyn* promoter fused to the *Sma*I site of pKOTW1. Fragments of various lengths were generated as indicated in the legend to Fig. 1.

The constructs used to analyze expression of *cynR* are given in Fig. 2. The *Hin*cII site described above was used for all constructs. Fragments of various lengths are shown in Fig. 2.

Labeling of *cynR* protein. *E. coli* K38 and plasmids pGP-1 and pT7-5 were obtained from S. Tabor (Harvard Medical School). The 1.3-kb *Cla*I-*Hin*cII fragment containing the entire *cynR* gene was isolated from pSJ104 (30), the *Cla*I site was filled in with DNA polymerase (Klenow fragment), and the blunt-ended fragment was inserted into the *Sma*I site of plasmid pT7-5 in both orientations to construct pSJ130 and pSJ131 (see Fig. 4a). The orientation of the fragment was determined by an *Ava*II-*Hin*dIII and *Ava*II-*Eco*RI double digest.

E. coli K38 containing pGP1-2 and either pSJ130 or pSJ131 was grown at 30°C with ampicillin (50 μg/ml) and kanamycin (40 μg/ml). At an OD_{660} of 0.3, 1 ml of cells was harvested and washed twice with minimal medium (8). The cells were resuspended in 10 ml of minimal medium containing thiamine (20 μg/ml) and 0.01% of each of 18 amino acids (minus cysteine and methionine) and incubated at 30°C for 100 min. The temperature of the culture was shifted to 42°C. After 15 min, rifampin was added (final concentration, 200 μg/ml). The cultures were incubated for an additional 20 min at 42°C, and then the temperature was shifted to 30°C for 20 min. The cultures were harvested by centrifugation and resuspended in 1 ml of 10 mM phosphate buffer (pH 7.0). Then 50 μl of cells was mixed with 50 μl of 2× sample buffer (31) and used for sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (SDS-PAGE).

Nucleotide sequence accession number. The nucleotide sequence shown in Fig. 3 has been assigned GenBank accession number M93053.

RESULTS

Regulation of the *cyn* operon. To investigate the regulation of the *cyn* operon, the promoter of the operon was fused to the promoterless *galK* gene on plasmid pKOTW1. A restriction site for *Hin*cII, located just downstream of the ATG initiation codon of *cynT*, was fused in front of *galK*, and fragments of various lengths upstream were generated by using various restriction enzymes (Fig. 1). Strains containing these plasmid constructs were assayed for galactokinase activity. The results in Table 1 indicated that a culture of SJ99 (Δ*cyn galK*) with a plasmid containing an insert of 0.36, 0.68, or 0.85 kb did not contain significant galactokinase activity when grown in either the presence or the absence of cyanate. However, SJ99 with a plasmid containing an insert of 1.12 kb or longer had significant galactokinase activity when grown in the presence but not the absence of cyanate. One explanation for these results is that the longer inserts contain a promoter and a sequence encoding a positively acting regulatory protein that is required for *cyn* operon expression. Alternative explanations are that the promoter as determined by S1 mapping is incorrect or that an unusually large segment of DNA is involved in regulating this

TABLE 1. Expression of the *cyn* operon promoter in various *cyn-galK* operon fusions[a]

Plasmid in strain SJ99	Galactokinase sp act[b] (nmol/min/mg of protein) with additional plasmid:	
	None or pACYC177	pSJ222
p0.36	<15	1,850
p0.68	<15	1,770
p0.85	<15	1,480
p1.12	1,260	1,450
p1.25	1,440	1,420
p1.35	1,400	1,510

[a] Enzyme assays were carried out on cultures grown in 1 mM cyanate. Enzymatic activity was not detectable in cultures grown in the absence of cyanate.
[b] Nanomoles of galactose phosphorylated per minute per milligram of protein.

operon. The 1.12-kb insert was sequenced and found to contain an ORF (see below).

ORF functions in *trans*. To determine whether the ORF has a *trans* effect on the regulation of the previously identified promoter of the *cyn* operon, the 1.35-kb insert of the plasmid p1.35 was subcloned into the plasmid pACYC177 to generate plasmid pSJ222. The plasmid pACYC177 contains the p15A origin of replication (5), which is compatible with the origin of plasmid pKOTW1. Plasmid pSJ222 was introduced into the strains carrying either plasmid p0.36, p0.68, or p0.85 that did not exhibit *galK* activity when cyanate was present in the previous experiment. The resulting strains were found to have inducible *galK* expression when cyanate was added to the growth medium (Table 1). Plasmid pACYC177 used as a control was found to have no effect on the induction of *galK* expression. These data indicated that the insert of plasmid pSJ222 encoded a *trans*-acting protein that was required together with cyanate for the expression of the *cyn-galK* fusions. This result suggests that the promoter previously mapped by nuclease S1 is probably correct. We propose the designation *cynR* for the gene encoding the *trans*-acting regulatory protein that is required for *cyn* operon expression. Since the *cynR* gene product is required for the expression of the *cyn* operon, the *cyn* operon is likely to be controlled by positive regulation.

Regulation of *cynR*. To investigate the regulation of the *cynR* gene itself, *cynR-galK* transcriptional fusions were used. The *cynR* promoter region was placed upstream of the promoterless *galK* gene on plasmid pKOTW1 (Fig. 2). When the fusion plasmids pR0.36 and pR0.68 were introduced into SJ99, the resulting strains expressed the same level of *galK* activity in the presence and absence of cyanate. The strains carrying pR0.36 and pR0.68 had activities of 310 and 350 nmol of galactose phosphorylated per min per mg of protein, respectively. However, in cells with a functional *cynR* gene, encoded by plasmid pSJ222, significantly decreased expression of the *cynR-galK* fusions was observed. These cells had less than 15 nmol of galactose phosphorylated per min per mg of protein, which is the limit that could be detected in this assay. These results indicated that the *cynR* gene product negatively autoregulated its own gene expression, which is a typical regulatory mechanism for positive regulatory genes (4, 13, 17, 18, 27). The presence of cyanate did not significantly affect *galK* expression.

Sequence of the ORF. By extending the DNA sequence upstream of the *cyn* operon (28), the nucleotide sequence of *cynR* was determined (Fig. 3). The most probable translational start site for the *cynR* gene is the ATG codon at position 1258. The ORF starting at this codon encoded a protein of 299 amino acid residues that would have a calculated molecular weight of 32,923. A potential ribosome-binding site, AGG, is located 6 nucleotides upstream from the initiation codon, ATG. The possible −35 and −10 promoter region for *cynR* identified by homology to the consensus sequence is underlined in Fig. 3. If this is the correct start of the ORF, the nucleotide sequence of the *cynR-cyn* operon region revealed a very short intergenic region (108 bp) between the coding region of *cynR* and the promoter-proximal *cynT* gene of the *cyn* operon. If this is the correct ORF, the short intergenic space would result in the overlapping of the putative −35 regions of the *cynR* promoter and the *cyn* operon promoter. Further analysis of the DNA sequence indicated that there is a 198-bp direct repeat at the 3′ end of the *cynR* gene. Repeat 1 and repeat 2 differed by 6 bp. Each direct repeat contains two inverted repeats that appeared to be similar to the repetitive extragenic palindrome (REP) sequence. The REP sequence is 35 nucleotides long and includes a highly conserved inverted repeat (26). These REP sequences as well as part of *cynR* were identified in the sequence of *codAB*, the genes next to *cynR* on the *E. coli* chromosome (19a). The sequence of *cynR* links the sequence of the *cyn* operon that overlaps the *lac* operon and *lacI* to the *codAB* operon, resulting in a 13.5-kb DNA of known sequence that encodes three operons and two regulatory proteins.

Homology of CynR protein to the LysR family. The protein data bank was searched to find homology to the predicted amino acid sequence of the *cynR* protein, and significant homology was found for *E. coli* IlvY and *Enterobacter* AmpR (Table 2). IlvY and AmpR have previously been identified as members of a large family of bacterial activator proteins, the LysR family (12). In addition to the primary structural homology among these activator proteins, there are other common regulatory features: (i) the regulatory protein and its inducer molecule are required for transcriptional activation of the operon genes; (ii) the level of the activator protein is regulated autogenously; and (iii) some of the operons (*ilv*, *lys*, *met*, and *amp*) have divergent transcription of the operon and the adjacent regulatory gene (14, 19, 22, 27). The LysR family of regulatory proteins are proposed to be DNA-binding proteins that interact with DNA through a helix-turn-helix motif located in the N-terminal region (11–16). After compilation and alignment of the protein sequences of the LysR family members, Henikoff et al. (12) proposed a potential consensus sequence for the LysR protein family as well as a helix-turn-helix consensus motif. Scoring of the comparison of protein sequences is dependent on the penalties assigned to deletions or additions of amino acid residues. In the analysis we have used, no deletions or additions were allowed; rather, domains of 15 or more residues of continuous sequence were compared. The similarities of the CynR sequence to the LysR consensus sequences as well as to selected members of the family are given in Table 2. Twenty amino acids constitute the consensus helix-turn-helix motif for the LysR family, and CynR contains 12 residues that are identical; of the remaining 8 positions, 4 are conservative mismatches. As can be seen from Table 2, most of the sequence similarity of the LysR family exists in the N-terminal half to two-thirds of the sequence. CynR has almost as much sequence similarity to the LysR family as LysR (data not shown) and has greater homology to some members than LysR has.

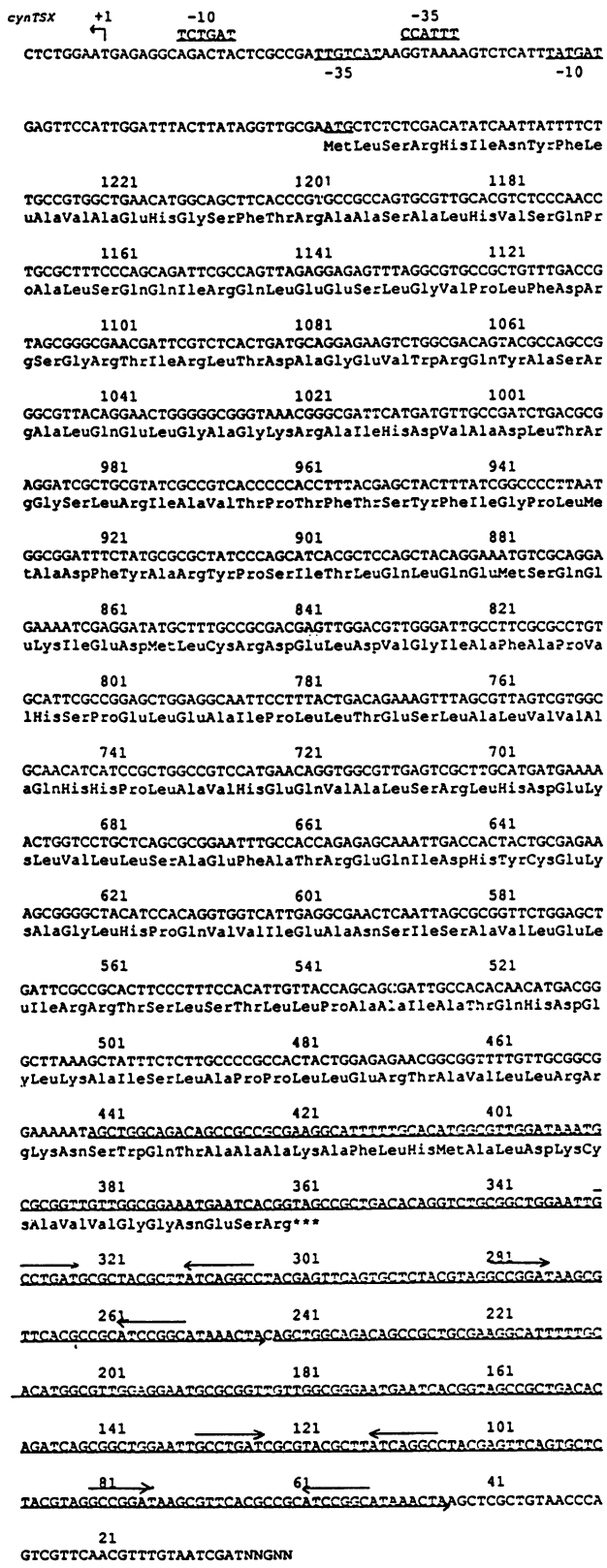

FIG. 3. DNA sequence and amino acid sequence of the ORF of the region of DNA located upstream of the *cyn* operon. The start of transcription of the *cyn* operon is indicated by +1 on the top line. The corresponding −10 and −35 sequences of the promoter are also indicated. The −10 and −35 sequences of the proposed promoter of *cynR* are indicated below the top line. The two long direct repeats are underlined, and the four REP sites are indicated by overlining.

TABLE 2. Comparisons of amino acid similarities of domains of *cynR* with the consensus sequence of the LysR family and selected members of the LysR family[a]

Protein	Domain[b] (aa residues)		SD[c]	% Similarity
	CynR	Comparison protein		
LysR family consensus	9–59	14–63	7.8	48.0
	112–127	112–127	3.4	43.8
LysR	4–247	7–250	10.8	26.6
CysB (*E. coli*)	25–55	26–56	5.9	51.6
	90–151	92–153	5.6	32.3
	166–232	169–235	4.7	29.9
CysB (*Salmonella typhimurium*)	25–55	26–56	5.6	48.4
	90–151	92–153	5.6	30.6
	167–232	170–235	4.7	30.3
IlvY (*E. coli*)	4–185	4–185	10.5	32.0
AmpR (*Enterobacter*)	9–58	14–63	7.5	46.0
	93–124	96–127	3.6	40.6
AmpR (*Rhodobacter*)	15–50	20–55	5.7	52.8
	138–186	138–186	3.7	28.6
OxyR (*E. coli*)	1–242	1–242	14.4	31.4

[a] Sequences were analyzed by using the Molecular Biology Information Resource site (Baylor College of Medicine, Houston, Tex.). Alignment and domains of similarity were defined with the program "homology," which is the Lawrence and Goldman (14) implementation of the Altschul and Erickson (2) algorithm. The scoring matrix used was that of Dayhoff (8a). The consensus sequence is that of Henikoff et al. (12).

[b] The minimum domain length was 15.

[c] The SD score is equivalent to the number of standard deviations by which a similarity score exceeds that expected by chance when searching sequences found in nature with a random sequence of average amino acid composition.

Expression of *cynR*. Numerous attempts were made to verify the start of transcription of the *cynR* gene by S1 mapping of the transcript from the chromosomal gene as well as the gene carried on a high-copy-number plasmid, but no product was detected. No mRNA could be detected by Northern (RNA blot) analysis. Presumably, the combination of a weak promoter and autoregulation is responsible for the low production of *cynR* mRNA. To verify that the CynR protein is of the size predicted from the ORF, the protein was expressed and analyzed. In previous experiments (28, 29), with maxicells used to express plasmid-encoded proteins, no product of *cynR* was observed, suggesting that it may be produced in very low abundance. To overcome this difficulty, a T7 RNA polymerase-pT7-5 promoter system was used (31), which consists of two compatible plasmids, pGP1-2 and pT7 derivatives. Plasmid pGP1-2 encodes T7 RNA polymerase under control of lambda p_L as well as the lambda gene encoding the repressor *c*I857. At 42°C, the repressor protein is inactivated and T7 RNA polymerase is synthesized. A second plasmid, pT7-5, contains a T7 pro-

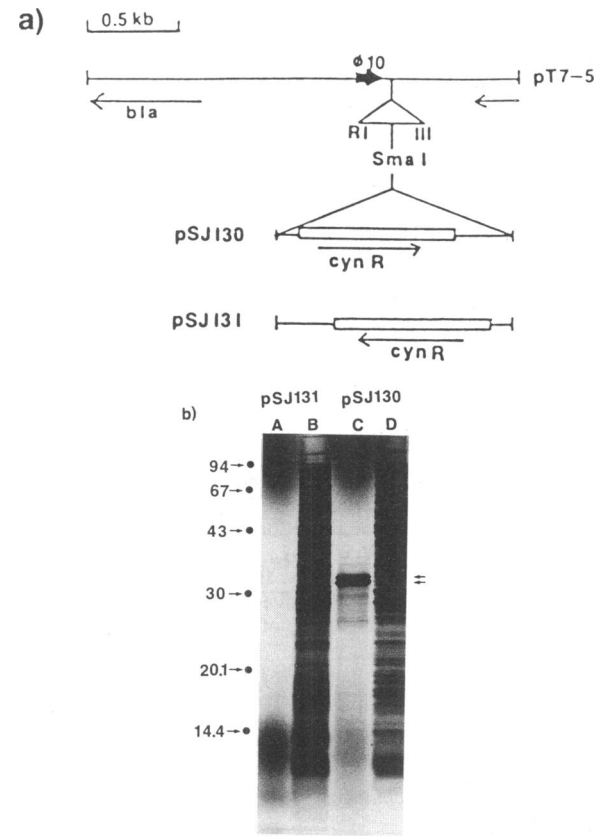

FIG. 4. Expression of *cynR* under control of a T7 promoter. (a) Map of pT7-5 and *cynR*-containing derivatives pSJ130 and pSJ131. The arrows indicate the direction of transcription. ϕ10 is a T7 promoter recognized by T7 RNA polymerase. *cynR* is the gene encoded by the proposed ORF upstream of the *cyn* operon. (b) Expression of *cynR*. Conditions for growth of cells, labeling of protein with [^{35}S]methionine, electrophoresis on a 15% polyacrylamide–SDS gel, and autoradiography are given in Materials and Methods. In lanes A and C, rifampin was added to inhibit the transcripts initiated by *E. coli* RNA polymerase. In lanes B and D, no rifampin was added. The positions of size markers are indicated to the left (in kilodaltons). The arrows on the right indicate the 32,000- and 33,000-dalton labeled bands.

moter followed by a polylinker and a T7 terminator. Plasmids pSJ130 and pSJ131 are derivatives of pT7-5 containing the *cynR* insert in opposite orientations.

E. coli K38 containing pGP1-2 as well as either pSJ130 or pSJ131 was constructed. T7 RNA polymerase synthesis was induced, and rifampin was added to selectively inhibit *E. coli* RNA polymerase (31). [^{35}S]methionine was added for 5 min to label proteins encoded by pSJ130 and pSJ131, and samples were analyzed by SDS-PAGE (31) and autoradiography. The sample from the strain containing pSJ130 contained two labeled proteins migrating with molecular masses of 32,000 and 33,000 Da (Fig. 4b). The smaller protein appears to be more abundant. The reason that two proteins are synthesized is possibly due to the T7 expression system producing a transcript longer than that produced from the *cynR* promoter. Alternatively, both proteins may be synthesized in vivo. These results suggest that the proposed ORF synthesizes a protein of the predicted size but that the protein is normally synthesized in very low abundance.

DISCUSSION

The observations reported in this article indicate that the *cyn* operon is regulated by a positively acting protein that, in the presence of cyanate, can activate transcription of the *cyn* mRNA. The *cynR* gene is negatively autoregulated. The sequence of *cynR* indicated that *cynR* is a member of the *lysR* family (12). Like *cynR*, most members of this family encode a positively acting regulatory protein, and, like several members of this family, including *lysR*, *cysB*, *nodD*, *ilvY*, and *metR*, the *cynR* product negatively regulates its own promoter while positively regulating an overlapping promoter on the complementary strand. While the function of the *cyn* operon is not fully understood, it appears to be responsible for the detoxification of cyanate. Positive regulation would ensure that the operon would not be synthesized unless cyanate was present. Autoregulation of the *cynR* gene has an obvious advantage in maintaining the concentration of the regulatory protein at an approximately constant level. By analogy with other members of the *lysR* family, we predict that *cynR* protein has two functionally different conformations, depending on whether cyanate is present. Without cyanate, we predict that CynR will bind to the regulatory site to prevent the transcription of the *cynR* gene without activating *cyn* operon expression. However, when cyanate binds to the CynR protein, we predict that it will shift the conformational equilibrium to the activated form, which will stimulate the transcription of the *cyn* structural genes while still negatively autoregulating its own expression. Thus, the *cynR* regulatory protein serves as a negative and/or positive control function, depending on whether the inducer, cyanate, is present.

The homology between *lysR* and *cynR* suggests that they could have evolved from a common ancestral gene or from each other. The presence of REP sequences downstream of both the *cynR* gene (Fig. 3) and the *lysR* gene (27) suggests that these may have been important in the evolution of these genes, since recombination can occur between REP sequences. The REP sequences could have played a role in the mediation of the chromosomal rearrangements or in duplication of DNA segments. The map positions of *cynR* (8 min) and *lysR* (61 min) are just opposite on the circular chromosome of *E. coli*, which might be the result of a duplication of the *E. coli* chromosome, as proposed previously (23). Alternatively, the presence of REP sequences could simply be coincidence, and the REP sequences could be involved in stabilizing the upstream RNA by protection from 3′-5′ exonuclease activity (20). The widespread occurrence of members of the *lysR* family suggests that evolutionarily it may be more advantageous to duplicate, move, and alter the binding site for an effector molecule as well as a DNA-binding site of a regulatory cassette consisting of a gene encoding a positively acting regulatory protein linked to its regulated promoter that to attempt to independently evolve a new regulatory system.

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