Mutual Regulation of Crl and Fur in Escherichia coli W3110*

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The small regulatory protein Crl controls the expression of curli. Recently we have shown that Crl interacts directly with one of the most global regulators of Escherichia coli, the stress-related α factor RpoS, suggesting a more global role for Crl. We show here by a proteomics analysis that the expression of at least nine cellular proteins was considerably modified when Crl was overexpressed. We assessed the part of transcriptional and post-transcriptional regulation for five of these genes. The results showed that Crl regulates the expression of another global regulator, the central regulator of iron homeostasis, Fur. A molecular analysis revealed that Crl and Fur affect their own and each other’s expression. We provide physical evidence for the binding of Fur to the crl and fur promoter regions. Crl modulated the affinity of Fur at the fur promoter but not at the crl promoter. The triad RpoS-Crl-Fur may thus represent the centerpiece of a global regulatory system of response to different stresses. *Molecular & Cellular Proteomics* 6:660–668, 2007.

The natural habitat of Escherichia coli is the gastrointestinal tract of humans and other animals. Adherence to host tissues and cells is critical to its survival in these environments. Bacterial cell surface filaments play important roles in adherence to and invasion of host cells. They are generated by the chaperone/usher pathway system (type I fimbriae), the type II secretion system (type IV pili), and the nucleation-dependent polymerization system (curli filaments). This adherence is often mediated by non-flagellar filamentous cell surface appendage, called fimbriae (or pili). Most strains of E. coli contain genes encoding type I (mannose-sensitive) fimbriae (1) and curli (aggregate fimbriae) (2), although not all strains produce these structures (3, 4).

Curli filaments are thin, coiled, aggregative fibers, first visualized on E. coli strains by Olsen et al. (5). Curli filaments mediate binding to soluble extracellular matrix proteins and the major histocompatibility complex class I molecules (6). They are associated with biofilm formation (7) and induce the proinflammatory response (7). The genes encoding curli, corresponding to the csg operon in *E. coli*, are conserved in Gram-negative bacteria (8). Their production is regulated by (i) different environmental conditions such as low temperature, low osmolarity, and stationary phase (3, 5) and (ii) a complex network of regulatory proteins, including OmpR (9), RpoS (9), CsgD (10, 11), H-NS (12, 13), integration host factor (10), and Crl (8, 12–14). Crl regulates the transcription of csgAB operon in an α²-dependent manner (8, 12, 13). In *E. coli*, this dependence results from specific protein-protein interactions between Crl and RpoS that promote binding of the α² holoenzyme to the csgA promoter (15). Crl accumulates in stationary phase cells at low temperature (30 °C), but there is much less accumulation at 37 °C.

In an attempt to understand the role of the Crl protein in stationary phase and at low temperature, we used two-dimensional gel electrophoresis to compare the expression profile of soluble proteins of the wild type *E. coli* strain with the same strain overexpressing the Crl protein. The expression of nine proteins, visualized as spots on 2D protein gels, was modified. Seven of them were identified by mass spectrometry, including the iron-regulatory protein Fur (ferric uptake regulatory protein). The transcriptional analysis of crl, fur, ompA, and mdh genes were studied in different genetic contexts (wild type, crl–, rpoS−, and fur− mutants) using the lacZ reporter gene. Molecular analyses, including EMSA 1 and Western blotting, suggested a possible protein-protein interaction between Crl and Fur. We propose a model of the underlying regulatory network including the three major regulatory proteins RpoS, Fur, and Crl.

**MATERIALS AND METHODS**

*Strains and Plasmids—*The genotypes of *E. coli* K12 strains and plasmids used in this study are listed in Table I. Media used were Luria-Bertani broth with 5 g/liter NaCl (LB) and LB containing 12 g/liter agar (LA) (16). Media were supplemented with 15 µg/ml chloramphenicol (Cm), 25 µg/ml kanamycin (Kan), or 100 µg/ml ampicillin as required. Strains were grown at 37 or 30 °C with shaking (200 rpm).

The fur mutation was transferred from *E. coli* QC1732 (17) to W3110 (wild type WT) and BL002 (ppoS::Kn') using P1vir-mediated

1 The abbreviations used are: EMSA, electrophoretic mobility shift assay; Cm, chloramphenicol; Kan, kanamycin; WT, wild type; NTA, nitrilotriacetic acid; 2D, two-dimensional.

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transduction (18). Transduction of the fur::Cm' allele was obtained by selection on Cm plates. The correct location of the mutation was verified by Southern blotting. All clones were stored at −80 °C in a 20% glycerol solution.

2D Gel Electrophoresis—50–100 μl of the overnight cultures of the different E. coli strains were plated on a LA plate supplemented with ampicillin and grown at 30 °C for 24 h. Bacterial cells were scraped and resuspended from plates in 1.5 ml of phosphate buffer (7 g/liter Na2HPO4, 3 g/liter NaH2PO4, and 4 g/liter NaCl), pelleted, and washed twice with phosphate buffer in a final volume of 1.5 ml. The pellets were frozen until protein extraction. Soluble protein extracts were prepared, and two-dimensional electrophoresis was performed as described in Lelong and Rabilloud (19). Briefly the cell pellets were resuspended in 200 μl of SDT solution (0.05 M Trizma (Tris) base), 0.2 M DTT, 0.3% SDS, and 1 mM EDTA). The extracts were boiled at 100 °C for 5 min and then cooled on ice. 20 μl of DR solution (1 mg/ml DNase, 0.25 mg/ml RNase, 0.05 M Tris-HCl, pH 7.5, and 0.05 M MgCl2) were added, and the extracts were incubated on ice for 10 min. Finally 800 μl of lysis buffer (9.9 M urea, 4% Nonidet P-40, 0.1 M DTT, and 2.2% Ampholine) were added. The mixture was vortexed vigorously to obtain a homogeneous solution. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad) and bovine serum albumin as standard. Protein samples were stored at −20 °C until use. 150 μg of each protein extract were focused isoelectrically on an 18-cm pH 4–7 immobilized pH gradient (homemade IPG (20)) for 65,000 V-h using the Multiphor II system (Amersham Biosciences) at 20 °C. In the second dimension, proteins were separated on a 10% SDS-PAGE gel running at 10 °C and 25 V for 1 h and then at 12.5 watts/gel for 5 h. Finally gels were stained using silver nitrate (19). Each experiment was performed at least three times for each strain using two independent cultures. The gels were scanned, analyzed, and quantified using Melanie software. The software quantifies spots as Gaussian shapes, and spot intensities are expressed as a fraction of the sum of the intensities of all detected spots. Only spots showing reproducible modifications (p value in Student’s t test <0.05, Table II) were retained and identified by mass spectrometry.

**Protein Digestion**—Protein bands were manually excised from preparative Coomassie Blue-stained gels. Excised gel bands were washed several times with destaining solutions (25 mM NH4HCO3 for 15 min and then with 50% (v/v) acetonitrile containing 25 mM NH4HCO3 for 15 min). Gel pieces were then dehydrated with 100% acetonitrile and dried. Gel pieces were then incubated with a reducing solution (25 mM NH4HCO3 containing 10 mM dithiothreitol) for 1 h at 56 °C and subsequently with an alkylation solution (25 mM NH4HCO3 containing 55 mM iodoacetamide) for 45 min at 37 °C. After reduction and alkylation, gels were washed several times with the destaining solutions and finally with pure water for 15 min before being treated again with 100% acetonitrile. Depending on the protein amount, 2–3 μl of 0.1 μg/μl modified trypsin (Promega, sequencing grade) in 25 mM NH4HCO3 were added over the gel spots. After 30 min of incubation, 7–10 μl of 25 mM NH4HCO3 were added to cover the gel spots before overnight incubation at 37 °C.

**MALDI-TOF MS Analyses and Identification of Proteins**—For MALDI-TOF MS analyses, a 0.5-μl aliquot of peptide mixture was mixed with 0.5 μl of matrix solution (cyano-4-hydroxycinnamic acid at half-saturation in 60% acetonitrile, 0.1% TFA (v/v)). The resulting solution was automatically spotted on a MALDI-TOF target plate, dried, and rinsed with 2 μl of 0.1% TFA.

Peptide mixtures were then analyzed with a MALDI-TOF mass spectrometer (Autoflex, Bruker Daltonics) in reflector/delayed extraction mode over a mass range of 0–4200 Da. For each sample, spectrum acquisition was obtained with an average of 200 laser shots after an external calibration using a mixture of four synthetic peptides (angiotensin II, 1046.54 Da; substance P, 1347.74 Da; bombesin, 1619.82 Da; and adrenocorticotropic hormone clip 18–39, m/z 2465.20 Da). Spectra were then annotated (XMass software, Bruker Daltonics), and the peptide mass fingerprints obtained were finally submitted to database searches against the Swiss-Prot TrEMBL database (50.1 version, restricted to E. coli) with an intraintr 1.9 version of MASCOT software. MASCOT search parameters used with MS data were: database, Swiss-Prot TrEMBL; enzyme, trypsin; one miscleavage allowed; variable modifications, acetyl (N terminus)/oxidation (Met)/carbamidomethyl (Cys)/false mass assignment +1; peptide tolerance, 100 ppm; monoisotopic; and [M + H]+ (protonated molecular ions). A 95% confidence level threshold was used for MASCOT protein scores. All the proteins identified (Table II) were in the top hits with a sequence coverage of more than 30%. In E. coli, Crl, Fur, CysK, OmpA, and Mdh do not belong to a multiprotein family. Therefore, their identification was unambiguous.
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**β-Galactosidase Assays**—Putative promoter regions were amplified using PCR to produce fragments extending from ∼200 bp upstream and ∼150 bp downstream of the start codon for *crl*, *fur*, *ompA*, and *mdh* using chromosomal DNA from the W3110 strain as template and the following primers: fur2 (5′-ggaattccaccagcgtcgtcaaactg-3′) and fur3 (5′-cgggatcccgggctatcatttcgaag-3′), crl7 (5′-cgggatcccggtccggtagttcatacc-3′) and crl8 (5′-tccaccagcccagaatt-3′), ompA1 (5′-cgggatcccgccacgagacaactttc-3′) and omp2 (5′-ggaattccgggcattgttgttgat-3′), and mdh1 (5′-cgggatcccgtcagccctgaagaaggct-3′) and mdh2 (5′-ggaattccgggatatggctcagatcg-3′). The fragments, containing terminal BamHI and EcoRI recognition sites, were cloned into the pRS550 plasmid (21) between these two restriction sites. The resulting plasmids were sequenced for verification and then transformed into the different strains (BL001, BL002, BL003, BL004, and BL005). 50–100 μl of the overnight cultures of the different *E. coli* strains were plated onto LA plates supplemented with ampicillin and grown at 30 °C for 24 h. Bacteria pellets were obtained as described in the previous paragraph. The pellet was frozen until analysis for β-galactosidase activity.

β-Galactosidase assays were performed as described by Miller (18). Specific activities were expressed as mol of produced *ortho*-nitrophenol/min/mg of protein. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad) and bovine serum albumin as standard. Basal activity levels were determined in each genetic context (WT, crl::Cmr', rpoS::Kn', fur::Cmr', crl::Cmr' rpoS::Kn', and rpoS::Kn' fur::Cmr') using the corresponding strain containing the pRS550 plasmid without insert. Values presented in Fig. 2 were the results of at least two measurements for three independent cultures.

**Overproduction and Purification of His<sub>6</sub>-Fur and His<sub>6</sub>-Crl**—The coding sequence of *crl* gene was cloned on a His<sub>6</sub> tag pQE-30 vector (Qiagen), yielding the pHis6-Crl plasmid as described previously (15). The coding sequence of *fur* gene was cloned in the same way: we PCR-amplified the coding region of the *fur* gene using chromosomal DNA from the W3110 strain as template and the primers fur5 (5′-actgataacaataccgccc-3′) and fur6 (5′-ttatttgccttcgtgcgc-3′). The PCR fragment was cloned into the His<sub>6</sub> tag pQE-30 vector, yielding the pHis6-Fur plasmid, which was sequenced for verification. The purification of His<sub>6</sub>-Crl protein has been described by Bougdour et al. (15). His<sub>6</sub>-Fur protein was purified using the same protocol (binding of crude extracts from *E. coli* strains M15 to a Ni<sup>2+</sup>-NTA column). The proteins were eluted under native conditions with imidazole. The major His<sub>6</sub>-Crl-protein fusion peak was recovered at 250 mM imidazole. As expected, His<sub>6</sub>-Fur (molecular mass, 16,652 Da) migrated with a relative mobility of around 16.6 kDa on SDS-PAGE as did wild type Fur (data not shown.). Proteins were purified by gel filtration on a PD-10 desalting column (Amersham Biosciences) equilibrated with storage buffer (50 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 150 mM KCl, 5 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, and 0.1 mM DTT). The proteins were >95% pure.
and C2 (5'/H11032/H11032/H11032/H11032/H11032/H11032). The PCR products, named F2F3-atctgctcttcgggtgtc-3'.

reaction mixtures containing 0.5 nM (0.097 pmol) C1C2 PCR product, or 6 nM (0.12 pmol) F2F3 PCR product, 3.5 M (0.8 μg) His6-Fur, and from 0.51 to 2.6

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cross-reaction was observed for the antibodies used in this study.

Electrophoresis and Immunoblot Analysis—Antibodies against Fur were produced in rabbits by injecting highly purified His6-Fur protein (Eurogentec). Crl antibodies were as described in Bougdour et al. (15). No cross-reaction was observed for the antibodies used in this study. Protein samples were analyzed by SDS-PAGE (16).

The immunoblot analysis of proteins electrotransferred (Bio-Rad system) onto nitrocellulose or polyvinylidene membranes (Amersham Biosciences) was performed with polyclonal antibodies raised against Fur (Amersham Biosciences), and staining intensity was quantified with ImageGauge (Fujifilm) software.

EMSA—DNA for binding assay was generated by PCR from chromosomal DNA and by using the primers F2 (5'-gtcatctgcctgctc-3'), F3 (5'-aatgtccgcggtttcaag-3'), C1 (5'-ggtgagtcagctctgct-3'), and C2 (5'-actgctctgggtgc-3'). The PCR products, named F2F3 (257 bp) and C1C2 (325 bp), were purified using Qiagen columns. Binding assays with His tag-purified proteins were carried out in 20-μl reaction mixtures containing 0.5 TA buffer (40 mM Tris base, 20 mM

acetic acid), 0.2 μg/ml heparin, 5 mM DTT, 50 mM KCl, 10% glycerol, 5 mM (0.097 pmol) C1C2 PCR product, or 6 mM (0.12 pmol) F2F3 PCR product, 3.5 M (0.8 μg) His6-Fur, and from 0.51 to 2.6 M His6-Crl for 30 min at room temperature. The samples were subjected to electrophoresis on a 5% non-denaturing polyacrylamide gel in 0.5 TA buffer. Free DNA and protein-DNA complexes were visualized by staining with ethidium bromide under UV light at 254/312/365 nm.

RESULTS

Overexpression of Crl Induces Modifications of the Expression of 9 Proteins—Crl is preferentially expressed at low temperature (30 °C), and expression begins at the entry into stationary phase (15). Furthermore because this regulator controls the transcription of the curli genes, which are involved in cell adhesion, the most appropriate conditions for studying the Crl regulon are stationary phase cells grown at 30 °C on solid medium. Comparison of the WT control strain (containing the PCRII®-Topo® plasmid without insert) with the identical strain carrying the crl gene (Crl') cloned on the Topo vector showed a modified intensity for nine spots on the 2D gel: three spots disappeared, four spots appeared, and two had an increased expression in the Crl' strain (Fig. 1).

Seven of the spots were identified by mass spectrometry and correspond to five proteins (Fig. 1 and Table II): Crl, Fur (ferric uptake regulator), CysK (cysteine synthase A), OmpA (outer membrane protein II), and Mdh (malate dehydrogenase). The Fur and Crl proteins were each present as two spots with the same molecular mass but different isoelectric points. The apparent molecular mass of Crl (15 kDa) was slightly lower than that of Fur (16 kDa) in agreement with the theoretical molecular masses (15.5 and 16.65 kDa, respectively). All proteins migrated with a molecular mass and isoelectric point similar to those predicted from the sequence (Table II). The functions of the identified proteins are diverse: CysK and Mdh are part of the central metabolism, OmpA is a porin (therefore involved in the response of the cell to environmental modifications), and Crl and Fur are regulators of gene expression. The most interesting proteins with respect to cellular regulation are thus the two transcriptional regulators Crl and Fur. In addition to changes in the accumulation of these two proteins in the mutant strains, we noted that they ran as two spots on the 2D gel. These post-translational modifications may play a role in regulation, but we were not able to characterize the chemical nature of these modifications.

Instead we set out to understand the molecular details of
the interactions between Crl and Fur and their physiological roles in our growth conditions (LB plates at 30 °C). Recently we have shown (15) that Crl acts by modulating the activity of RpoS, the σ factor of stationary phase and general stress conditions, via a direct protein-protein interaction. This model had also been suggested earlier by Pratt and Silhavy (22). Fur regulates the expression of numerous genes in response to changes in iron availability. To determine whether the expression changes observed by 2D electrophoresis were due to transcriptional regulation, dependent on Crl and/or RpoS and/or Fur, or were a consequence of post-transcriptional regulation, we measured the transcription of the four genes (crl, fur, mdh, and ompA) in different genetic contexts (wild type, crl−, rpoS−, fur−, crl−rpoS−, and rpoS−fur−).

The promoter strengths of these five genes differed greatly as shown in Fig. 2e. In the WT context the transcription of the crl gene was close to our limit of detection. For this gene, only substantial increases in promoter activity could be reliably interpreted. The expression of mdh was only affected in the context of the fur−rpoS− double mutant. The transcription of ompA was clearly dependent on RpoS. Such control by σs has never been described, but no extensive transcriptional

![Fig. 2. Transcriptional activity of crl, fur, mdh, and ompA promoters. β-Galactosidase activities were measured as described under “Materials and Methods.” The histograms in a–e represent promoter activities in each genetic context. The values are the average of at least three independent experiments, and each aliquot was assayed three times. e shows the activities of all promoters in the WT strain. pRS550 is the reporter vector without a promoter insert and represents the detection limit of our assay. SA, specific activity; ONP, ortho-nitrophenol.](image-url)
analysis of this promoter has ever been done, particularly not under the conditions of our experiments. Most strikingly, Crl and Fur regulated their own and each other’s transcription (Fig. 4, A and B); Crl repressed its own transcription and slightly activated the transcription of fur. Fur positively auto-regulated itself and repressed the transcription of crl.

In summary, using a proteomics analysis and measuring transcription profiles of target genes, we observed in vivo a new reciprocal transcriptional regulation network between Crl and Fur. We therefore wanted to understand, in vitro, the mechanism governing this reciprocity.

Crl Modulates the Binding Ability of Fur on DNA by a Direct Protein-Protein Interaction—Crl regulates transcription of target genes by binding to specific sites upstream or overlapping the promoter, whereas Crl physically interacts with RpoS (and not with σ70) and increases its activity (15). The regulation of Crl expression by Fur has not been described yet, but we identified putative binding sites for Fur within the Crl promoter region. Because fur is transcribed from a σ70 promoter, a direct activation of fur transcription by Crl is impossible. However, because Fur autoregulates its own transcription, Crl might act by interaction with Fur. To investigate this possibility and confirm Fur binding to the Crl promoter, we tested in vitro the influence of Crl on the binding of Fur to two DNA regions, crl and fur promoter regions. We used the purified His-tagged proteins for these EMSA experiments.

We first analyzed purified proteins by immunoblot assays using polyclonal antibodies raised against Crl or Fur. The analysis of the eluted fractions of the purification of His6-Crl with anti-Fur antibodies showed that Fur co-elutes with His6-Crl from the Ni2+ column (Fig. 3A). As a control, a cleared extract from the same strain, but not expressing a fusion protein, was applied to the Ni2+-NTA column. The immunoblot analysis of the imidazole eluted fraction did not reveal the presence of Fur, suggesting that the interaction between Crl and Fur is specific. We also did the reciprocal experiment, probing the eluted fraction containing the His6-Fur protein with an anti-Crl polyclonal antibody (Fig. 3B). No Crl was detected in the His6-Fur fraction. This negative result is the expected consequence of the mutual transcriptional regulation described in the previous paragraph: overexpression of Fur represses the already low transcription of crl, thereby reducing the concentration of Crl below the detection limit of our Western blot. The protein purification therefore gave a first hint at a possible direct interaction between Crl and Fur.

To investigate the molecular mechanism of the mutual regulation of Crl and Fur, we measured the interaction of His6-Fur with the two promoters in the presence and absence of Crl. We incubated a 257- or a 325-bp DNA fragment, encompassing the fur or crl promoter region, respectively, with purified proteins and analyzed the complexes on native polyacrylamide gels (Fig. 4). The extent of binding in each lane was quantified using ImageGauge software (Fujifilm) (Fig. 4, B and D). Fig. 4 (A and C) shows that His6-Fur bound to the promoter region of the fur gene, confirming previous experiments, but Fur also bound to the crl promoter. Control experiments (Fig. 4F) showed that His6-Crl alone does not interact with DNA. Hist6-Crl, added to equimolar concentrations with Hist6-Fur, modified the interaction of Hist6-Fur with the promoter region. Very little or no such effect was observed for the crl promoter fragment. At the fur promoter region, when we added 0.76 μM Hist6-Crl (3.6 μM Fur in the reaction), a slower migrating complex appeared, and the proportion of free DNA decreased 2-fold when 0.88 μM or more Hist6-Crl was included in the reaction. A control experiment in which Hist6-Crl was replaced by BSA at the same concentration ensured that the increased binding was not due to protein aggregation or excluded volume effects (Fig. 4E).

**DISCUSSION**

Effects of Crl Overexpression—Crl had been described as the protein that regulates curli expression. Further experiments have suggested that Crl may act in coordination with RpoS to modulate the bgl operon expression (23), and recently we have shown that Crl interacts directly with RpoS to control the expression of the csgBA operon (15). The combination of these data suggests a pleiotropic role of Crl in the cell. In this study and in Lelong et al. (24), we confirmed this hypothesis by studying global expression patterns in different genetic contexts. Using a proteomics approach and crl and/or rpoS deletion mutants, we showed that the expression of about 60 proteins is modified by RpoS and/or Crl (24). In the present study we focused on the consequences of an over-expression of Crl, and we observed modifications of the expression of 10 proteins. Transcriptional analysis showed that for some of these proteins, OmpA and Mdh, the regulation is
FIG. 4. Crl binds to Fur at the fur and crl promoters. A and C, 5 nM crl or 6 nM fur promoters were run on a 5% polyacrylamide gel alone (lane 1) or after incubation with 3.6 μM purified His$_6$-Fur (lane 2) or with 3.6 μM purified His$_6$-Fur and increasing concentrations of purified His$_6$-Crl, 0.51, 0.76, 0.88, 1.75, and 2.6 μM (lanes 3, 4, 5, and 6, respectively). B and D, signals of bound DNA (dashed lines) and unbound DNA (solid lines) were quantified and normalized by the total signal of DNA in each lane of the gels (A and C). E, as a control of the specificity of the protein-DNA interaction, BSA was added to the same concentration as purified His$_6$-Fur and His$_6$-Crl. Lanes 1 and 6, fur and crl promoter DNA (5–6 nM) incubated with 3.6 μM purified His$_6$-Fur, respectively; lanes 2 and 7, DNA incubated with 3.6 μM purified His$_6$-Fur and 2.6 μM purified His$_6$-Crl; lanes 3 and 8, DNA incubated with 3.6 μM purified His$_6$-Fur and 3.6 μM BSA; lanes 4 and 9, DNA incubated with 2.6 μM purified His$_6$-Crl and 3.6 μM BSA; lanes 5 and 10, DNA alone. F, lanes 1 and 3, fur and crl promoter DNA alone, respectively; and lanes 2 and 4, DNA incubated with 2.6 μM purified His$_6$-Crl.
post-transcriptional. No effects on transcription were observed for these genes, although the protein concentrations clearly changed as observed on 2D protein gels. We have to conclude that post-transcriptional processes dominate the regulation of expression of these genes.

The most interesting effects of the Crl overexpression or crl mutant concern the expression of crl itself and of the Fur protein. Our analysis revealed a yet unknown regulatory relationship between Crl and Fur, and we examined some of the molecular details of this regulation. We showed that the overexpression of Crl leads to increased fur expression and that the deletion of crl gene diminishes the transcription of the fur gene. The most important transcriptional regulation of fur is, however, exerted by Fur itself. In our experimental conditions, growth at 30 °C on LB plates, both Crl and Fur activated the expression of fur and repressed the expression of crl. EMSA experiments suggested the presence of a Fur binding site in the crl promoter region. Fur binding to this site was unaffected by Crl. Fur is known as a transcriptional repressor that acts by binding to promoter region, thereby preventing or diminishing the binding of RNA polymerase. In the case of the regulation of Crl expression by Fur, such a model agrees with our observations.

However, identical experiments with the fur promoter fragment indicated that Crl enhances the affinity of Fur for the sites on its own promoter probably by direct protein-protein interaction. This interaction was corroborated by the co-purification of Fur with His6-Crl and the activation of fur transcription by Crl and Fur observed in β-galactosidase assays. It is known that Fur can activate gene expression (17). Recently the molecular details of a particular case of activation by Fur have been shown to involve a small RNA, RyhB (25). In the case of Crl we have not yet identified the molecular mechanism of the observed regulation.

**Role of Crl in the Global Regulation of Gene Expression**—Crl seems to be at the interface of two central regulators, RpoS and Fur, both of which are involved in the response to environmental changes. These two proteins do not seem to be connected directly but rather via Crl. Crl is known to be preferentially expressed at 30 °C and on solid medium. These environmental factors may be important for the virulence of a strain. Crl, in turn, controls the activity of two other important virulence genes, rpoS and fur. We imagine therefore that the triad RpoS-Crl-Fur plays an important role in detecting environmental signals and transducing such information to the expression of virulence factors. Crl should thus be considered not only as the regulator of curli synthesis but rather as a central modulator involved in the response to environmental changes, particularly those related to virulence and/or those regulated by RpoS (24).

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