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Mutual Regulation of Crl and Fur in *Escherichia coli* W3110*

Cécile Lelong‡§, Magali Rolland‡, Mathilde Louwagie¶, Jérôme Garin¶, and Johannes Geiselmann‡

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 σ 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 (aggregative fimbriae) (2), although not all strains produce these structure (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 a σ^S -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 σ^S 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 EMSA¹ 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 μ g/ml chloramphenicol (Cm), 25 μ g/ml kanamycin (Kn), or 100 μ g/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 (*rpoS*::Kn') using P1(vir)-mediated

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

TABLE I
Strains and plasmids

Genotypes		Ref.
<i>E. coli</i> strains		
W3110	λ , IN ^a (<i>mD-rmE</i>)1, <i>rph1</i>	26
BL001	W3110, <i>crl::Cm</i> ^r	27
BL002	W3110, <i>rpoS::Kn</i> ^r	15
BL004	W3110, <i>fur::Cm</i> ^r	This work
BL003	W3110, <i>crl::Cm</i> ^r <i>rpoS::Kn</i> ^r	15
BL005	W3110, <i>rpoS::Kn</i> ^r <i>fur::Cm</i> ^r	This work
QC1732	<i>fur::Cm</i> ^r	28
M15	F ⁻ Lac ⁻ Thi ⁻ Mtl ⁻	Qiagen
Plasmids		
pQE-30 UA	Amp ^r	Qiagen
pCRII-Topo	Amp ^r Kn ^r	Invitrogen
pTopo- <i>crl</i>	PCRII-Topo/ <i>crl</i>	15
pRS550	<i>bla-kan-T14-BamHI-SmaI-EcoRI-lacZ</i> ⁺	21
pCrl	pRS550 vector containing the promoter region of the <i>crl</i> gene	This work
pfur	pRS550 vector containing the promoter region of the <i>fur</i> gene	This work
pompA	pRS550 vector containing the promoter region of the <i>ompA</i> gene	This work
pMdh	pRS550 vector containing the promoter region of the <i>mdh</i> gene	This work

^a Inversion.

transduction (18). Transduction of the *fur::Cm*^r 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 an 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 Na_2HPO_4 , 3 g/liter NaH_2PO_4 , 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) 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 MgCl_2) 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 NH_4HCO_3 for 15 min and then with 50% (v/v) acetonitrile containing 25 mM

NH_4HCO_3 for 15 min). Gel pieces were then dehydrated with 100% acetonitrile and dried. Gel pieces were then incubated with a reducing solution (25 mM NH_4HCO_3 containing 10 mM dithiothreitol) for 1 h at 56°C and subsequently with an alkylating solution (25 mM NH_4HCO_3 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 $\mu\text{g}/\mu\text{l}$ modified trypsin (Promega, sequencing grade) in 25 mM NH_4HCO_3 were added over the gel spots. After 30 min of incubation, 7–10 μl of 25 mM NH_4HCO_3 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, *m/z* 1046.54 Da; substance P, *m/z* 1347.74 Da; bombesin, *m/z* 1619.82 Da; and adrenocorticotrophic 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*; 30,482 sequences) with an intranet 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 $[\text{M} + \text{H}]^+$ (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.

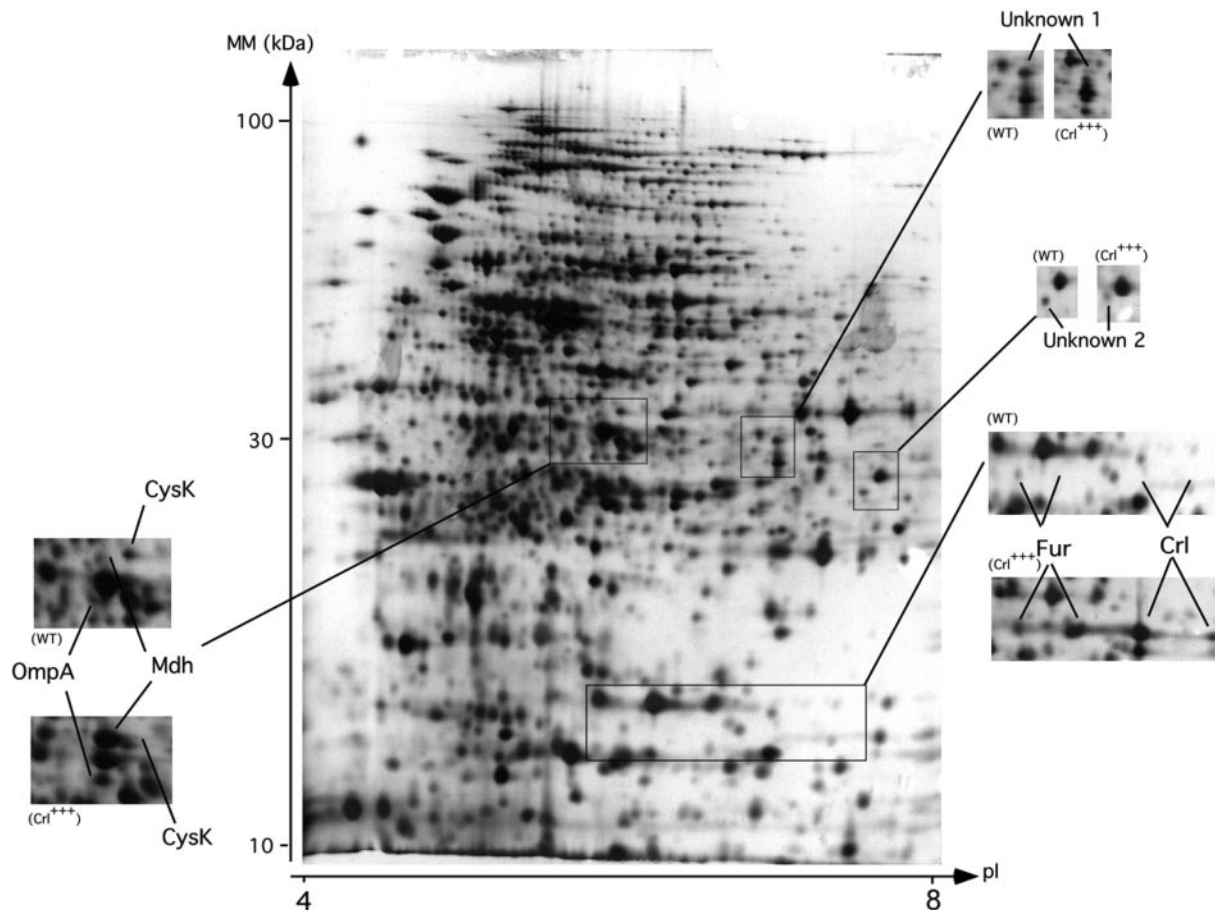


FIG. 1. Protein pattern of WT strain compared with that of the strain overexpressing Crl by 2D gel electrophoresis. 50–100 μ l of an overnight culture were spread on LB agar plates and incubated at 30 °C for 24 h. The confluent lawn of bacteria was collected, and the protein extracts, prepared as described under “Materials and Methods,” were separated by 2D gels electrophoresis. The *insets* focus on parts of each gel, comparing crude extracts of the WT strain (containing the PCR11-Topo plasmid without insert) and the strain overexpressing Crl. *MM*, molecular mass.

β -Galactosidase Assays—Putative promoter regions were amplified using PCR to produce fragments extending from \approx 200 bp upstream and \approx 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′-ggaattccaccagcgtcgtcaaaactg-3′) and *fur3* (5′-cgggatcccgggctatcatttcgaag-3′), *crl7* (5′-cgggatcccgtccggtagttcataacc-3′) and *crl8* (5′-tccaccagcccagaatt-3′), *ompA1* (5′-cgggatcccggccaccagacaacttcc-3′) and *omp2* (5′-ggaattccgggcattgtttgat-3′), and *mdh1* (5′-cgggatcccgtcagccctgaagaaggct-3′) and *mdh2* (5′-ggaattccgggatggctcagatcg-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 μ l 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 μ 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::Cm^r*, *rpoS::Kn^r*, *fur::Cm^r*,

crl::Cm^r rpoS::Kn^r, and *rpoS::Kn^r fur::Cm^r*) 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 His₆-Fur and His₆-Crl—The coding sequence of *crl* gene was cloned on a His₆ 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′-actgataacaataaccgcc-3′) and *fur6* (5′-ttatttcctcgtgcgc-3′). The PCR fragment was cloned into the His₆ tag pQE-30 vector, yielding the pHis₆-Fur plasmid, which was sequenced for verification. The purification of His₆-Crl protein has been described by Bougdour *et al.* (15). His₆-Fur protein was purified using the same protocol (binding of crude extracts from *E. coli* strains M15 to a Ni²⁺-NTA column). The proteins were eluted under native conditions with imidazole. The major His₆-protein fusion peak was recovered at 250 mM imidazole. As expected, His₆-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-HCl, pH 7.5, 0.1 mM EDTA, 150 mM KCl, 5 mM CaCl₂, 5 mM MgCl₂, and 0.1 mM DTT). The proteins were >95%

TABLE II
Protein quantification and identification by mass spectrometry

2D gel analysis					
Protein	Function	Phenotype or ratio Crl ⁺⁺⁺ /WT	<i>p</i> value (Student's <i>t</i> test)	pI/molecular mass (kDa)	
				Observed	Calculated
Crl	Regulatory protein for curli	Appearance	0.018	7.4/15.5	6.3/15.5
Crl	Regulatory protein for curli	Appearance	0.049	6.8/15.5	6.3/15.5
Fur	Ferric uptake regulator	12.9	0.04	6.35/16	6.65/16.6
Fur	Ferric uptake regulator	Appearance	0.03	5.9/16	6.65/16.6
CysK	Cysteine synthase A	Disappearance	0.001	5.9/40.3	5.7/34.3
OmpA	Outer membrane protein	15.07	<0.001	5.8/38.9	5.96/37.03
Mdh	Malate dehydrogenase	Appearance	0.008	5.8/40.5	5.56/32.2
1	Not identified	Disappearance	0.015	6.8/40.3	
2	Not identified	Disappearance	0.045	7.4/37	

Mass spectrometry results					
Protein	Swiss-Prot accession no.	Identification method	Peptides matched	Score	Coverage
					%
Crl	P24251	MS	10/30	123	57
Crl	P24251	MS	10/46	102	57
Fur	P06975	MS	7/39	55	51
CysK	P11096	MS	12/30	93	34
OmpA	P02934	MS	13/52	92	33
Mdh	P61889	MS	11/27	98	33

pure as judged by silver-stained protein gels. Glycerol was added to a final concentration of 10% (v/v), and the samples were stored at -20°C until use. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad) and bovine serum albumin as standard.

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 Crl and Fur as described above. The blots were developed with ECL (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'-gttcttcaggccagct-3'), F3 (5'-aatgtcccgggtttcaag-3'), C1 (5'-gtggagctgaatcgctat-3'), and C2 (5'-atctgctctctgggtgtc-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 \times TA buffer (40 mM Tris base, 20 mM acetic acid), 0.2 $\mu\text{g/ml}$ heparin, 5 mM DTT, 50 mM KCl, 10% glycerol, 5 nM (0.097 pmol) C1C2 PCR product, or 6 nM (0.12 pmol) F2F3 PCR product, 3.5 μM (0.8 μg) His₆-Fur, and from 0.51 to 2.6 μM His₆-Crl for 30 min at room temperature. The samples were subjected to electrophoresis on a 5% non-denaturing polyacrylamide gel in 0.5 \times 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 $^{\circ}\text{C}$), and expression begins at the entry into stationary phase (15). Furthermore because this regulator controls the transcription of the curli genes, which are in-

involved in cell adhesion, the most appropriate conditions for studying the Crl regulon are stationary phase cells grown at 30 $^{\circ}\text{C}$ 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

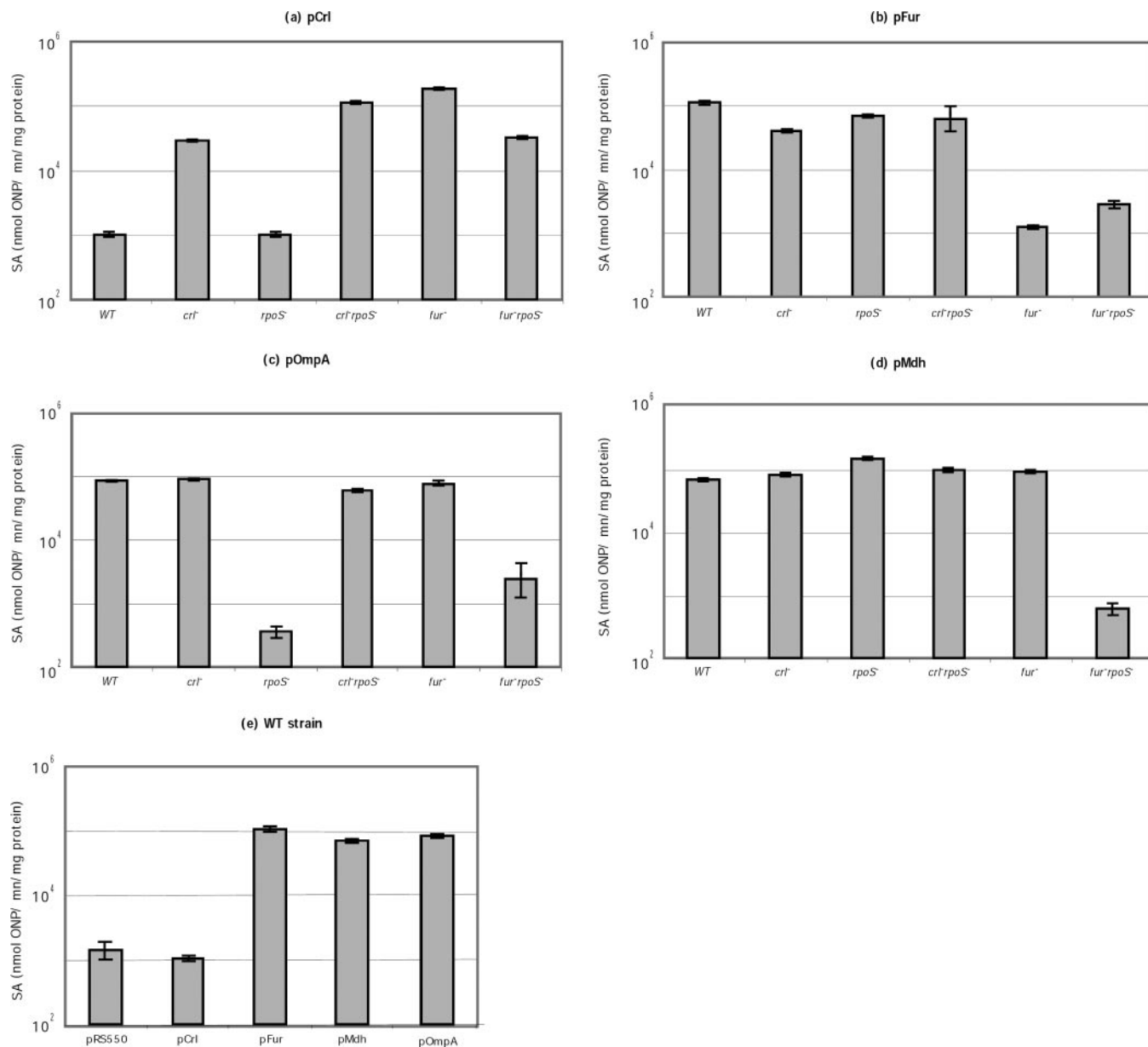


FIG. 2. **Transcriptional activity of *crl*, *fur*, *mdh*, and *ompA* promoters.** β -Galactosidase activities were measured as described under "Materials and Methods." The histograms in a–e represent promoter activities in each genetic context. The values are the average of at least three independent 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.

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 σ^S has never been described, but no extensive transcriptional



FIG. 3. Co-purification of Fur with His₆-Crl revealed by immunoblotting using antibodies raised against Crl or Fur. Immunodetection of Fur (A) and Crl (B) separated on a 12% acrylamide gels is shown. Lanes A1–A4 contain crude extracts of WT, *crl*[−], *fur*[−], and *rpoS*[−] strains, respectively. Lanes A5–A8 and B1–B4 contain elution fractions from different loads on Ni²⁺-NTA columns: lanes A5 and B1, elution with 250 mM imidazole of a column loaded with crude extract from cells that did not express the fusion protein; lanes A6 and B2, elution with 250 mM imidazole of a column loaded with crude extract from cells that expressed His₆-Crl protein; lanes A7 and B3, washing fractions containing 20 mM imidazole before elution; lanes A8 and B4, elution with 250 mM imidazole of a column loaded with crude extract from cells expressing the His₆-Fur protein. In lane A8, Fur runs as a double band, corresponding to native Fur and His₆-Fur. The faster running band probably corresponds to a degradation product of His₆-Fur in the overexpression strain. Ab, antibody; MW, molecular mass.

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 autoregulated 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—Fur regulates transcription of target genes by binding to specific sites upstream or overlapping the promoter, whereas Crl physically interacts with RpoS (and not with σ^{70}) 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 σ^{70} 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. His₆-Crl, added to equimolar concentrations with His₆-Fur, modified the interaction of His₆-Fur with the *fur* 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 μ M His₆-Crl (3.6 μ M Fur in the reaction), a slower migrating complex appeared, and the proportion of free DNA decreased 2-fold when 0.88 μ M or more His₆-Crl was included in the reaction. A control experiment in which His₆-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 overexpression 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

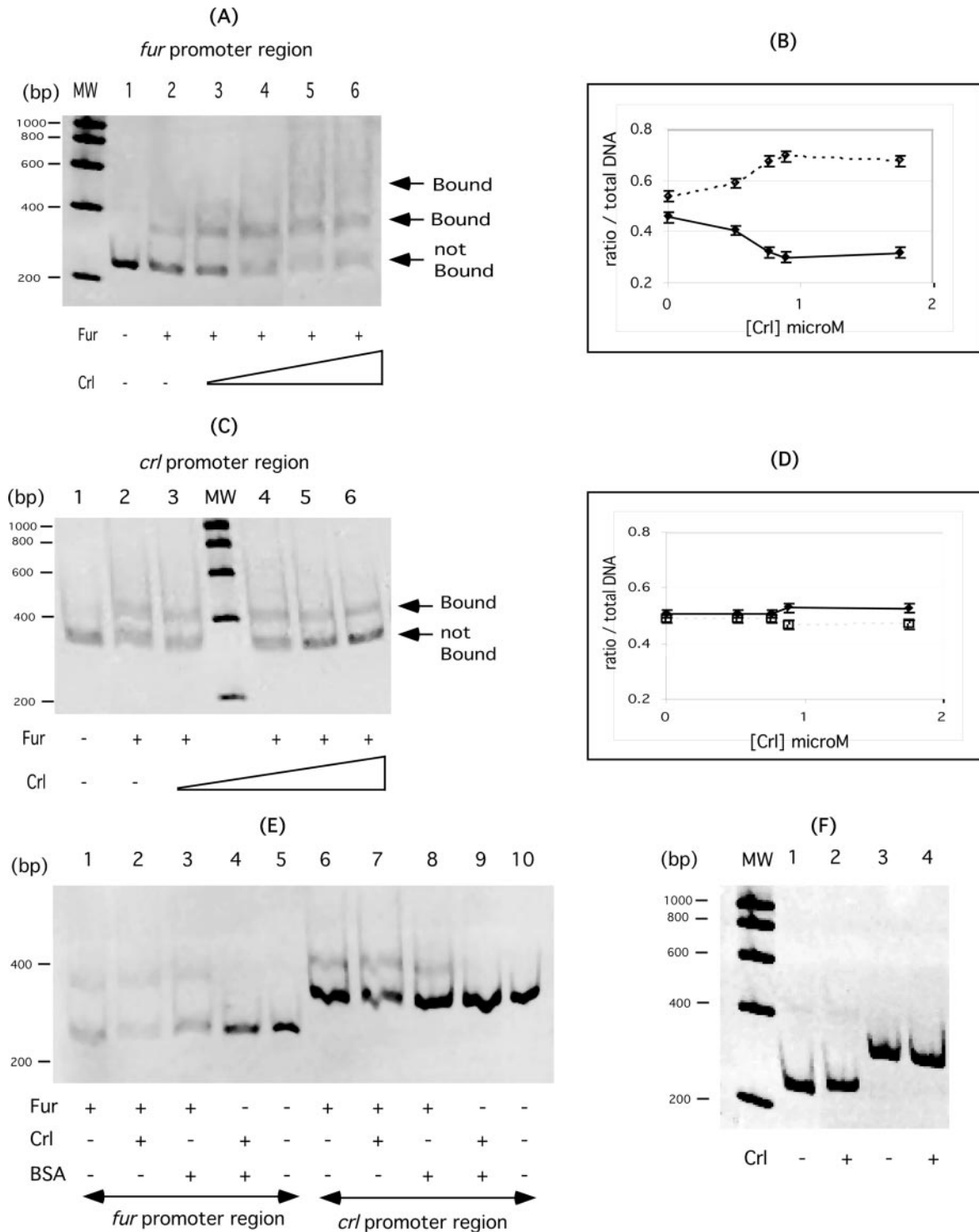


FIG. 4. **Crl binds to Fur at the *fur* and *crl* promoters.** A and C, 5 nm *crl* or 6 nm *fur* promoters were run on a 5% polyacrylamide gel alone (lane 1) or after incubation with 3.6 μ M purified His₆-Fur (lane 2) or with 3.6 μ M purified His₆-Fur and increasing concentrations of purified His₆-Crl, 0.51, 0.76, 0.88, 1.75, and 2.6 μ M (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 His₆-Fur and His₆-Crl. Lanes 1 and 6, *fur* and *crl* promoter DNA (5–6 nm) incubated with 3.6 μ M purified His₆-Fur, respectively; lanes 2 and 7, DNA incubated with 3.6 μ M purified His₆-Fur and 2.6 μ M purified His₆-Crl; lanes 3 and 8, DNA incubated with 3.6 μ M purified His₆-Fur and 3.6 μ M BSA; lanes 4 and 9, DNA incubated with 2.6 μ M purified His₆-Crl and 3.6 μ M 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 μ M purified His₆-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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