The Crl-RpoS Regulon of Escherichia coli*§

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The RpoS subunit of RNA polymerase controls the expression of numerous genes involved in stationary phase and in response to different stress conditions. The regulatory protein Crl increases the activity of RpoS by direct interaction with the RpoS holoenzyme. To define the extent of the Crl regulon, we used two-dimensional SDS-PAGE to measure the role of Crl in regulating the expression of the Escherichia coli proteome in stationary phase at 30 °C. By comparing the proteome of four strains (wild type, crl−, rpoS−, and crl−rpoS−), we observed that the intensity of 74 spots was modified in at least one mutant context. 62 spots were identified by mass spectrometry and correspond to 40 distinct proteins. They were classified in four main categories: DNA metabolism, central metabolism, response to environmental modifications, and miscellaneous. Three proteins were specifically involved in quorum sensing: TnaA (the tryptophanase that converts tryptophan to indole), WrbA (Trp repressor-binding protein), and YgaG (homologous to LuxS, autoinducer-2 synthase). Because little is known about the regulation of Crl expression, we investigated the influence of diffusible molecules on the expression of Crl. Using Western blotting experiments, we showed that, at 30 °C, a diffusible molecule(s) produced during the transition phase between the exponential and stationary phases induces a premature expression of Crl. Indole was tested as one of the potential candidates: at 37 °C, it is present in the extracellular medium at a constant concentration, but at 30 °C, its concentration peaks during the transition phase. When indole was added to the culture medium, it also induced prematurely the expression of Crl at both the transcriptional and translational levels in a Crl-dependent manner. Crl may thus be considered a new environmental sensor via the indole concentration. *Molecular & Cellular Proteomics 6:648–659, 2007.

Bacteria can grow indefinitely as long as they find sufficient nutrients in their environment. Their rapid division results in the depletion of nutrients, which in turn leads to a starvation-induced growth arrest called stationary phase. During the transition between the exponential and stationary phases, the metabolism and global physiology of the cell change dramatically. These changes are largely provoked by modifications in the pattern of gene expression: numerous genes are no longer expressed (many of the housekeeping genes, for example), and others, necessary for survival in starvation conditions, are expressed. This transition is controlled by several regulatory proteins, including transcriptional regulators. One of the most important of these is the σ factor RpoS, which, bound to the RNA polymerase core enzyme (E), allows the recognition and binding to specific promoter regions and subsequent transcription of these genes. RpoS is also induced in response to many other stress conditions such as oxidative stress, high temperature, or modification of the osmolarity of the medium (1, 2). The expression of RpoS is controlled at different stages: transcriptional, translational, and post-translational (for a review, see Ref. 3).

A small protein, Crl, stimulates the activity of RpoS, leading to an increased transcription rate of some genes of the RpoS regulon in stationary phase (4). We have recently shown that Crl interacts directly with RpoS, and this interaction promotes the binding of the holoenzyme (Eσr) to the csgBA promoter (5). These latter genes code for the subunits of the curli proteins that form fibers at the cell surface called fimbriae, which are involved in cell-cell attachment and adhesion to extracellular matrices. We have demonstrated that Crl is expressed in particular conditions: low temperature (30 °C) and stationary phase as Arnqvist et al. (6) have already shown for curli. Pratt and Silhavy (4) have shown with different mutants, rpoS−, crl−, and crl−rpoS−, that the genes of the curli operon are not the only genes regulated by Crl and RpoS: for example, poxB and ompF are also regulated by Crl and RpoS. RpoS is known to regulate the expression of many proteins, between 60 and 100 depending on the authors.

Using a proteomics approach (2D1 gel electrophoresis) we show here that Crl, in coordination with RpoS, specifically regulated the expression of 63 proteins in advanced stationary phase at low temperature. We identified by mass spectrometry 31 of these proteins, and we classified them into four main functional categories: response to environmental modification, central metabolism, DNA metabolism, and miscellaneous. We focused in particular on the detection of environ-

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1 The abbreviations used are: 2D, two-dimensional; WT, wild type.
molecular signals, and we showed that the Crl regulon mediates a signal transduction event initiated by indole, a signaling molecule thought to be important during the interaction of bacteria with eukaryotic cells.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The genotypes of *Escherichia coli* K12 strains and plasmids used in this study are listed in Table I. Media were used were Luria-Bertani broth with 5 g/liter NaCl (LB) and LB plates containing 12 g/liter agar (LA) (7). All clones were stored at −80 °C in a 20% glycerol solution.

Crude Extract Preparation—50–100 μl of the overnight cultures of the different *E. coli* strains were plated on an LA plate 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. The soluble protein extracts were prepared, and two-dimensional electrophoresis was performed as described previously (8). Briefly the cell pellets were resuspended in 200 μl of SDS solution (0.05 M Tris, 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 mM 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 samples were stored at −20 °C until use. Protein concentrations were determined before and after storage using the Bradford protein assay kit (Bio-Rad) and bovine serum albumin as standard.

2D Gel Electrophoresis—150 μg of each protein extract were focused isoelectrically on an 18-cm pH 4–7 immobilized pH gradient (homemade IPG (9)) for 65,000 V-h using the Multiphor II system (Amersham Biosciences) at 20 °C. In the second dimension, proteins were separated on a 12% SDS-PAGE gel running at 10 °C and 25 V for 1 h and then at 12.5 watts/gel for 5 h. Finally the gels were stained using silver nitrate (8). The gels were digitized on an Agfa Arcus II scanner. The experiments were repeated at least three times for each strain using two independent cultures. The spots were quantified using Melanie software. The software quantifies spots as Gaussian molecule thought to be important during the interaction of bacteria with eukaryotic cells.

**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 submitted to drying. 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 alkylating 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 incubation overnight at 37 °C.

**MALDI-TOF MS Analyses and Identification of Proteins**—For MALDI-TOF MS analyses, a 0.5–μl aliquot of the 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.

MALDI-TOF MS spectra were recorded automatically using an Autoflex MALDI mass spectrometer (Bruker Daltonics) in reflector/delayed extraction mode. The spectra were acquired in the mass range 0–4200 Da and calibrated externally 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 adrenocorticotropic hormone clip 18–39, m/z 2465.20 Da). For each sample, spectrum acquisition was obtained with an average of 200 laser shots and a resolution of about 8500. Peak lists in Extensible Markup Language (XML) data format were created using XMass software (Bruker Daltonics) and were searched using the MASCOT search engine. The Swiss-Prot TrEMBL database was searched. Peaks above the threshold were considered as significant. The following search parameters: peptide tolerance of 0.1 Da, mass accuracy of 100 ppm, and fixed carbamidomethylation of cysteine, variable oxidation of methionine and acetylation of N terminus. All peptide masses were assumed to be monoisotopic and [M + H]+ (protonated molecular ions). No restrictions were applied on protein molecular weight and pI value. A 95% confidence level threshold was used for MASCOT protein scores. When a protein is characterized by a MASCOT score higher than 70 and a coverage higher than 20%, the protein is automatically validated. When one of these criteria is not met, a manual inspection of the data is needed based on the error between the experimental and theoretical mass values from each peptide of the peptide mass fingerprint, the number of miscleavages, and the annotation. For more details, see Supplemental Table 1.

**LC-MS/MS Analyses**—For LC-MS/MS analyses, tryptic peptides were resuspended in 0.5% aqueous trifluoroacetic acid. The samples were injected into a CapLC (Waters) nano-LC system and first pre-concentrated on a PepMap C18 precolumn. The peptides were then eluted onto a C18 column. The chromatographic separation used a
gradient from solution A (2% acetonitrile, 98% water, 0.1% formic acid) to solution B (80% acetonitrile, 20% water, 0.08% formic acid) over 35 min at a flow rate of 200 nl/min. The LC system was directly coupled to a Q-TOF Ultima mass spectrometer (Waters). MS and MS/MS data were acquired and processed automatically using MassLynx 4.0 software. An intranet 1.9 version of MASCOT software was used for database searching with the following parameters: database, Swiss-Prot TrEMBL; enzyme, trypsin; one miscleavage allowed; variable modifications, acetyl (N terminus)/oxidation (Met)/carbamidomethyl (Cys)/false mass assignment + 1; peptide tolerance, 0.4 Da; MS/MS tolerance, 0.4 Da; monoisotopic; peptide charge, 2 + and 3 +; data format, .pkf; instrument, ESI-Q-TOF. Proteins, which were identified with at least two peptides both showing a score higher than 40, were validated without any manual validation. For proteins identified by only one peptide having a score higher than 40, the peptide sequence was checked manually. Peptides with scores higher than 20 and lower than 40 were systematically checked and/or interpreted manually to confirm or cancel the MASCOT suggestion.

Preparation and Growth in Conditioned Medium—A sufficient volume of an overnight E. coli WT, crl- or rpoS- culture (grown in LB medium) was added to 50 ml of fresh LB medium to obtain an initial A600 of 0.1 and was grown at 30 °C with shaking. A600 of each culture was measured all along the growth. After 3.6, or 24 h of growth, the culture was centrifuged at 4000 × g for 30 min at 4 °C. We added 5 g/liter tryptone and 2.5 g/liter yeast extract, corresponding to 0.5 × LB medium concentration, to each recovered supernatant. The pH was adjusted to 7.5. The conditioned medium was then filtered through a 0.2-μm membrane and stored at 4 °C until use.

An overnight E. coli W3110 (wild type strain) culture (grown in LB medium) was added to an initial A600 of 0.1 to each conditioned medium and grown at 30 °C with shaking. A600 was followed during growth. For Western blot analysis, 10 ml of the culture were centrifuged after 3, 6, or 24 h of growth for 4000 × g for 30 min at 4 °C. The pelleted cells were stored at −20 °C until crude extract preparation as described in the previous paragraph.

Immunoblot Analysis—To measure Crl expression, culture aliquots were collected throughout growth in appropriate media, i.e. conditioned media, 0.5 × LB, or LB containing 1 mM indole. Crude extracts were prepared as described above. Protein concentration was measured using the Bradford assay kit (Bio-Rad). 20 μg of each crude extract was separated on a 12% SDS-PAGE gel and then electrotransferred (Bio-Rad system) onto nitrocellulose or polyvinylidene membranes (Amersham Biosciences). The immunoblot analysis was performed with polyclonal antibodies raised against Crl (5). The blots were developed with ECL (Amersham Biosciences), and staining intensity was quantified with ImageGauge (FujiFilm) software. A nonspecific band recognized systematically by antibodies raised against Crl was used to normalize the band intensity of the Crl protein. The intensity of this nonspecific band is proportional to the total quantity of crude extract loaded on the gel as shown by a serial dilution of the crude extract (data not shown). Furthermore, for any particular growth phase, the intensity of the nonspecific band is identical for all strains tested.

Indole Assays—Indole was quantified in the supernatant of different E. coli WT, crl- or rpoS- cultures during growth in LB medium (10). Briefly an overnight culture was used to inoculate 20 ml of LB medium, and the cells were grown at 30 and 37 °C. 1-ml aliquots were removed every 1.5 h and centrifuged to remove the cells. 200 μl of recovered supernatant were added to 200 μl of 0.5 N perchloric acid and centrifuged again to remove the precipitated proteins. The amount of indole was measured by the addition of 400 μl of Ehrlich’s reagent (11). After incubation at 37 °C for 30 min, the absorbance was read at 571 nm. The indole concentration was then determined with reference to a standard curve in the range of 1–10 μM indole.

RESULTS AND DISCUSSION

2D Protein Gels Identify Part of the RpoS and Crl Regulons

To identify E. coli proteins regulated by RpoS and Crl proteins, we cultured wild type E. coli W3110 and their isogenic crl-, rpoS- and crl-rpoS- mutants (Table I) on LB plates. Crude protein extracts from the four strains, WT, crl-, rpoS- and crl-rpoS-, grown on an LB plate at 30 °C for 24 h were prepared as described under “Experimental Procedures.” The whole cell extracts were analyzed on 2D protein gels. Approximately 1000 spots were detected on a gel image. The experiment was performed three times with two sets of independently grown cultures. The gels of the wild type crude extracts were used as standards, and each spot observed on mutant extracts was compared with the standard. Only spots showing the same phenotype in three independent experiments were retained and quantified using the Melanie software (Fig. 1 and Table II). Fig. 1a lists the 74 spots satisfying the following criteria: their intensity increased, decreased, or even disappeared in comparison with the wild type strain in at least one of the three mutant strains (Fig. 1b and Table II). An enlargement of a portion of the gel illustrates this result. A detailed analysis of the spots showed that many proteins are regulated directly or indirectly either by RpoS or by both Crl and RpoS. 62 of 74 spots were identified by mass spectrometry and correspond to 40 distinct proteins. 15 proteins were represented by several spots (between two and four); this was probably due to uncharacterized post-transcriptional modifications. Protein carbonylation, for example, is an irreversible modification that is typical of “aging” proteins and increases considerably during bacterial growth arrest. Because all our samples were from cultures in advanced stationary phase it may be not surprising that we observed a relatively large proportion of protein modifications due to carbonylation or other reactions. We did not further pursue the characterization of these different protein forms.
We classified the proteins into four main categories depending on their roles in the cell: (i) response to environmental modifications, (ii) central metabolism, (iii) DNA metabolism, and (iv) miscellaneous. Some proteins belong to two categories. A comprehensive summary of the observed expression changes is given in Table II. Proteins were further characterized as being dependent only on RpoS or dependent on both RpoS and Crl. Some of the proteins are already known to be

**Fig. 1.** a, 2D gel electrophoresis of the WT strain. The spots changing intensity in a mutant strain are circled and numbered. Their identifications are summarized in Table II. b, the four smaller panels are an illustration of the observed phenotypes in different genetic contexts. MM, molecular mass.
### Table II

Proteins differentially expressed in the three mutant strains, crl\(^{-}\), rpoS\(^{-}\), and crl-rpoS\(^{-}\), with the wild type strain (W3110)

Spot intensities were quantified using Melanie software on three independent experiments. Only reproducible phenotypes, with a Student’s \(t\) test value of \(p \leq 0.05\), are shown. Four spots have a \(p\) value \(> 0.05\) and are indicated with a footnote. PTS, phosphotransferase system; SU, subunit.

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Protein name</th>
<th>Ratio</th>
<th>Protein function</th>
<th>Functional category</th>
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<tr>
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<td>Not identified</td>
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<td>0.15</td>
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<tr>
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<td>FadL</td>
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<td>TnaA</td>
<td>1.36</td>
<td>2.49</td>
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</table>

"The Crl-RpoS Regulon of \(E. coli\)"
directly or indirectly regulated by RpoS. They will be detailed later. Proteins were considered to be regulated by Crl and RpoS when (i) they displayed changes in expression in crl and in rpoS mutants or (ii) when the phenotype observed in the double mutant, crl/rpoS, was different from those of the single mutants, crl or rpoS. 

### Proteins Involved in Central Metabolism—
27 spots, corresponding to 18 proteins, are involved in the central metabolism. This concerns glycolysis (PfkA, FbaB, and PykF), the Krebs cycle (GltA, SdhB, Aat, SucA, and SucD), fatty acid metabolism and transport (FadL, AccB, and AccC), amino acid metabolism and transport (GlnH, WrbA, and TnaA), and sugar metabolism and transport (MalE, Crr, and UgpB) (Table II and Fig. 2). Nine proteins were dependent on RpoS only, and 10 were dependent on both RpoS and Crl. None of them were regulated by Crl only. All the proteins involved in the utilization of carbon sources (amino acid or sugar) were under the control of both RpoS and Crl. The role and regulation of TnaA (tryptophanase) and WrbA (Trp repressor-binding protein), proteins that can also be classified in the category "response to environmental changes," will be discussed below.

### Proteins Involved in DNA Metabolism and Protection—
Nine spots, corresponding to six proteins, were more or less directly necessary for DNA metabolism. GyrB (DNA gyrase), Ndk (nucleoside-diphosphate kinase), and GidA (glucose-inhibited division) are dependent only on RpoS and Ssb (single strand-binding protein), Ppa (inorganic pyrophosphatase), and Dps (DNA protection) were Crl- and RpoS-dependent. Dps, which protects DNA against oxidative stress, could also be attributed to the category response to environmental changes (13–15). Ssb and GyrB are also known to participate in the repair of DNA damage.

### Proteins Involved in the Response to Environmental Changes—
28 spots, corresponding to 15 proteins, are involved in the response to environmental modifications. Only five of them were only dependent on RpoS. Three of those, Crr, OmpF, and FadL, are transporter proteins or porins (16–18). Four others transporters were dependent on both Crl and RpoS: MalE, UgpB, CstA, and OmpX. Among the environ-
mental modifications, most of the identified proteins are also involved in the response to environmental stresses: UspA and YbdQ, which were both Crl- and RpoS-dependent, are universal stress proteins; OsmC and OsmY are induced during drastic osmotic changes (19, 20); GrxB (glutaredoxin) and Bfr and Dps (bacterioferritins) are necessary for the cellular response to starvation conditions (21) or oxidative stress (22), respectively. Some of these proteins were already known to be regulated by RpoS: in a negative manner via an indirect mechanism such as ompF (23) and uspA (24) or directly and in a positive manner such as dps (14), osmY (20, 25–27), p oxB (28, 29), wrbA (30), osmC (31), bfr (32), fadL (24), ugpB (33), or tnaA (32). Only two of them were known to be regulated by Crl and RpoS: poxB and ompF (4). Three other genes were directly regulated by RpoS: talA (34), fbaB (32), and ygaU (34).

Two proteins, YgaG, the homolog of LuxS (autoinducer-2 synthase), and TnaA (tryptophanase), are known to be involved in cell-to-cell signaling, more specifically in quorum sensing (10, 32, 35, 36). Both were dependent on Crl and RpoS. Quorum sensing enables a population of bacteria to

Fig. 2. Schematic representation of the metabolic pathways including the proteins that are differentially expressed in mutant contexts. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; F1,6bisP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; 4P, 4-phosphate; 7P, 7-phosphate.
collectively control gene expression, thereby coordinating activities that are productive only at a high population density (for a review, see Ref. 37). This process is accomplished through the production, secretion, and detection of small chemical signals called autoinducers. One such molecule involved in intercellular signaling is indole, and two of the genes that we identified participate in the indole metabolism. It has already been shown that both WrbA (Trp repressor-binding protein) and TnaA are controlled by RpoS (32, 38). Recently Patridge and Ferry (39) have shown that WrbA is involved in the two-electron reduction of quinone, protecting against oxidative stress. TnaA, a tryptophanase, degrades tryptophan, resulting in the formation of indole. Indole has been proposed to act as an extracellular signal in stationary phase in E. coli (10, 36). Production of indole, via the enzymatic activity of TnaA, is also induced during biofilm formation (30). Because of all these data we decided to classify TnaA and WrbA in the category response to environmental changes.

These proteomics data prompted us to investigate the interrelation between Crl and quorum sensing. Very little is known about the regulation of Crl expression. Bougdour et al. (5) have shown that Crl is preferentially expressed at 30 °C and during the transition between the exponential and stationary phases of growth. It is therefore clear that the expression of Crl responds to environmental signals. However, the exact signal transduction pathway connecting the environmental signal to the control of Crl expression has not been elucidated yet. In the previous paragraph, we showed that the absence of Crl modifies the expression of proteins involved in quorum sensing. We therefore decided to investigate in more detail the influence of diffusible molecules on the expression of Crl.

**Crl Expression Is Modified in Conditioned Media**

As a starting point for assessing the environmental signal initiating the signal transduction pathway leading to Crl activation we measured the influence of the composition of the growth medium on Crl expression. In particular, we wanted to test the influence of potential diffusible molecules produced by the bacteria themselves. We therefore grew the WT strain (W3110) in conditioned media that had already served as a substrate for the growth of strains with different genetic backgrounds.

The growth curves of the W3110 strain in conditioned media obtained at the transition stage of crl− and WT cultures were similar to the growth curve in 0.5× LB medium (data not shown). In a reproducible way, in the conditioned medium prepared from an rpoS− strain, we observed a decrease of A600 after the beginning of the transition phase. However, in stationary phase (after 24 h of growth), cells reached the same A600 as in other conditioned media. No difference was observed in conditioned media derived from an exponentially growing culture (3 h) or from stationary phase (24 h) (data not shown).

We then measured Crl protein expression in the W3110 strain after growth in different conditioned media by immunoblot analysis with polyclonal antibodies raised against His6-Crl (5). All along the growth in conditioned media recovered after 3 h of growth the expression of Crl was identical to the that observed in fresh LB or 0.5× LB (data not shown). In conditioned media recovered after 6 h (transition stage) and 24 h (later stationary stage), the observed phenotypes were similar: for both of these media the expression rate of Crl was increased (Fig. 3) in comparison with growth in LB (5) or 0.5× LB. After 3 h of growth, no or very little Crl was detected in our conditions in LB medium (5) or 0.5× LB medium (Fig. 3, lane 2). In conditioned media, the expression of Crl increased 10–15 times compared with the control condition in 0.5× LB (Fig. 3, lane 5 compared with lane 2). After 6 h of growth, little or no increase was detectable in conditioned media recovered from the wild type strain compared with the expression after 3 h of growth (Fig. 3, lane 9 compared with lane 5). In contrast, the conditioned media recovered from crl− and rpoS− strains led to an increase of Crl expression by a factor of 2 and 1.5 compared with the expression after 3 h of growth in the same media, respectively (Fig. 3, lanes 7 and 8 compared with lanes 3 and 4). In summary, Crl expression was prematurely induced by a diffusible molecule, the production of which was under the control of Crl and RpoS during the transition between exponential and stationary phases of growth.

**Indole Concentration**—Our proteomics analysis had shown that TnaA expression was induced in stationary phase in crl− and rpoS− strains. Nishino et al. (38) and Lacour and Landini (32) have shown that RpoS controls the expression of TnaA. In the previous paragraph, we showed that Crl expression is modulated by a diffusible molecule. Indole is the product of the degradation of tryptophan by TnaA. Indole therefore seems to be a likely candidate of the diffusible molecule at the inducing signal transduction pathway leading to Crl activation. We specifically addressed this possibility by measuring the effect of exogenous indole on the expression of Crl.

**Indole Modifies Crl Expression**—We first measured the indole concentration in media during the growth at two temperatures, 30 and 37 °C. At 37 °C, whatever the strain (WT, crl−, or rpoS−), the indole concentration was constant, around 3 μM, during the entire growth (Fig. 4a). At 30 °C, we observed an increase of the indole concentration at the transition stage (around 6 h of growth) in all strains tested. This peak occurred just 1 h before the peak of Crl expression. We hypothesized therefore that indole may act as an inducer of Crl expression. To test this hypothesis, we measured the transcription of the crl gene after the addition of different concentrations of indole (100 nM to 1 mM). From 100 nM to 5 μM of indole, no effect was observed on crl transcription (data not shown). In any genetic background, the transcription of the crl gene was increased in the presence of at least 50 μM indole. Fig. 4c shows the expression profile of crl after the addition of 1 mM indole. The
temporal profile of cfr transcription corresponded to the protein levels measured by Western blot (5): the promoter was activated at the end of exponential phase and in early stationary phase.

We confirmed the role of indole for Crl expression by analyzing the intracellular concentration of Crl by immunoblots. We compared Crl protein expression during growth in the absence or presence of 1 mM indole. The results were similar at all stages of growth (exponential, transition, and stationary phases): the presence of an excess of indole increased Crl expression in all cases (Fig. 4). In summary, indole is a diffusible molecule produced by the cells that induced Crl expression during the transition between the exponential and stationary phases of growth at 30 °C in a Crl- and RpoS-dependent manner.

**Concluding Remarks**

Until now, the role of Crl was thought to be restricted to the regulation of curli expression. However, our global analysis showed that Crl modulates the expression of a considerable portion of the RpoS regulon. Our data showed that Crl is involved in regulating many aspects of cellular metabolism through its interaction with RpoS, the central regulator of stationary state. We have shown previously that Crl and RpoS interact directly to promote the expression of the curli operon (5). In this study, we showed that this same interaction is probably involved in the regulation of the expression of many other genes via RpoS.

We analyzed the modifications of protein expression during stationary state on solid medium (Petri dish) at 30 °C, i.e., growth conditions known to induce expression of RpoS and Crl. Many of the identified target genes are specific to the stationary state or exhausted medium. The vast majority of changes have already been observed in the rpoS mutant, but Crl seems to modulate or increase these effects. The regulatory influence of Crl was clearly visible, but the effect of the Crl regulation was completely masked in the rpoS/H11002 strain because the action of Crl depends on interactions with RpoS and therefore on the presence of RpoS.

We also noticed that many transporters are controlled by Crl: MalE, UgpB, and GinH. Furthermore among the genes involved in the response to environmental modification, we identified several proteins involved in quorum sensing: YgaG, TnaA, and WrbA (40). We further investigated this aspect of environmental sensing via Crl. Lacour and Landini (32) have shown that the indole molecule acts as a signal in stationary phase cells in an RpoS-dependent manner. It is also known that RpoS controls tnaA expression (32, 38). Here we further
detail one of the ways in which Crl expression is controlled: Crl is sensitive to diffusible molecule(s) produced during the transition state between the exponential phase and the stationary phase. Such molecules are called quorum-sensing signals and play a major role in cell-to-cell communication during stationary phase. 

**Fig. 4.** a, indole assays in the supernatant of cultures grown at 37 °C (triangles) and 30 °C (circles). Values are the average of at least two independent experiments in three strain backgrounds: WT, rpoS−, and crf−. A typical growth curve at 30 °C is shown (squares). b, immunoblot analysis of WT crude extract recovered after 6 h of growth in LB medium supplemented (lane 2) or not (lane 1) with 1 mM indole. Lane 3 contains crude extract of crf− strain grown in LB medium for 6 h. The amount of Crl was quantified by normalizing the Crl band (17 kDa) to the nonspecific band (72 kDa) indicative of gel loading. c, bioluminescence per cell of the pSBluc-crl vector transformed in different genetic backgrounds: WT, crf−, and rpoS−. The circles and triangles show the growth curves and the stars and squares show the reporter gene activities without and in the presence of 1 mM indole, respectively. Dashed and solid lines are fits to the data with and without indole. The error bars are the S.E. rel., relative.
and in different processes such as biofilm formation or virulence. We showed that indole is one of the inducers that modulate the expression of Crl. Crl may thus be considered a new sensor of the environment via indole. Because Crl modulates the activity of RpoS, this signal modulates the expression of many other genes involved in cell-to-cell communication via the RpoS regulon. Numerous experiments have shown that the expression of RpoS is affected by quorum-sensing molecules, such as lactones (3), although a clear picture of this mode of environmental sensing by RpoS has not yet emerged. Our results add another component to this highly regulated system. Quorum sensing may not only affect the expression directly but also via Crl. We will now test the influence of other diffusible molecules, such as autoinducer-2, on Crl expression. Crl may turn out to be a central intermediary between environmental sensing and the stress response.

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