Effect of Growth Temperature on Crl-Dependent Regulation of σ^S Activity in *Salmonella enterica* Serovar Typhimurium[▽]

Véronique Robbe-Saule, Ingrid Carreira,[†] Annie Kolb, and Françoise Norel*

Unité de Génétique moléculaire and CNRS URA2172, Institut Pasteur, Paris, France

Received 30 January 2008/Accepted 22 April 2008

The small regulatory protein Crl favors association of the stationary-phase sigma factor σ^S (RpoS) with the core enzyme polymerase and thereby increases σ^S activity. Crl has a major physiological impact at low levels of σ^S . Here, we report that the Crl effects on σ^S -dependent gene expression, the H₂O₂ resistance of *Salmonella enterica* serovar Typhimurium, and the resistance of this organism to acidic pH are greater at 28°C than at 37°C. Immunoblot experiments revealed a negative correlation between σ^S and Crl levels; the production of Crl was slightly greater at 28°C than at 37°C, whereas the σ^S levels were about twofold lower at 28°C than at 37°C. At both temperatures, Crl was present in excess of σ^S , and increasing the Crl level further did not increase the H₂O₂ resistance level of *Salmonella* and the expression of the σ^S -dependent gene *katE* encoding the stationary-phase catalase. In contrast, increasing the σ^S level rendered *Salmonella* more resistant to H₂O₂ at 28°C, increased the expression of *katE*, and reduced the magnitude of Crl activation. In addition, the effect of Crl on *katE* transcription in vitro was not dependent on temperature. These results suggest that the effect of temperature on Crl-dependent regulation of the *katE* gene and H₂O₂ resistance are mediated mainly via an effect on σ^S levels. In addition, our results revealed that σ^S exerts a negative effect on the production of Crl in stationary phase when the cells contain high levels of σ^S .

Bacteria frequently encounter limited-nutrient conditions and are exposed to other stressful conditions in their natural habitats, causing them to grow and divide slowly and to enter stationary phase. Stationary-phase physiology, particularly multiple-stress resistance, and even cell morphology are determined by the general stress response. This response is controlled at the molecular level by a sigma subunit of RNA polymerase, σ^S (for reviews, see references 12 and 14). In enterobacteria, RNA polymerase is composed of a core enzyme, E, with the subunit structure $\alpha_2\beta\beta'\omega$, which associates with one of the seven different σ subunits to form the holoenzyme (E σ). Each σ subunit targets the RNA polymerase to a different set of promoters, thereby modulating the gene expression patterns. The RNA polymerase holoenzyme containing the σ^{70} subunit is responsible for the transcription of most genes during exponential growth. When a cell enters stationary phase or when it is under specific stress conditions during the exponential growth phase (high osmolarity, low pH, or high and low temperature), σ^S , which is encoded by the *rpoS* gene, accumulates in the cell, associates with the core enzyme, and directs the transcription of genes essential for the general stress response and for stationary-phase survival (for reviews, see references 12 and 14). Regulation of σ^S occurs at the transcriptional and posttranscriptional levels and involves numerous regulators (for a review, see reference 14).

Sigma factors compete for binding to a limited amount of

the core polymerase (9, 12, 18). σ^{70} is abundant throughout the growth cycle and shows the highest affinity of all sigma factors for core polymerase in vitro (12). In contrast, the level of σ^S reaches only about one-third of the σ^{70} level upon entry into stationary phase and exhibits the lowest affinity for E of all sigma factors in vitro (12). The cell uses at least two strategies to ensure the switch between σ^{70} and σ^S in the RNA polymerase and to allow reprogramming of gene expression during entry into stationary phase. Several factors (Rsd, 6S RNA, ppGpp, and DksA) indirectly increase σ^S competitiveness upon entry into stationary phase by decreasing σ^{70} effectiveness (for a review, see reference 14). In addition, increasing the performance of σ^S involves the unconventional regulatory protein Crl.

The *crl* gene product is a regulator of σ^S activity in *Escherichia coli* (1, 8, 19, 30) and *Salmonella* (21, 22). The Crl protein binds σ^S in vitro (1) and facilitates RNA polymerase holoenzyme E σ^S formation (30), thereby enhancing σ^S effectiveness (8, 21, 22, 30). The physiological effects of Crl are greatest at low levels of σ^S (22). In addition to both transcriptomic and proteomic analyses, gene fusions experiments have indicated that members of the *rpoS* regulon exhibit differential sensitivity to Crl activation (6, 16, 22, 30). Also in vitro, the magnitude of Crl activation of gene transcription varies according to the promoter (8, 22). In *Salmonella enterica* serovar Typhimurium strain ATCC 14028, Crl is required for development of the rdar morphotype (21), a colony morphology caused by production of curli and cellulose and correlated with biofilm formation (24). However, the Δcrl mutation did not have any effect on the general stress resistance of ATCC 14028 unless σ^S levels were reduced by using a derivative of ATCC 14028 in which *rpoS* had the inefficient translation start codon TTG (22).

In this study, we showed that the effect of a *crl* mutation on the general stress resistance of ATCC 14028 is revealed upon

* Corresponding author. Mailing address: Institut Pasteur, Unité de Génétique Moléculaire, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France. Phone: 33 140613122. Fax: 33 145688960. E-mail francoise.norel@pasteur.fr.

[†] Present address: Universidade Católica-Ecola Superior de Biotecnologia, Extensão de Caldas da Rainha, Rua Mestre Mateus Fernandes, 2500-237 Caldas da Rainha, Portugal.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
<i>S. enterica</i> serovar Typhimurium strains		
ATCC 14028	Wild type	American Type Culture Collection
ATCC <i>crpS</i>	ATCC 14028 Δ <i>rpoS</i> ::Cm	21
ATCC <i>crl</i>	ATCC 14028 Δ <i>crl</i> ::Cm	21
ATCC <i>katE-lacZ</i>	ATCC 14028 Tn5	22
ATCC <i>crl katE-lacZ</i>	ATCC 14028 Δ <i>crl</i> ::Cm, Tn5B21-2.4	22
ATCC <i>crpS katE-lacZ</i>	ATCC 14028 Δ <i>rpoS</i> ::Cm, Tn5B21-2.4	22
ATCC <i>dps</i>	ATCC 14028 Δ <i>dps</i> ::Cm	This study
ATCC <i>dps-lacZ</i>	ATCC 14028 <i>dps-lacZY</i>	This study
ATCC <i>crl dps-lacZ</i>	ATCC 14028 Δ <i>crl</i> ::Cm, <i>dps-lacZY</i>	This study
ATCC <i>crpS dps-lacZ</i>	ATCC 14028 Δ <i>rpoS</i> ::Cm, <i>dps-lacZY</i>	This study
Plasmids		
pACYC184	Cm ^r Tet ^r , cloning vector	3
pACYC184-Km	Cm ^r Km ^r , identical to pACYC184 except that a 1.4-kb BamHI fragment carrying the Km cartridge from pUC4K is inserted into the BamHI site in the <i>tet</i> gene	This study
pAC <i>crl</i> -1	Tet ^r , pACYC184 with the promoterless <i>crl</i> gene cloned into the <i>cat</i> gene (<i>crl</i> is transcribed from the <i>cat</i> promoter)	21
pAC <i>crl</i> -1-Km	Km ^r , identical to pAC <i>crl</i> -1 except that a 1.4-kb BamHI fragment carrying the Km cartridge from pUC4K is inserted into the BamHI site in the <i>tet</i> gene	This study
pSTK4	Cm ^r , pACYC184:: <i>rpoS</i>	15
pUC4K	Cb ^r , Km resistance cartridge	Pharmacia

entry into stationary phase by growing cells at 28°C instead of 37°C. We showed that the effects of Crl on the hydrogen peroxide (H₂O₂) resistance of ATCC 14028 and on gene transcription are greater at 28°C than at 37°C because σ^S levels are lower at 28°C than at 37°C. Upon entry into stationary phase, Crl is more abundant than σ^S , and σ^S levels, but not Crl levels, are limiting for expression of σ^S -dependent genes. Finally, we underscored a feedback control of Crl effects; in stationary phase, when the level of σ^S is high and Crl may be dispensable, σ^S exerts a negative effect on Crl production. This control of Crl production by σ^S , together with the negative control of σ^S stability by Crl (19, 30), likely ensures tight control of, and a negative correlation between, σ^S and Crl levels.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *S. enterica* serovar Typhimurium strains used in this study are listed in Table 1. Transposon Tn5B21, a derivative of Tn5 that confers tetracycline resistance, was constructed to facilitate the creation of *lacZ* gene fusions (28). *S. enterica* serovar Typhimurium ATCC 14028 derivatives carrying Tn5B21 insertions in σ^S -dependent genes have been described previously (11, 22). Bacteriophage P22HT105/*lnt* was used to transfer mutations between *Salmonella* strains by transduction (26). Green plates, for screening for P22-infected cells or lysogens, were prepared as described previously (29). The plasmids used in this study are listed in Table 1. Strains were routinely cultured at 37°C in Luria-Bertani medium (LB) (25). Antibiotics were used at the following concentrations: carbenicillin, 100 μ g ml⁻¹; chloramphenicol, 15 μ g ml⁻¹ for the chromosomal resistance gene and 30 μ g ml⁻¹ for the plasmid resistance gene; kanamycin, 50 μ g ml⁻¹; and tetracycline, 20 μ g ml⁻¹.

Stress resistance assays. For stress resistance assays, cells were grown to stationary phase in LB at 37 and 28°C (optical density at 600 nm [OD₆₀₀], 3.5 to 4). For the oxidative shock survival assay, cells were washed and resuspended in 0.9% NaCl to an OD₆₀₀ of 1.0 (about 10⁹ CFU per ml), and H₂O₂ was added to a final concentration of 5, 15, or 30 mM, as indicated below. For the acid shock survival assay, cells were diluted 1:1,000 in LB (pH 3). In all experiments, aliquots of bacteria were removed at various times, and the numbers of colony-forming cells were determined on LB plates.

DNA manipulations. Standard molecular biology techniques were used (23, 25). DNA was sequenced by Genomexpress (France). Oligonucleotides were obtained from Eurogentec (France).

Construction of the *dps* mutant and the chromosomal *dps-lac* fusion. We created a chromosomal mutation in the *dps* gene of *Salmonella* (strain ATCC*dps*) (Table 1) using PCR-generated linear DNA fragments and the λ Red recombination method as described by Datsenko and Wanner (5). We used primers *dps*-P1 (5'-CCTGGGACACAAACATCAAGAGGATATGAGATTATGAGTACCGGTGTAGGCTGGAGCTGCTTC-3') and *dps*-P2 (5'-CTGCAACTCGAAGTATTTCAGGGTAGAGATAGATTATTCGATGCATATGAATATCCTCCTTAG-3') for disruption of the *dps* gene. Mutant candidates were characterized by using a combination of PCRs performed with locus-specific primers and common test primers (5). Finally, isogenic strains were constructed by P22HT105-mediated transduction of the mutations into the appropriate strains. We constructed a single-copy *dps-lacZY* transcriptional gene fusion from mutant ATCC*dps* (Table 1) using a conditional plasmid containing promoterless *lacZY* genes and the FLP recognition target (FRT) site as described previously (7). We then used PCR assays to ensure integration of the plasmid in the correct location and to determine the presence of multiple plasmid integrants (using common test primers, such as those described by Ellermeier et al. [7]). We also used flanking locus-specific primers to amplify junction fragments that were subsequently analyzed by DNA sequencing. Isogenic strains were constructed by P22HT105-mediated transduction of the mutations into the appropriate strains.

Electrophoresis and immunoblot analysis of proteins. Whole-cell extracts were prepared and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Silhavy et al. (27). The amount of protein in whole-cell lysates was determined using a DC protein assay kit (Bio-Rad). Equal amounts of protein were loaded in all slots. The molecular sizes of the proteins were estimated using molecular size standards (Fermantas, France). Antibodies against the Crl protein of *Salmonella* have been described previously (21). Rabbit antibodies against the σ^S protein of *S. enterica* serovar Typhimurium were obtained from Coynault et al. (4). Proteins were transferred to Hybond P membranes (Amersham Life Sciences) and incubated with the polyclonal rabbit antibody serum as previously described (4). Bound antibodies were detected using a secondary anti-rabbit antibody linked to peroxidase and an ECL plus Western blot detection system kit (Amersham Life Sciences). Quantification was performed using a PhosphorImager (Molecular Dynamics).

Enzymatic assays. β -Galactosidase activity was measured as described by Miller (17) and was expressed in Miller units.

FIG. 1. Crl effect on expression of σ^S -dependent genes at 28 and 37°C. A collection of *lacZ* transcriptional fusions in σ^S -dependent genes was constructed in *S. enterica* serovar Typhimurium strain ATCC 14028 and its Δcrl derivative ATCCcrl (22). These fusions were initially isolated from a bank of *Salmonella* mutants harboring random Tn5B21 insertions (11). The σ^S -dependent gene fusions (2.4 to G57) are labeled according to the names of the initial Tn5B21 mutants (11). The strains harboring the fusions were grown to stationary phase in LB at 37 and 28°C (OD_{600} , 3.5 to 4), and β -galactosidase activity was measured by using the method of Miller (17). The Crl effect is the ratio of the β -galactosidase activity measured in the wild-type strain to that in the *crl* mutant.

RESULTS

Greater effects of Crl on gene expression at 28°C than at 37°C. We previously showed that a Δcrl mutation does not have any effect on the general stress resistance of *S. enterica* serovar Typhimurium ATCC 14028 in LB at 37°C (22), but the development of the *rdar* morphotype in a Δcrl mutant was affected (21). Development of the *rdar* morphotype is efficient at low temperature and osmolarity (i.e., at 28°C in LB without salt [LB0]). To determine whether growth conditions modulate Crl effects, we first compared the effects of the *crl* mutation on σ^S -dependent gene expression in LB at 28 and 37°C and in LB0 at 28°C.

We used 25 chromosome-borne *lacZ* gene fusions in σ^S -dependent genes that were previously isolated from a bank of *Salmonella* mutants harboring random Tn5B21 insertions (11, 22). Expression of the σ^S -dependent fusions in ATCC 14028 was slightly lower in LB at 28°C than at 37°C (data not shown), but the magnitude of Crl activation in LB was greater at 28°C than at 37°C (Fig. 1). At 37°C, the induction levels caused by Crl varied from 1.3 for fusion 2.4 located in the *katE* gene (11) to 4.1 for fusion G57 located in the *katN* gene (20). At 28°C, expression of *katE* and expression of *katN* were induced 2.8- and 8.1-fold, respectively, by Crl (Fig. 1). The growth rate of *Salmonella* in LB was reduced by 30% at 28°C compared to 37°C, but the stationary-phase cultures reached similar optical densities at the two temperatures (OD_{600} , ca. 4.5). At 28°C, the growth rate was further reduced by 20% in LB0 compared to LB. However, the levels of induction of expression of the fusions by Crl at 28°C were in the same range in both LB0 and LB (data not shown). These results indicated that temperature had an effect on the magnitude of Crl-dependent activation of gene expression.

Greater physiological effects of Crl at 28°C than at 37°C. σ^S is required for bacterial resistance to various stresses during stationary phase (the so-called general stress resistance) (12, 14). The σ^S -dependent gene *katE* encodes a catalase, an en-

zyme that detoxifies H_2O_2 and contributes to the resistance of bacteria to oxidative stress. In stationary-phase LB cultures of *Salmonella*, KatE is the major catalase (2, 20), and in the experimental conditions used, KatE played a major role in the resistance of ATCC 14028 to H_2O_2 (Fig. 2D).

In stationary-phase LB cultures of ATCC 14028, transcription of *katE* was less efficient at 28°C than at 37°C (Fig. 3A) and more sensitive to Crl activation at 28°C than at 37°C (Fig. 1 and 3A). These findings were confirmed at the protein level by measurement of catalase activity (data not shown). To determine whether the effects of Crl and temperature on the level of expression of *katE* were relevant at the physiological level, we monitored the effect of Crl on the H_2O_2 resistance of ATCC 14028 at 28 and 37°C. Upon exposure to 5 mM H_2O_2 , the wild-type and *crl* strains survived well, and even the *katE* mutant was mildly affected (data not shown). At higher H_2O_2 concentrations (15 and 30 mM) (Fig. 2AB), the impact of Crl on the level of H_2O_2 resistance of ATCC 14028 was dependent on the growth temperature. At 37°C, the *crl* mutation had no significant effect on the H_2O_2 resistance of ATCC 14028, but it decreased the H_2O_2 resistance of this strain at 28°C.

Although the *katE* mutant was highly sensitive to H_2O_2 , the *rpoS* mutant was even more sensitive (Fig. 2D), indicating that other σ^S -dependent H_2O_2 defense mechanisms were involved in addition to the detoxifying KatE enzyme. The σ^S -dependent gene *dps* encodes a ferritin-like protein involved in *Salmonella* resistance to oxidative stress (10). As observed for the other σ^S -dependent gene fusions (Fig. 1), the *dps-lacZ* gene fusion was more sensitive to Crl activation at 28°C than at 37°C (Fig. 3A). The Dps protein was involved in the H_2O_2 resistance of ATCC 14028, but in the conditions used, its contribution was less marked than that of KatE (Fig. 2D). Lowering the growth temperature also increased the effects of the *crl* mutation on the resistance of ATCC 14028 to acidic pH (Fig. 4). Altogether, these results showed that the physiological effects of Crl are greater at 28°C than at 37°C.

Production of Crl and σ^S at 37 and 28°C. The smaller stimulatory effect of Crl at 37°C than at 28°C might be due to the fact that Crl is less abundant at high temperature. At 28°C, Crl was synthesized during the early exponential growth phase, but the Crl protein levels increased during the late exponential and stationary growth phases (21). A similar pattern of Crl production was observed in LB at 37°C, when the accumulation of Crl slightly preceded that of σ^S (Fig. 5A and B). The levels of Crl were maximal upon entry into stationary phase and showed significant reductions in overnight cultures; they were less than 1.5-fold greater at 28°C than at 37°C, and the effect was maximal at an OD_{600} of 3.5 to 4 (Fig. 6A). The σ^S levels were about twofold lower at 28°C than at 37°C as cells entered stationary phase (OD_{600} , 2.5 to 3) (Fig. 6B). Therefore, temperature had a more pronounced effect on the amount of σ^S than on the amount of Crl, and the levels of Crl and σ^S were negatively correlated.

Crl levels are not a limiting factor for σ^S -dependent gene expression. The amount of Crl in cells was only slightly larger at 28°C than at 37°C. However, if Crl is present at a limiting level in the cell, even a small increase in the Crl concentration might increase the Crl effects. A high level of Crl expression can be obtained with pACcrl-1, a plasmid in which *crl* is transcribed from the *cat* promoter of the vector (21). When the Crl

FIG. 2. Impact of a *crl* knockout mutation on H_2O_2 resistance at 28 and 37°C. The resistance of *Salmonella* strains to H_2O_2 was determined in stationary-phase LB cultures grown at 37 and 28°C (OD_{600} , 3.5 to 4). (A and B) Resistance of wild-type strain ATCC 14028 (wt) (triangles) and ATCC*crl* (*crl*) (circles) to H_2O_2 at concentrations of 15 mM (A) and 30 mM (B) at 37°C (open symbols) and 28°C (filled symbols). (C) Effect of the pACYC184 vector and the pSTK4 and pAC*crl*-1 plasmids (carrying *rpoS* and *crl*, respectively) on the H_2O_2 resistance of ATCC 14028 (squares and diamonds) and ATCC*crl* (triangles and circles) at 28°C. (D) Resistance at 28°C of ATCC 14028 (triangles) and its *rpoS* (squares), *katE* (circles), and *dps* (diamonds) mutant derivatives to H_2O_2 at concentrations of 15 mM (open symbols) and 30 mM (filled symbols). The measurements were repeated twice, and the results of a representative experiment are shown.

level was increased by introducing pAC*crl*-1, the H_2O_2 resistance of the *crl* strain at 28°C was restored to the wild-type level, but pAC*crl*-1 did not further increase H_2O_2 resistance in ATCC 14028 (Fig. 2C). We could not use pAC*crl*-1 to increase the Crl level in strains harboring the *katE-lacZ* fusion due to the tetracycline resistance marker of Tn5B21 (Table 1). We used pAC*crl*-1-Km, which is identical to pAC*crl*-1 except for the tetracycline resistance gene, in which a kanamycin resistance cartridge was inserted (Table 1). pAC*crl*-1-Km did not significantly increase the expression level of the *katE-lacZ* gene fusion (Fig. 3B, compare bars 5 and 6). These results suggested that under the growth condition used, Crl levels are not limiting in the cell. Consistent with this hypothesis, quantitative immunoblots using standard curves with purified His-Crl and His- σ^S proteins indicated that there were 15 to 20 fmol of σ^S and 40 to 50 fmol of Crl per μ g of total proteins in LB stationary-phase cultures of ATCC 14028 grown at 37°C (OD_{600} , 3.8). Thus, in stationary phase at 37°C there is a two- to three-fold excess of Crl over σ^S . At the entry into stationary phase, the σ^S level is lower than the level in late stationary phase, and thus the excess of Crl is even greater (Fig. 5A and B). The amount of σ^S found in LB stationary-phase cultures of ATCC 14028 is similar to the amount previously found in *E. coli* MC4100 (13).

σ^S levels mediate temperature-dependent regulation of the Crl effect on H_2O_2 resistance. The results presented above indicate that the major stimulatory effect of Crl on σ^S activity at low temperature does not result from a higher level of Crl. However, Crl might be more active at low temperature. To obtain further insight into this possibility, we performed in vitro single-round transcription assays with the *katE* promoter at 37 and 25°C (data not shown). Many more transcripts were formed at 37°C than at 25°C, a finding consistent with the fact that promoter melting is facilitated by high temperature. Crl activation was visible at both temperatures, especially at low E/ σ^S ratios, as previously demonstrated (22). However, the magnitude of the Crl activation at *katE* did not change with temperature. Therefore, we concluded that under the conditions used, Crl activity is not dependent on temperature.

The magnitude of the Crl effect is greater at low levels of σ^S (21, 22). Therefore, the greater effects of Crl at 28°C than at 37°C (Fig. 1, 2, and 4) might result from the decreased level of σ^S at 28°C compared to the level at 37°C (Fig. 6). Consistent with this hypothesis, pSTK4, which increases the level of σ^S (21) (Fig. 7), reduced the magnitude of the Crl effect on the H_2O_2 resistance of ATCC 14028 (Fig. 2C) and the expression of the *katE-lacZ* fusion (Fig. 3B) at 28°C. In addition, ATCC 14028 containing the pACYC184 vector was less resistant to

FIG. 3. Crl-dependent activation of *katE* and *dps* transcription at 28 and 37°C. (A) Effect of Crl on *katE* and *dps* gene transcription at 28 and 37°C. *Salmonella* wild-type (wt), Δ *crl*, and Δ *rpoS* strains harboring the *katE-lacZ* and *dps-lacZ* fusions were grown in LB at 37 and 28°C, and β-galactosidase activity was measured in the stationary phase of growth (OD₆₀₀, 3.5 to 4). (B) Effect of pSTK4 and pAC*crl*-1-Km on *katE* expression at 28 and 37°C. Expression of the *katE-lacZ* gene fusion was measured in *S. enterica* serovar Typhimurium ATCC 14028 and its Δ *crl* mutant harboring the pACYC184 and pACYC184-Km vectors and plasmids pSTK4 and pAC*crl*-1-Km that carry *rpoS* and *crl*, respectively (Table 1). *Salmonella* strains were grown in LB at 37 and 28°C, and β-galactosidase activity was measured in the stationary phase of growth (OD₆₀₀, 3.5 to 4). Bars 1, ATCC*katE-lacZ*-(pACYC184); bars 2, ATCC*crl katE-lacZ*-(pACYC184); bars 3, ATCC-*katE-lacZ*-(pSTK4); bars 4, ATCC*crl katE-lacZ*-(pSTK4); bars 5, ATCC*katE-lacZ*-(pACYC184-Km); bars 6, ATCC*crl katE-lacZ*-(pACYC184-Km).

H₂O₂ and expressed lower levels of *katE* at 28°C than at 37°C, whereas similar levels of H₂O₂ resistance and *katE* gene expression were found at the two temperatures in the presence of pSTK4 (Fig. 2D and 3B and data not shown). The effect of pSTK4 on *katE* was evident mainly at the onset of stationary phase and resulted in earlier expression of the *katE-lacZ* fusion, even at 37°C (data not shown). These results suggested that Crl had a marked effect on the H₂O₂ resistance at 28°C because under these conditions, σ^S levels were the limiting factor for maximal expression of σ^S-dependent genes, including *katE*; consequently, Crl plays a major role by increasing the amount of Eσ^S.

RpoS has a negative effect on Crl production in stationary phase. Crl levels were lower in overnight cultures than in stationary-phase cultures, whereas the levels of σ^S in these two types of cultures did not differ significantly (Fig. 5B, compare

FIG. 4. Effect of Crl on acid stress resistance of *Salmonella* at 28 and 37°C: acid stress resistance of wild-type strain ATCC 14028 (wt) (triangles) and ATCC*crI* (*crI*) (circles) at 37°C (open symbols) and 28°C (filled symbols). The *Salmonella* strains were grown to stationary phase (OD₆₀₀, 3.5 to 4) in LB at 37 and 28°C and were subjected to acid stress (LB, pH 3). The results of representative experiments are shown. Similar results were obtained in repeat experiments.

time points 7 and 8). Interestingly, the levels of Crl in overnight cultures were about twofold higher in the *rpoS* mutant than in wild-type strain (Fig. 5C, time point 8). Moreover, increased production of σ^S from pSTK4 reduced production of Crl in stationary phase and overnight cultures of ATCC 14028 (Fig. 7). This suggests that *rpoS* negatively regulates Crl production in stationary phase, when the level of σ^S is high.

FIG. 5. Levels of Crl and σ^S during growth of *Salmonella* strains in LB at 37°C. ATCC 14028 and ATCC*rpoS* were grown in LB at 37°C. Exponential-phase cultures (OD₆₀₀, 0.5) of *Salmonella* were diluted into LB prewarmed at 37°C to prolong the exponential phase. (A) The growth phase was determined by measuring the culture OD₆₀₀. Aliquots were removed at various time points (time points 1 to 8) and analyzed by immunoblotting with anti-Crl (B and C) and anti-σ^S (B) antibodies. Ten micrograms of total protein was loaded in each slot. The same samples were used for the experiments whose results are shown in panels B and C. (B) Levels of σ^S and Crl in ATCC 14028. (C) Levels of Crl in ATCC*ΔrpoS* (lanes -) compared to the levels in ATCC 14028 (lanes +).

FIG. 6. Effect of temperature on the levels of Crl and σ^S . ATCC 14028 was grown in LB at 37 and 28°C. Aliquots were removed at various times (aliquots 1 to 5 for the strain grown at 37°C and aliquots 6 to 9 for the strain grown at 28°C) and analyzed by immunoblotting with anti-Crl (A) and anti- σ^S (B) antibodies. The growth phase was determined by measuring the culture OD₆₀₀. Relative quantification of σ^S and Crl in the extracts was determined as a function of OD₆₀₀. Five micrograms of total protein was loaded in each slot. For relative quantification of σ^S , a fixed amount of a His₁₂- σ^S protein was loaded into each slot. This protein migrated slower than the wild-type σ^S protein. The relative amount of σ^S was determined by dividing the intensity of the band corresponding to σ^S in the extract by the intensity of the band corresponding to the His₁₂- σ^S protein. For relative quantification of Crl, a fixed amount of a *Salmonella* extract containing a mutated Crl protein (Crl*) was loaded in each slot. The Crl* protein migrated slower than the wild-type Crl protein. The relative amount of Crl was determined by dividing the intensity of the band corresponding to Crl in the extract by the intensity of the band corresponding to the Crl* protein.

DISCUSSION

The Crl protein has been proposed to be a thermosensor that favors expression of *rpoS*-regulated genes at low temperature because Crl levels in *E. coli* MC4100 were very low at 37°C compared to the levels at 30°C (1). However, in a recent study, Crl levels in MC4100 were found to be only mildly affected by temperature (30). In agreement with the later report for *E. coli*, we found that the Crl levels in *S. enterica* serovar Typhimurium ATCC 14028 were only slightly increased at 28°C compared to those at 37°C (Fig. 6). Nevertheless, the physiological effects of Crl were greater at 28°C than at 37°C (Fig. 1 to 4). In particular, the expression of the *katE* gene, which encodes a σ^S -dependent stationary-phase catalase, was induced by less than 1.5-fold by Crl at 37°C and by 2- to 3-fold at 28°C. Consistently, the *crl* mutation had no effect on the H₂O₂ resistance of ATCC 14028 at 37°C but significantly decreased the H₂O₂ resistance of ATCC 14028 at 28°C.

Crl binds σ^S (1) and facilitates RNA polymerase holoenzyme E σ^S formation (30). The plasmon surface resonance data indicate that Crl binds σ^S with a 1:1 stoichiometry (unpublished results). One hypothesis to explain the effect of temperature on Crl-mediated activation is that Crl levels are limiting in vivo compared to σ^S levels, so that a small increase in the Crl level at 28°C compared with that at 37°C results in greater Crl effects. However, there was a two- to threefold excess of Crl over σ^S in stationary phase in ATCC 14028 grown at

FIG. 7. Negative effect of σ^S on Crl levels. Strains ATCC 14028 and ATCC14028 containing the pACYC184 vector and pSTK4, which carries *rpoS*, were grown in LB at 37°C. Aliquots were removed in exponential phase (LOG) (OD₆₀₀, 0.45), stationary phase (STA) (OD₆₀₀ for lane 1, 4; OD₆₀₀ for lanes 2 and 3, 4.7), and after overnight growth (ON) (OD₆₀₀ for lane 1, 4; OD₆₀₀ for lanes 2 and 3, 4.7) and analyzed by immunoblotting with anti-Crl and anti- σ^S antibodies. Ten micrograms of total protein was loaded in each slot. Lanes 1, ATCC-*rpoS*(pACYC184); lanes 2, ATCC 14028(pACYC184); lanes 3, ATCC 14028(pSTK4).

37°C in LB (and the excess was even greater during entry to stationary phase). In addition, increasing Crl levels in ATCC 14028 did not result in higher expression of the *katE* gene, nor did they result in greater H₂O₂ resistance (Fig. 2 and 3). This suggests that Crl levels are not a limiting factor for the induction of σ^S -dependent genes. We also showed that Crl activity in vitro is not dependent on temperature (data not shown). This result is consistent with the finding of Typas et al. (30), who showed that in vitro transcription from the *synp9* promoter is similar at 30 and 37°C. Altogether, these results indicate that the major stimulatory effect of Crl at low temperature is not due to the fact that Crl is more active or more abundant at low temperature.

We previously showed that *katE* expression is more sensitive to Crl in ATCC14028_{LT2}, a derivative of ATCC 14028 that contains an *rpoS* allele with the inefficient TTG translation start codon and reduced σ^S levels (22). We reasoned that the higher induction ratio for *katE* expression by Crl at 28°C than at 37°C and the marked effect of the *crl* mutation on the H₂O₂ resistance of ATCC 14028 might result from a lower level of σ^S . Consistent with this hypothesis, the level of σ^S in ATCC 14028 was about twofold lower at 28°C than at 37°C (Fig. 6), and this likely resulted in the lower levels of *katE* expression and H₂O₂ resistance at 28°C than at 37°C (Fig. 2 and 3). Indeed, increasing the σ^S level at 28°C resulted in greater H₂O₂ resistance in ATCC 14028 (Fig. 2C) and a higher level of *katE* expression (Fig. 3B). Altogether, these results indicate that σ^S levels are the limiting factor at 28°C and that they mediate the observed effect of temperature on the magnitude of Crl activation. This does not exclude the possibility of additional effects of temperature at particular promoters, for instance at promoters with low melting ability. There may also be other environmental conditions under which the balance of Crl and σ^S levels is set such that the presence of more Crl makes an important difference.

Consistent with a major role for Crl at low levels of σ^S , the maximal level of Crl is reached when σ^S begins to accumulate (Fig. 5), and Crl has a negative effect on σ^S production (19, 30). We further show here that when the level of σ^S is high in stationary phase and Crl may be dispensable, σ^S exerts a negative effect on Crl production (Fig. 5 and 7). We do not know the molecular mechanism responsible for this phenomenon, which reveals that the levels of σ^S and Crl are intimately linked and negatively correlated.

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