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Negative Control of *rpoS* Expression by Phosphoenolpyruvate: Carbohydrate Phosphotransferase System in *Escherichia coli*

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The σ^S (or σ^{38}) subunit of RNA polymerase, encoded by the *rpoS* gene, is a crucial regulator in the transcriptional control of a set of genes under stressful conditions, such as nutrient starvation. The expression of *rpoS* is regulated in a complex manner at the levels of transcription, translation, and stability of the product. Although a number of factors involved in the regulation of *rpoS* expression have been identified, the underlying molecular mechanisms are not fully understood. In this study, we identified the Crr (or $EIIA^{Glc}$) protein as a novel factor that plays an important role not only in the transcriptional control but also in the translational control of *rpoS* expression. Crr is an important component in glucose uptake through the well-characterized phosphoenolpyruvate:carbohydrate phosphotransferase system. The results of a series of genetic analyses revealed that Crr negatively controls *rpoS* translation and transcription. The observed transcriptional control by Crr appears to be mediated by cyclic AMP. However, it was found that Crr negatively controls *rpoS* translation rather directly. These results suggest a possible linkage between the control of *rpoS* expression and carbon metabolism.

When *Escherichia coli* cells are exposed to several stressful conditions preventing rapid growth, such as nutrient starvation, the expression of a set of genes is induced to allow the cells to survive under the harsh conditions (8). The σ^S (or σ^{38}) subunit of RNA polymerase, encoded by the *rpoS* gene, has been shown to function as a central regulator in the transcriptional control of such genes (11). Since the gene expression is, at least, mainly dependent on the cellular σ^S content, it is important to determine how σ^S content is regulated during cell growth.

rpoS expression is severely repressed at the logarithmic growth phase, while it is induced during entry into the stationary phase (5, 23). The regulation is conducted in a complex manner at the levels of transcription, translation, and stability of σ^S (10). Recent extensive studies have revealed that several proteinaceous factors as well as small molecules are involved in the regulation. *rpoS* transcription was proposed to be negatively regulated by a cyclic AMP (cAMP) receptor protein-cAMP complex, based on the fact that σ^S is significantly accumulated in the cells of *cya* and *crp* mutants (9, 10). Posttranscriptional mechanisms also, and more importantly under certain conditions, determine the cellular σ^S content. It has been revealed that an RNA-binding protein, HF-I, encoded by the *hfq* gene, and a nucleoid protein, H-NS, are involved in *rpoS* translation positively and negatively, respectively (1, 15, 27). In rapidly growing cells, σ^S is markedly unstable, being degraded by an ATP-dependent protease complex, ClpPX (18). This rapid turnover requires the functions of both H-NS and RssB (or SprE) (1, 14, 17, 27), the latter protein belonging to the response regulator family in a two-component signal transduction system. Although the genes involved in the regulation of *rpoS* expression have been identified to some extent, the underlying molecular mechanisms remain to be elucidated. The questions of how cells sense the external and internal growth conditions and how such signals are transduced in the cells and then regulate *rpoS* expression through the factors described above have not been answered, in spite of their biological significance.

In this study, we identified the Crr protein as a novel factor that is crucial for the translational as well as transcriptional control of *rpoS* expression. The Crr protein (or $EIIA^{Glc}$) is an important component in glucose uptake in the phosphoenolpyruvate:carbohydrate phosphotransferase system (PEP:PTS) (16). In this system, the phosphate group of PEP is transferred through successive phosphorelay reactions involving enzyme I (EI), histidine protein (HPr), and glucose-specific enzyme II (EII^{Glc}), and then extracellular glucose is phosphorylated by EII^{Glc} concomitantly with its uptake. Crr is a cytoplasmic component of EII^{Glc} and is known to be crucial not only for glucose uptake but also for the regulation of several cellular functions in carbon metabolism, such as inducer exclusion and modulation of adenylate cyclase activity. We first isolated an *E. coli* mutant in which the expression of *rpoS* is derepressed even at the logarithmic growth phase. The following genetic analyses revealed that the function of the *crr* gene is impaired by a transposon insertion in the mutant, suggesting that Crr is deeply involved in the negative control of *rpoS* expression. In this case, the phosphorylation of Crr is also crucial in glucose uptake. Crr appears to be involved in both the translational and the transcriptional control of *rpoS* expression. We will discuss the underlying molecular mechanisms and the physiological role of Crr in the control of *rpoS*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. They are all derivatives of MC4100 (2). Several mutant strains were constructed by P1 transduction (13). Cells were grown at 37°C mainly in Luria broth (13). An overnight culture was inoculated into fresh

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype
MC4100	F$^-$ Δ*(arg-lac)U169 araD139 rpsL150 ptsF25 flbB5301 rbsR deoC relA1*
CU263	MC4100(λ*rpoS-lacZ* PF977)
CU264	MC4100(λ*rpoS-lacZ* PF212)
CU329	CU263 *crr2-3*::mini-Tn*10cam*
CU330	CU263 *crr*::*kan*
CU334	CU263 *clpX*::*kan*
CU344	CU263 *nupC510*::Tn*10*
CU345	CU263 *nupC510*::Tn*10* Δ*ptsHI*
CU348	CU263 *ptsG*::Tn*5*
CU351	CU263 *crr*::*kan rssB*::*cam*
CH11	MC4100(λ*rpoS-lacZ* OF[a])
CH12	MC4100(λ*katE-lacZ*)
NM5	CH12 *crr*::*kan*
NM7	CH11 *crr*::*kan*
NM9	CU264 *crr*::*kan*
NM11	CU264 *hfq1*::Ω
NM12	CU264 *rssB*::*kan*
NM13	CU264 *clpX*::*kan*
NM20	CU264 *crr2-3*::mini-Tn*10cam*
NM21	CU264 *crr2-3*::mini-Tn*10cam hfq1*::Ω
NM23	CU263 *cya*::*kan*
NM24	CU264 *cya*::*kan*
NM25	CH11 *cya*::*kan*

[a] OF, operon fusion.

medium, and cells were grown until logarithmic phase, whereupon the culture was appropriately diluted with fresh medium and further grown (see Fig. 2) and then used for all experiments at the mid-logarithmic phase. Antibiotics were added as necessary.

Construction of *rpoS-lacZ* fusion genes. *rpoS-lacZ* fusion genes were constructed essentially by the method of Hirano et al. (6). The *rpoS-lacZ* operon fusion was constructed as follows. A 1.6-kb *Cla*I-*Dra*I fragment encompassing *rpoS* promoters was purified from pKTF101 (22). After treatment with T4 DNA polymerase, the resultant fragment was inserted into the previously blunt-ended *Hin*dIII site of pMS434HS. An *E. coli* strain harboring λpF13 was transformed with the resultant plasmid carrying the *rpoS-lacZ* operon fusion, and a lambda phage lysate was prepared from the transformant by UV irradiation. MC4100 was infected with the phage lysate, and then lysogens were selected from blue plaques on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as candidates carrying the fusion gene on the chromosome. The lysogens thus purified were further scored for the Lac$^+$ phenotype, and then one such lysogen, named CH11, was used in this study.

rpoS-lacZ PF977 was constructed as follows. A 2.4-kb *Cla*I-*Eco*47III fragment encompassing the *rpoS* gene lacking the C-terminal portion, as well as the promoter region, was first purified from pKTF101 (22). After treatment with T4 DNA polymerase, the fragment was inserted into the *Hin*cII site of pUC119 (25) to construct the *rpoS-lacZ*α protein fusion. From the resultant plasmid, a 2.6-kb *Hin*dIII-*Bgl*I fragment encompassing the protein fusion was purified by partial digestion with *Bgl*I (the *Bgl*I site is located within *lacZ*α), and then the fragment was inserted between the *Hin*dIII and *Bgl*I sites of pMS434HS (the *Bgl*I site corresponds to the identical site of the *rpoS-lacZ*α protein fusion) to yield pCU71. The resultant *rpoS-lacZ* protein fusion, including the N-terminal 325 codons of the *rpoS* open reading frame, was named PF977 and subsequently transferred to the chromosome of MC4100, as described above. *rpoS-lacZ* PF212 was constructed by means of the same procedures except that a 1.6-kb *Cla*I-*Hin*cII fragment encompassing the N-terminal 70 codons of the *rpoS* open reading frame as well as the promoter region was used for the construction.

The *katE-lacZ* operon fusion was constructed as for the *rpoS-lacZ* operon fusion, except that a 1.25-kb *Hin*dIII-*Pst*I fragment encompassing the *katE* promoter was used.

Transposon insertion mutagenesis. Transposon insertion using mini-Tn*10cam* was carried out essentially according to the method of Kleckner et al. (7). Cells of MC4100 were grown in Luria broth at 37°C to the mid-logarithmic phase. A portion of the culture was infected with a lambda phage lysate of λNK1324 and then plated onto a Luria agar plate containing 25 μg of chloramphenicol per ml. Chloramphenicol-resistant (Cm^r) transductants were pooled and infected with the P1*vir* phage to prepare a P1 phage lysate. The resultant phage lysate was stored and used for P1 transduction.

Assaying of β-galactosidase activity. Assaying of β-galactosidase activity was carried out by the method of Miller (13).

Immunoblotting analysis. Total cellular proteins were prepared by precipitation with trichloroacetic acid (final concentration, 5%) and then collected by centrifugation. After a wash with ice-cold acetone, the precipitate was dissolved in 1% (wt/vol) sodium dodecyl sulfate (SDS)–50 mM Tris-HCl (pH 8)–1 mM EDTA buffer. The protein concentration was accurately determined for each sample using a Micro BCA protein assay reagent kit (Pierce Chemical Co., Rockford, Ill.). Appropriate amounts of total cellular proteins were separated by SDS-polyacrylamide gel electrophoresis, followed by immunoblotting with anti-σ^S and anti-CbpA polyclonal antisera.

RESULTS

Isolation of transposon-insertional mutations that result in derepression of *rpoS*. To monitor *rpoS* expression appropriately in different genetic backgrounds, in this study we constructed three types of *rpoS-lacZ* fusion genes on the chromosome (Fig. 1). An *rpoS-lacZ* operon (transcriptional) fusion contains only the *rpoS* promoter region, fused to the *lacZ* gene, so it can be used to monitor the transcriptional control of *rpoS*. Two protein (translational) fusion genes, named PF212 and PF977, were also constructed. Note that these fusions include the N-terminal 70 and 325 codons of the *rpoS* open reading frame, respectively. The former contains a *cis*-acting element required for translational control in addition to the *rpoS* promoter region, whereas the latter contains all regulatory elements required for *rpoS* control at the levels of transcription, translation, and stability of the gene product (15). These two protein fusions, PF212 and PF977, were used appropriately to evaluate the translational efficiency and overall output of *rpoS* expression, respectively.

Using PF977 as a monitoring probe, an attempt was first made to isolate mutants exhibiting derepressed expression for *rpoS* in order to identify a novel factor(s) involved in the regulatory mechanism for *rpoS* expression. PF977 should be advantageous for comprehensively isolating mutants affecting

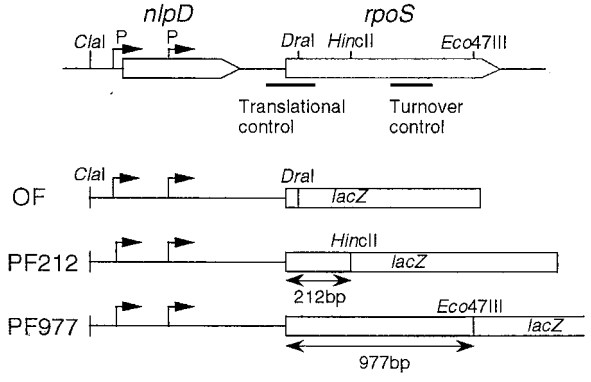

FIG. 1. Schematic representation of a set of *rpoS-lacZ* fusion genes used in this study. The *rpoS* operon is diagrammed at the top. The *rpoS* open reading frame is indicated. P, promoter of *rpoS*. *cis*-acting elements required for translational control and turnover control and restriction sites used for the construction of the *rpoS-lacZ* fusion genes are indicated. The structures of three types of *rpoS-lacZ* fusion genes are diagrammed below.

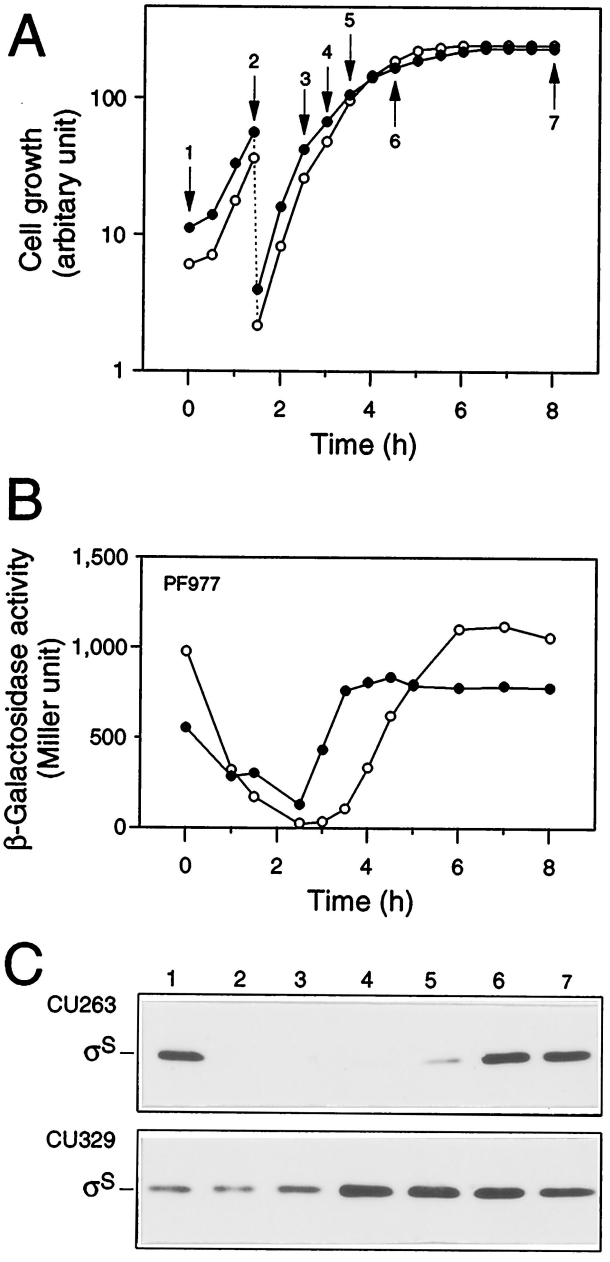

FIG. 2. Expression of *rpoS* during cell growth. Strains CU263 (open circles) and CU329 (closed circles), each carrying *rpoS-lacZ* PF977, were grown at 37°C in Luria broth. Both cell growth (A) and β-galactosidase activity expressed by PF977 (B) were measured. Protein samples were prepared at the indicated time points in panel A, and then each 20 μg of total proteins was subjected to immunoblotting with anti-σ^S antiserum (C).

rpoS expression at any regulatory step(s). Indeed, on tetrazolium-lactose plates (19), the wild-type strain carrying PF977 on the chromosome gives red colonies (indicating low β-galactosidase activity), whereas the *hns*::*neo* derivative, in which *rpoS* expression is known to be derepressed, gives white ones (indicating high β-galactosidase activity). Thus, mutants exhibiting derepressed expression of *rpoS* should give white or pink colonies on tetrazolium-lactose plates due to the enhanced β-galactosidase activity.

Cells of CU263, carrying PF977 on the chromosome, were mutagenized by random insertion of mini-Tn*10cam* carrying a Cm^r marker (7). From among $\sim 7 \times 10^4$ Cm^r transductants, we selected 20 white or pink colonies on tetrazolium-lactose plates containing chloramphenicol. The following immunoblotting analysis revealed that 2 of these 20 mutants significantly accumulated σ^S in their cells even at the logarithmic phase, whereas in the remaining 18 mutants the σ^S content showed only a three- to fourfold induction compared to that in the parental wild-type strain at the logarithmic phase. These two mutants were genetically purified by repeated P1 transduction into the fresh CU263 background and designated CU328 and CU329. In this study, we focus our attention on the latter mutant, CU329, to investigate the underlying regulatory mechanism for *rpoS* expression (the former mutant will be dealt with elsewhere).

σ^S is accumulated in CU329 even at the logarithmic phase. To clarify the phenotype more precisely, we monitored the expression of PF977 in CU329 by measuring β-galactosidase activity during cell growth. Cells were grown at 37°C in Luria broth, and β-galactosidase activity was measured at appropriate intervals. The expression of PF977 in the wild-type background decreased once after inoculation of an overnight culture and then increased concomitantly with cell growth, reaching the maximum level at the onset of the stationary phase (Fig. 2A and B). PF977 expression in CU329 was found to be ~20-fold higher than that in wild-type cells only around the logarithmic phase (Fig. 2A and B). This kinetic profile was similar to that observed for a *clpX*::*kan* mutant (data not shown), in which σ^S is known to be accumulated (18). Immunoblotting analysis also confirmed this particular phenotype; that is, σ^S content increased approximately 10- to 20-fold in CU329 around the logarithmic phase (Fig. 2C). These results indicated that *rpoS* expression in CU329 is indeed derepressed even at the logarithmic phase. It should be noted that the accumulation of CU329 at the logarithmic phase was less significant, i.e., three- to fourfold accumulation, when cells were grown in M9 synthetic medium (data not shown). The following analyses were thus carried out using Luria broth exclusively as the culture medium.

The *crr* gene is involved in the control of *rpoS* expression. To clarify the gene disrupted by the mini-Tn*10cam* insertion in CU329, we first cloned a DNA segment encompassing the insertion from the chromosomal DNA using the *cam* (Cm^r) gene of the transposon as a selective marker. The following DNA sequencing of the flanking region of the *cam* gene revealed that the transposon is inserted just within the *crr* gene encoding the A subunit of glucose-specific enzyme II ($EIIA^{Glc}$). The insertion point is the 95th codon of the open reading frame consisting of 169 codons. We thus named the relevant insertional mutation *crr2–3*::mini-Tn*10cam*. To further confirm that the *crr* mutation indeed affects *rpoS* expression, we constructed a derivative of CU263, named CU330, carrying a well-characterized *crr*::*kan* mutation (21). The resultant strain also exhibited both enhanced expression of PF977 and accumulation of σ^S at the mid-logarithmic phase (Fig. 3A; compare vector controls). In addition, the wild-type *crr* gene was able to complement the mutational phenotype in CU330 with regard to

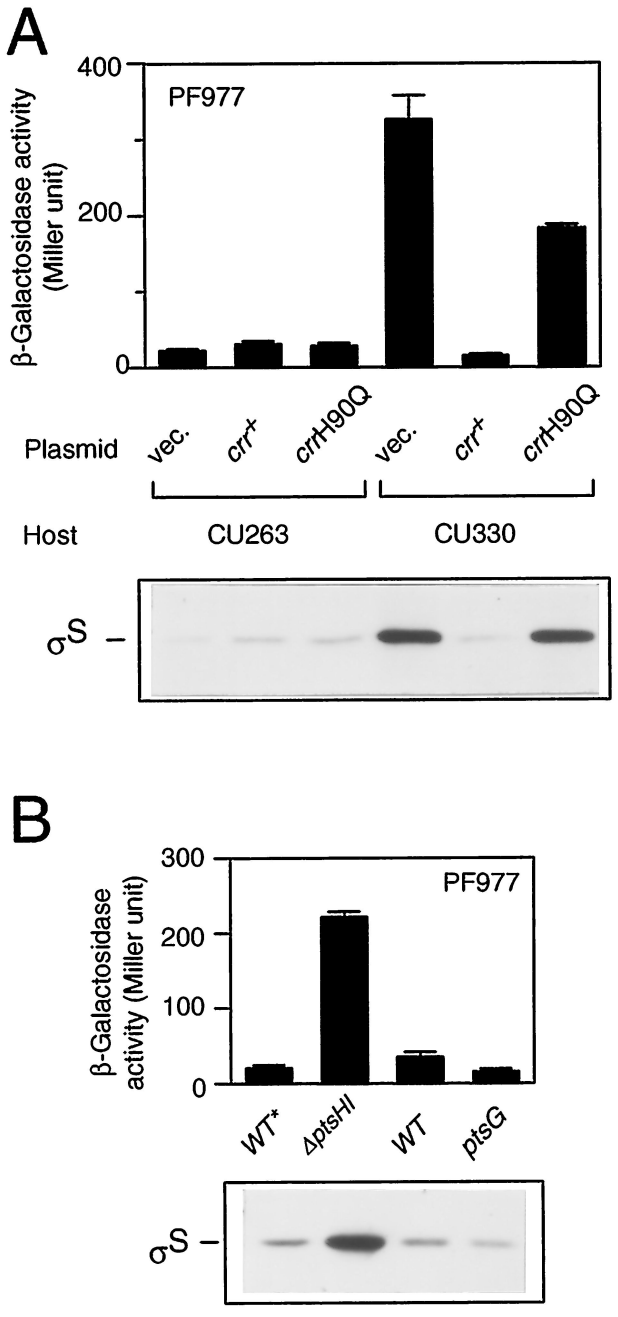

FIG. 3. Phosphorylation of Crr is crucial for *rpoS* regulation. (A) Complementation assay of the *crr*::*kan* mutant. Strains CU263 (wild type) and CU330 (*crr*::*kan*) carrying plasmid pTSV28 (vector [vec.]), pSTCRR (*crr*$^+$), or pST172 (*crr*H90Q) were grown at 37°C in Luria broth supplemented with 25 μg of chloramphenicol per ml. At the mid-logarithmic phase, β-galactosidase activity (upper panel) and σ^S content (lower panel) were measured. (B) Effect of the *ptsHI* or *ptsG* mutation on *rpoS* expression. Strains CU344 (wild type [WT*]), CU345 (Δ*ptsHI*), CU263 (wild type), and CU348 (*ptsG*::Tn*5*) were grown at 37°C in Luria broth. Note that CU344 and CU345 carry the *nupC510*::Tn*10* allele, which was used as a selectable marker to construct the Δ*ptsHI* mutant. At the mid-logarithmic phase, β-galactosidase activity expressed by PF977 (upper panel) and σ^S content (lower panel) were measured. β-Galactosidase activity data are means with standard deviations for four independent assays. Each 20 μg of total proteins was used for immunoblotting.

both the enhanced expression of PF977 and the accumulation of σ^S (Fig. 3A). Thus, we concluded that the *crr* gene product somehow negatively controls *rpoS* expression at the logarithmic phase.

Phosphorylation of the Crr protein is crucial for negative regulation of *rpoS* expression. The Crr protein ($EIIA^{Glc}$) plays an important role in glucose uptake through the PEP:PTS system (16). In this system, the phosphate group of PEP is transferred to glucose through successive phosphorelay reactions involving enzyme I (EI), histidine protein (HPr), and glucose-specific enzyme II (EII^{Glc}). Extracellular glucose is transported into cells through a coupled phosphorylation reaction catalyzed by EII^{Glc}. To determine whether the phosphorylation of Crr is crucial for the negative regulation of *rpoS* expression, we carried out the following two lines of experiments. First, we examined the complementation ability of a mutant *crr* gene (*crr*H90Q), in which the phosphorylated His residue is replaced by Gln so that the mutant Crr protein is no longer phosphorylated (21). The introduction of the *crr*H90Q allele into CU330 failed to fully complement the mutational phenotype of CU330, although both the expression of PF977 and the cellular content of σ^S decreased slightly (Fig. 3A). Second, we examined whether a certain lesion of *ptsHI* affects *rpoS* expression. Since the phosphorylation of Crr is dependent on both EI and HPr, which are encoded by the *ptsI* and *ptsH* genes, respectively, the Crr protein cannot be phosphorylated in *ptsHI* mutant cells (16). Both the expression of PF977 and the σ^S content appeared to increase in the Δ*ptsHI* mutant cells (Fig. 3B, left pair). This result also supported the view that the phosphorylation of Crr is important for regulation of *rpoS* expression. These lines of evidence demonstrated that the phosphorylation of the Crr protein is crucial for the negative regulation of *rpoS* expression.

Since during glucose uptake the function of the Crr protein ($EIIA^{Glc}$) is coupled to $EIICB^{Glc}$, which is encoded by the *ptsG* gene (16), both subunits of EII^{Glc} could be required for the negative regulation of *rpoS* expression. To examine this possibility, *rpoS* expression in a *ptsG* mutant was investigated. Neither the expression of PF977 nor the σ^S content were ever increased by the *ptsG*::*kan* mutation, and instead, both were reduced twofold (Fig. 3B; right pair), indicating that only the A subunit, i.e., not the CB subunit, of EII^{Glc} is involved in the negative regulation of *rpoS* expression.

σ^S accumulated due to the *crr* mutation is transcriptionally active. Since σ^S positively controls the expression of a set of genes whose functions are induced under certain harsh conditions, it is important to determine whether the σ^S accumulated in the *crr* mutant is transcriptionally active or not. In the *crr*::*kan* background, we thus examined the expression of the *katE* and *cbpA* genes, whose transcription is known to be dependent on the σ^S function (12, 26). A set of strains (Fig. 3A) carrying a *katE-lacZ* operon fusion on the chromosome was constructed, and *katE-lacZ* expression was measured at the logarithmic phase. The results show that *katE* promoter activity was increased fivefold by the *crr*::*kan* mutation (Fig. 4A). The wild-type *crr* gene was able to complement this particular phenotype, but the *crr*H90Q allele was not. *cbpA* expression was also induced by the *crr*::*kan* mutation (Fig. 4B). Thus, these results of immunoblotting analysis of the CbpA protein essentially led to the same conclusion; that is, the σ^S accumu-

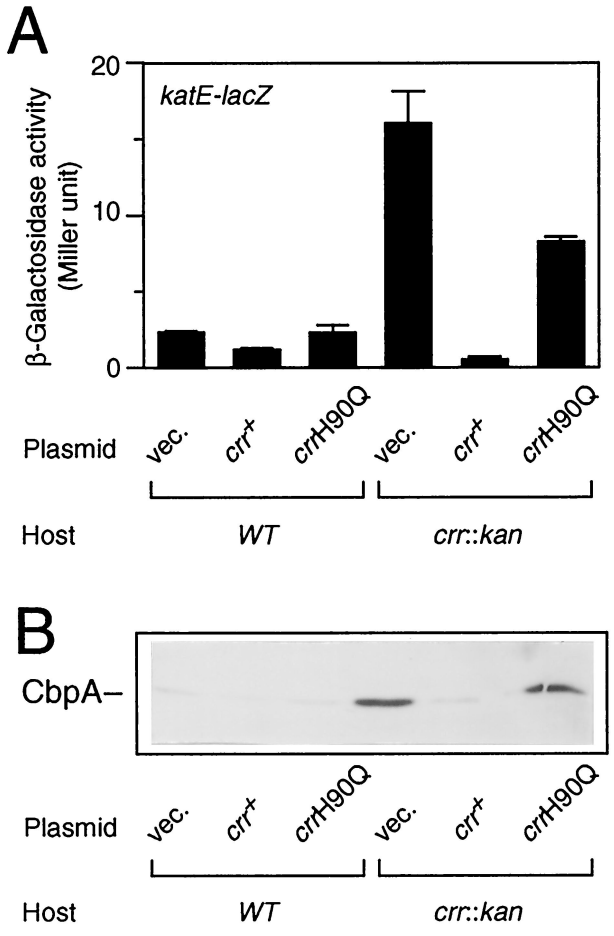

FIG. 4. Expression of *katE* and *cbpA* in the *crr*::*kan* mutant. (A) Strains CH12 (wild type [WT], *katE-lacZ*) and NM5 (*crr*::*kan katE-lacZ*), each harboring plasmids as described for Fig. 3A, were grown at 37°C in Luria broth supplemented with chloramphenicol (25 μg/ml). At the mid-logarithmic phase, β-galactosidase activity expressed by the *katE-lacZ* operon fusion was measured. The data are means with standard deviations for four independent assays. (B) A set of transformants as described for Fig. 3A was grown at 37°C in Luria broth supplemented with 25 μg of chloramphenicol per ml. Immunoblotting with anti-CbpA antiserum was carried out as described for Fig. 3A.

lated in the *crr* mutant cells indeed has the ability to allow the transcription of its target genes.

The *crr* mutation affects *rpoS* expression at the level of transcription. *rpoS* expression is regulated at the levels of transcription, translation, and stability of the protein (10). Which step(s) does Crr negatively regulate? To answer this question, we examined the phenotypic effects of the *crr* mutation on three types of *rpoS-lacZ* fusion genes: operon fusion, PF212, and PF977. As mentioned above (Fig. 1), PF212 allows the monitoring of the effects of certain mutations on translational efficiency, other than the stabilization of σ^S. Indeed, the expression of PF212 was affected by the *hfq1*::Ω mutation (24) but not by the *clpX*::*kan* or *rssB*::*kan* mutation (data not shown). While the *crr*::*kan* mutation affected the *rpoS* promoter activity slightly (twofold or less), it significantly induced expression of PF212 (fivefold) as well as that of PF977 (eightfold) (Fig. 5A). The half-life of σ^S in the *crr* mutant, as determined by immunoblotting analysis of chloramphenicol-treated cells, was approximately 2.5 min, which is quite similar to that observed for wild-type cells (Fig. 5B). Moreover, the *crr*::*kan rssB*::*cam* double mutant showed higher σ^S content than that in either single mutant (Fig. 5C), suggesting that the *crr*::*kan* mutation does not affect the stability of σ^S. Thus, Crr seems to be partly involved in the negative regulation of *rpoS* expression at the level of its transcription. However, it should be noted that phosphorylated Crr is able to activate the enzymatic activity of adenylate cyclase and that the cellular concentration of cAMP is positively controlled by Crr (16). Therefore, the effect of the *crr* mutation on *rpoS* transcription described above is not surprising, since the cAMP receptor protein-cAMP complex has been shown to negatively regulate *rpoS* transcription in vivo (9, 10). To confirm such an indirect effect of the *crr* mutation on *rpoS* transcription through adenylate cyclase activity, we examined the effect of the addition of cAMP on the derepressed expression of *rpoS* in the *crr* mutant. Cells harboring the *rpoS-lacZ* operon fusion were grown in Luria broth in either the absence or the presence of 5 mM cAMP, and then β-galactosidase activity was measured at the mid-logarithmic phase. As shown in Fig. 6, the expression of the operon fusion was completely restored to the wild-type level in both the *crr* and the *cya* backgrounds by the addition of cAMP. This indicated that the effect of the *crr* mutation on *rpoS* transcription is rather indirect due to modulation of adenylate cyclase activity.

Phosphorylated Crr is involved in translational control of *rpoS*. We next examined whether Crr is involved in *rpoS* translation indirectly through modulation of the cellular concentration of cAMP. CU330 cells were grown in Luria broth at 37°C in either the presence or the absence of 5 mM cAMP, and then σ^S content was determined by immunoblotting analysis. As shown in Fig. 7A, σ^S content decreased only slightly (~10% or less) in the *crr* mutant upon the addition of cAMP. In contrast, the σ^S content of the *cya*::*kan* mutant decreased to the normal level upon the addition of cAMP. Moreover, expression of PF212 in the *crr*::*kan* background only slightly decreased upon the addition of cAMP, whereas that in the *cya*::*kan* background clearly decreased to the normal level (Fig. 7B). These results strongly suggested that Crr is involved in *rpoS* translation rather directly or, at least, not via the modulation of adenylate cyclase activity.

We further addressed the issue of whether the translational control of *rpoS* requires the phosphorylation of Crr. The strains harboring *crr* plasmids described in the legend to Fig. 3 were grown in the presence of 5 mM cAMP, and then σ^S contents were determined by immunoblotting. It should be noted that with the addition of cAMP one is able only to estimate precisely the effect of a mutation on translational control by eliminating the effect on transcriptional control. As shown in Fig. 7C, the *crr*H90Q allele was unable to complement the mutational phenotype of CU330. The slight reduction in σ^S content, as shown in Fig. 3A, would be due to the overproduction of the mutated Crr by the multicopy plasmid. The results demonstrated that the phosphorylation of Crr is crucial for translational control of *rpoS*.

Epistasis analysis with the *hfq* mutation. The results described above suggested that Crr is involved in translational regulation of σ^S. It was reported that an RNA-binding protein,

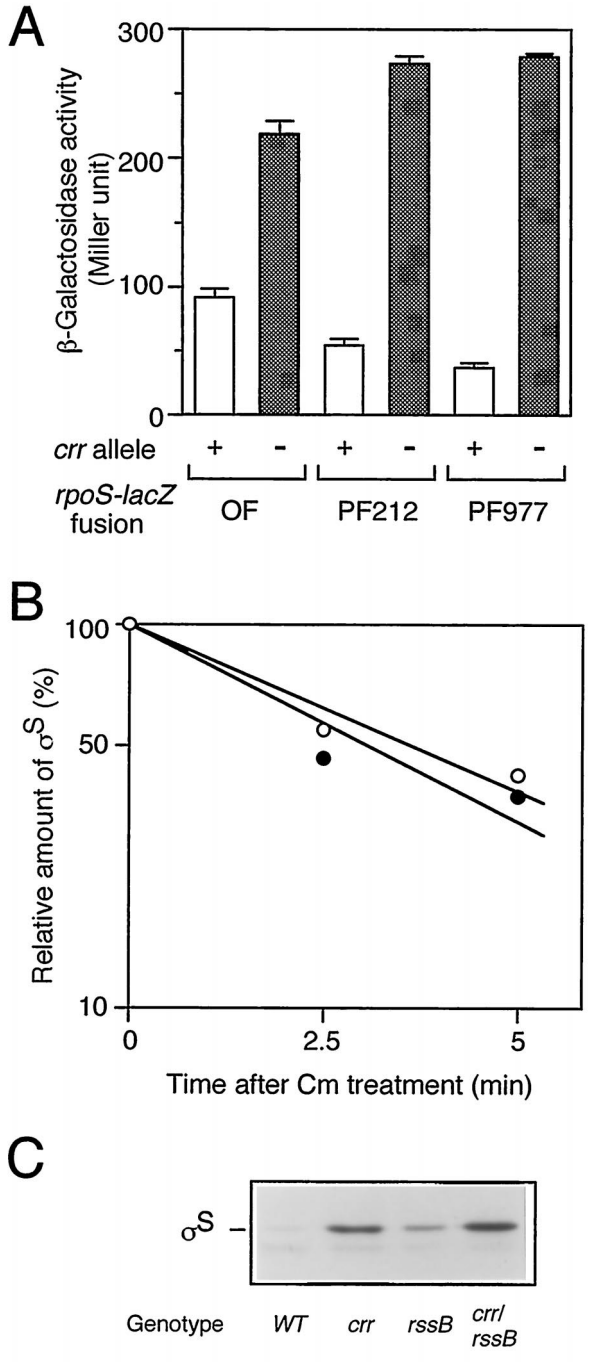

FIG. 5. (A) Effect of the *crr*::*kan* mutation on a set of *rpoS-lacZ* fusion genes. Strains carrying the three types of *rpoS-lacZ* genes described for Fig. 1 in either *crr*$^+$ (+) or *crr*::*kan* (−) background were grown at 37°C in Luria broth. At the mid-logarithmic phase, β-galactosidase activity was measured. The data are means with standard deviations for four independent assays. OF, operon fusion. (B) Effect of the *crr*::*kan* mutation on the half-life of σ^S. Strains CU263 (wild type; open circles) and CU330 (*crr*::*kan*; closed circles) were grown at 37°C in Luria broth. At the mid-logarithmic phase, cells were treated with 25 μg of chloramphenicol per ml. At the indicated intervals, cells were harvested and subjected to immunoblotting using anti-σ^S antiserum. The amount of σ^S was expressed relative to that determined for the sample at time zero. (C) Cellular σ^S content in the *crr*::*kan rssB*::*cam* double mutant. Strains carrying the indicated mutations were grown at 37°C in Luria broth. At the mid-logarithmic phase, cells were harvested and subjected to immunoblotting using anti-σ^S antiserum. WT, wild type.

HF-I, the *hfq* gene product, is required for *rpoS* translation (15). We thus finally examined the genetic relationship between the *hfq* and *crr* genes with respect to *rpoS* translation. A set of strains carrying the *hfq1*::Ω and/or *crr2–3*::mini-Tn*10cam* mutations were constructed, and the expression of PF212 was measured. The expression of PF212 in the *hfq1*::Ω background was apparently lower than that in wild-type cells, a finding which is in good agreement with the previous report (15), whereas that in the *crr2–3*::mini-Tn*10cam* background was fivefold higher. In *hfq crr* double-mutant cells, the β-galactosidase activity was slightly lower than that in wild-type cells and similar to that in the *hfq* single mutant (Fig. 8). This particular phenotype was also confirmed by immunoblotting (Fig. 8). There are two possible explanations. First, the *hfq* mutation is epistatic to the *crr* mutation because the strong effect of the *crr* mutation was diminished. Second, the two gene products act independently because the phenotype exhibited by the double mutant was similar to that of the wild-type strain. It is difficult to determine which explanation is correct.

DISCUSSION

In this study, we demonstrated that Crr negatively controls *rpoS* expression. The *crr* mutation affects *rpoS* expression mainly at the level of translation rather than that of transcription, since the addition of cAMP did not drastically affect the σ^S content of the *crr* mutant even while *rpoS* transcription was decreased to the wild-type level (Fig. 6 and 7). Although Crr has been shown to be able to modulate several cellular functions in carbon metabolism, such as inducer exclusion and adenylate cyclase activity, this is the first evidence that Crr participates in the translational process.

How does Crr negatively control *rpoS* translation? Two proteins, HF-I and H-NS, have previously been reported to be factors involved in the translational control of σ^S (1, 15, 27).

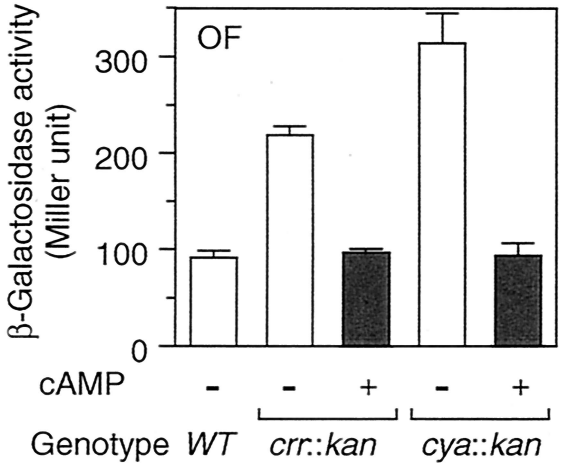

FIG. 6. Effect of cAMP on *rpoS* transcription. Strains CH11 (wild type [WT]), NM7 (*crr*::*kan*), and NM25 (*cya*::*kan*), each carrying the *rpoS-lacZ* operon fusion (OF), were grown at 37°C in Luria broth either in the absence (−) or the presence (+) of 5 mM cAMP. At the mid-logarithmic phase, β-galactosidase activity expressed by the *rpoS-lacZ* operon fusion was measured. The data are means with standard deviations for four independent assays.

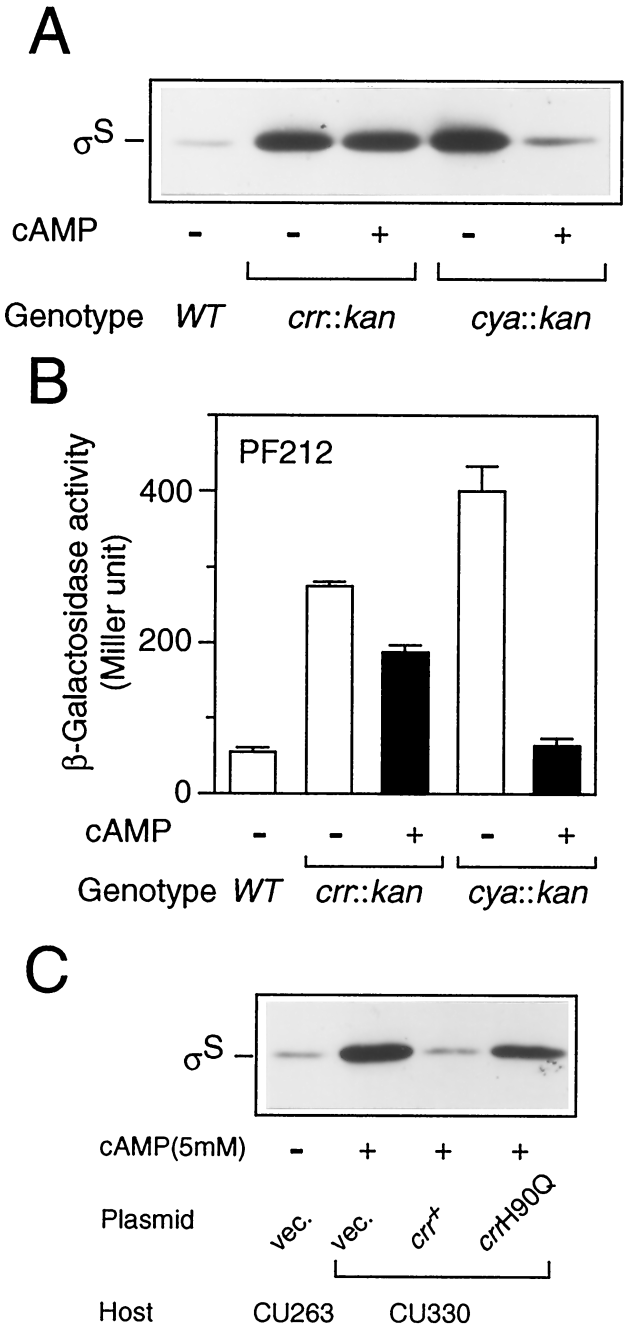

FIG. 7. The phosphorylated Crr is involved in *rpoS* translation. (A) Effect of cAMP on cellular σ^S content. Strains CU263 (wild type [WT]), CU330 (*crr*::*kan*), and NM23 (*cya*::*kan*) were grown at 37°C in Luria broth in either the absence (−) or the presence (+) of 5 mM cAMP. At the mid-logarithmic phase, total protein samples were prepared and each 20 μg was subjected to immunoblotting with anti-σ^S antiserum. (B) Effect of the addition of cAMP on *rpoS-lacZ* PF212 expression. Strains CU264 (wild type), NM9 (*crr*::*kan*), and NM24 (*cya*::*kan*), each carrying *rpoS-lacZ* PF212, were grown, and then β-galactosidase activity expressed by *rpoS-lacZ* PF212 was measured. The data are means with standard deviations for four independent assays. (C) Strains CU263 (wild type) and CU330 (*crr*::*kan*) harboring plasmid pTSV28 (vector [vec.]), pSTCRR (*crr*$^+$), or pST172 (*crr*H90Q) were grown at 37°C in Luria broth supplemented with 25 μg of chloramphenicol per ml in either the absence (−) or the presence (+) of 5 mM cAMP. At the mid-logarithmic phase, σ^S content was measured by immunoblotting.

HF-I is required for efficient *rpoS* translation, probably through melting of the preformed complex secondary structure of *rpoS* mRNA inhibiting the translation (3, 15). H-NS is, instead, a negative regulator of *rpoS* translation (1, 27), and we have no information as to the underlying molecular mechanism. One possible explanation is that Crr covalently modifies HF-I to modulate its function, although no modification of HF-I has been reported. Alternatively, Crr may somehow prevent the formation of the translational initiation complex required for *rpoS* translation. Since Crr is known to interact with many other proteins, such as sugar transporters and adenylate cyclase, in carbon metabolism, it is likely that Crr is also able to interact with the translational machinery or HF-I. In this regard, it is notable that ribosomal protein S7 has a consensus sequence implicated in the interaction with Crr (20). Crr may repress *rpoS* translation by modulating the function of S7, since S7 is known to repress its own translation through direct binding to mRNA (4). Of course, more complicated explanations cannot be excluded at present. In any event, further extensive genetic and biochemical analyses are necessary to clarify how Crr negatively regulates *rpoS* translation.

The phosphorylation of Crr appears to be important for the negative control of *rpoS* translation (Fig. 3 and 7). Since phosphorylated Crr is produced during successive phosphorelay reactions in PEP:PTS, not only Crr but also the overall system is apparently important for the control. The fact that the system absolutely depends on PEP, an important intermediate in carbon metabolism, leads us to the attractive idea that PEP:PTS regulates *rpoS* expression by monitoring the amounts of available nutrients throughout cell growth. However, this possibility seems unlikely because the phosphorylation state of Crr did not drastically fluctuate with the growth phase but rather

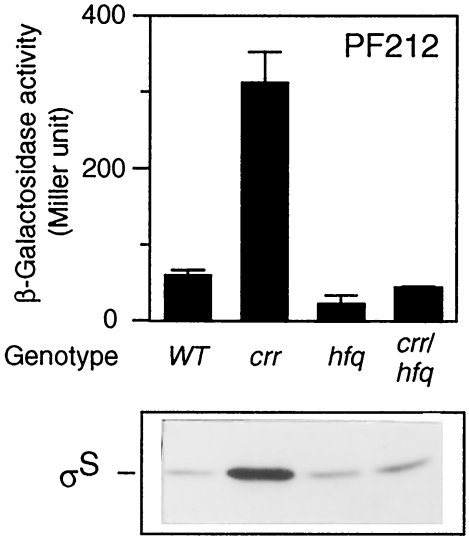

FIG. 8. Effect of an *hfq* mutation on *rpoS* expression in *crr* background. Strains CU264 (wild type [WT]), NM20 (*crr2–3*::mini-Tn*10cam*), NM11 (*hfq1*::Ω), and NM21 (*crr2–3*::mini-Tn*10cam hfq1*::Ω), each carrying *rpoS-lacZ* PF212 on the chromosome, were grown at 37°C in Luria broth. At the mid-logarithmic phase, β-galactosidase activity expressed by *rpoS-lacZ* PF212 (upper panel) and σ^S content (lower panel) were measured. β-Galactosidase activity data are means with standard deviations for four independent assays.

with the amount of extracellular glucose. Indeed, σ^S content in wild-type cells was not affected significantly by the addition of glucose (data not shown), which is known to dramatically decrease the levels of phosphorelated species of Crr (21). Therefore, alternatively, the intactness of PEP:PTS may be crucial for maintaining a lower level of *rpoS* expression. To ensure rapid proliferation at the logarithmic growth phase, the cells require a large amount of energy and several metabolic intermediates. In this situation, efficient transport of extracellular carbohydrates into cells by means of PEP:PTS should be necessary. When PEP:PTS is impaired by certain mutations, the cells may be unable to satisfy the requirement for adaptation to rapid growth conditions and subsequently *rpoS* expression is induced. If this is the case, the effect of the *crr* mutation on *rpoS* expression should be more significant under conditions permitting a higher growth rate, a hypothesis which is consistent with our observation that the effect of the *crr* mutation in a rich medium was stronger than that in a synthetic medium.

rpoS expression is enhanced when cells enter conditions preventing efficient proliferation, such as nutrient starvation, high osmolarity, and low pH, while it is repressed at the fast-growth logarithmic phase. Many proteinaceous factors involved in regulation of *rpoS* expression have been identified, and all of them except HF-I were found to negatively control *rpoS* expression (see the introduction), suggesting that the control of *rpoS* expression is achieved mainly through negative regulation at the logarithmic phase. When the growth conditions are sufficient for rapid proliferation, these negative regulators are activated by individual signals and subsequently repress *rpoS* expression at the transcription, translation, or protein stabilization step. If the growth conditions are not appropriate for rapid proliferation, a part of the negative regulatory mechanism may be unable to work well, resulting in derepressed expression of *rpoS* to some extent. The intactness of PEP:PTS may be recognized by cells as one of such cues for repression of *rpoS* expression.

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