JOURNAL OF BACTERIOLOGY, Mar. 1998, p. 1438–1445 Vol. 180, No. 6
0021-9193/98/$04.00+0
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Altered *srf* Expression in *Bacillus subtilis* Resulting from Changes in Culture pH Is Dependent on the Spo0K Oligopeptide Permease and the ComQX System of Extracellular Control

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Received 19 September 1997/Accepted 15 January 1998

The expression of the *srf* operon of *Bacillus subtilis*, encoding surfactin synthetase and the competence regulatory protein ComS, was observed to be reduced when cells were grown in a rich glucose- and glutamine-containing medium in which late-growth culture pH was 5.0 or lower. The production of the surfactin synthetase subunits and of surfactin itself was also reduced. Raising the pH to near neutrality resulted in dramatic increases in *srf* expression and surfactin production. This apparent pH-dependent induction of *srf* expression required *spo0K*, which encodes the oligopeptide permease that functions in cell-density-dependent control of sporulation and competence, but not CSF, the competence-inducing pheromone that regulates *srf* expression in a Spo0K-dependent manner. Both ComP and ComA, the two-component regulatory pair that stimulates cell-density-dependent *srf* transcription, were required for optimal expression of *srf* at low and high pHs, but ComP was not required for pH-dependent *srf* induction. The known negative regulators of *srf*, RapC and CodY, were found not to function significantly in pH-dependent *srf* expression. Late-growth culture supernatants at low pH were not active in inducing *srf* expression in cells of low-density cultures but were rendered active when their pH was raised to near neutrality. ComQ (and very likely the *srf*-inducing pheromone ComX) and Spo0K were found to be required for the extracellular induction of *srf-lacZ* at neutral pH. The results suggest that *srf* expression, in response to changes in culture pH, requires Spo0K and another, as yet unidentified, extracellular factor. The study also provides evidence consistent with the hypothesis that ComP acts both positively and negatively in the regulation of ComA and that both activities are controlled by the ComX pheromone.

Certain strains of *Bacillus subtilis* produce surfactin, a secondary metabolite composed of seven amino acids and a β-hydroxy fatty acid which together constitute an eight-membered cyclic lipopeptide. Surfactin is one of several microbially produced biosurfactants which are amphipathic molecules having many potential commercial applications (7). It is also endowed with antibacterial, antimycoplasma, antiviral, and hemolytic activity (1, 2, 47, 51, 52). Production of surfactin requires the products of the *srf* operon, encoding the three subunits of surfactin synthetase that catalyze the thiotemplate mechanism of nonribosomal peptide synthesis to incorporate the seven amino acids into the surfactin lipopeptide (5, 12, 15, 27, 28, 48–50). *srf* also contains the competence regulatory gene *comS*, which lies within and out-of-frame with the second gene of the operon, *srfB* (6, 18). A possible objective accomplished by this unusual association is the coregulation of the production of a lytic agent (surfactin) with a physiological state (genetic competence) designed for the uptake of a substance released from lysed cells (DNA).

The production of surfactin is but one example of a situation where an antibiotic biosynthesis gene or operon is activated by a regulatory system coupled to the accumulation of cell-derived extracellular signals. The production of streptomycin by *Streptomyces griseus*, of phenazine by pseudomonads, and of carbepenems by *Erwinia* spp. is regulated by extracellular factors mediating quorum sensing (13). The biological and/or ecological objective for regulating antibiotic production in this fashion would be to eliminate competition for scarce resources and coordinate the high level of production of antimicrobial agents among the members of a large, concentrated population so as to maximize the concentration and, thus, the impact of the secreted product. Biosurfactants, like the lipopeptide surfactin, might be produced by high-cell-density populations of *Bacillus* spp. so as to dispatch a lipophilic agent in high concentration to disperse hydrophobic aggregates, rendering their substituents susceptible to degradation and assimilation. Lipopeptides with similar structure to that of surfactin promote swarming motility in *Serratia marcescens* and, perhaps, *Proteus mirabilis*, a process necessarily carried out by dense populations of cells (14, 26).

Two pheromones, ComX and CSF, accumulate to high concentrations in late-growth cultures and are known to stimulate the transcription of *srf* (23, 25, 44, 45) (Fig. 1). ComX activates the signal transduction system composed of the two-component regulatory proteins ComP and ComA (25, 53, 54). The histidine protein kinase ComP donates a phosphate to the response regulator ComA, which, thus activated, stimulates the transcription of the *srf* operon (17, 30, 32–34, 40). CSF (competence-stimulatory factor) is encoded by the *phrC* gene, which is a member of the *phr* family of genes that encodes extracellular peptide factors that participate in the regulation of sporulation and other late-growth processes in *B. subtilis* by inhibiting the activity of aspartyl phosphate phosphatases of the Rap family (21, 35–37, 39, 44). CSF is believed to be imported via the oligopeptide permease encoded by *spo0K* (38, 41) and to negatively control the activity of the aspartyl phosphate phosphatase RapC. RapC is believed to negatively regulate *srf* transcription by removing the phosphate from ComA-P, thereby rendering ComA inactive (23, 44).

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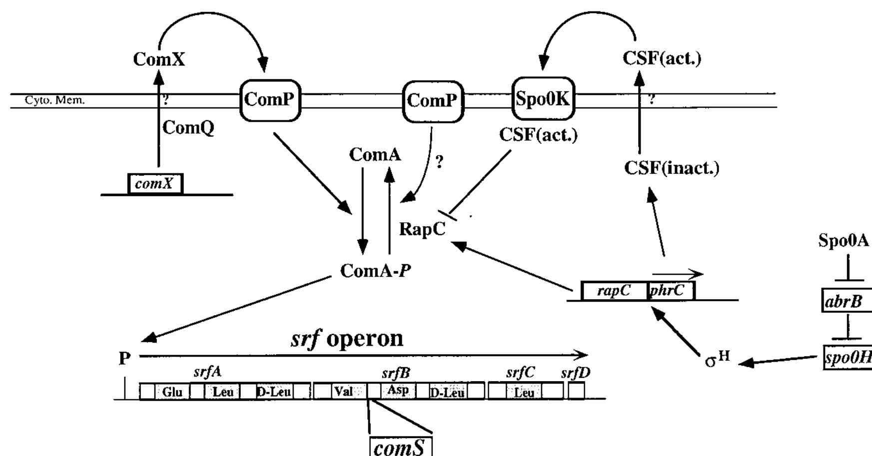

FIG. 1. Diagram showing the relationships among the regulatory factors governing *srf* transcription initiation. The *srf* operon as well as the regions of the *srf* genes encoding the amino-acid-activating modules of the peptide synthesis enzyme surfactin synthetase is shown. The *comS* gene, encoding the regulator of competence development, lies within the *srfB* gene of *srf*. Two pheromone-dependent pathways are shown. One pathway requires the ComX peptide which is processed by ComQ and which activates *srf* transcription through its interaction with ComP. ComP activates ComA by donating a phosphate, but it might also exert negative control by dephosphorylating ComA in the absence of ComX (see Discussion). The pheromone CSF activates *srf* transcription by inhibiting the ComA-phosphate phosphatase RapC. Import of processed, active CSF [CSF(act.)] requires the oligopeptide permease Spo0K. The inactive form of CSF [CSF(inact.)] is encoded by *phrC*, transcription of which requires the σ^H form of RNA polymerase. σ^H is encoded by *spo0H*, the transcription of which is regulated by Spo0A. Spo0A represses the *abrB* gene, which encodes a negative regulator of *spo0H* transcription. Cyto. Mem., cytoplasmic membrane.

srf transcription is also regulated through the DNA-binding protein CodY in response to changes in the nutritional environment (42, 43). High external concentrations of amino acids promote CodY-dependent repression of *srf* transcription initiation. The CodY protein has been shown to interact specifically with the *srf* promoter DNA (42).

The addition of excess glucose and glutamine to growth medium inhibits the expression of many late-growth genes including *srf* (4, 10, 11). We have recently reported that increasing the culture medium pH of late-exponential-phase cultures of *B. subtilis* relieves glucose-glutamine-dependent repression of genes that require the alternative RNA polymerase sigma subunit σ^H (4). We have examined the effect of culture pH on the production of surfactin by *B. subtilis* grown in excess glucose and glutamine and show that raising the culture pH relieves glucose-glutamine repression of surfactin production. This is the result of elevated *srf* transcription and is dependent on ComQ, ComA, and Spo0K but does not involve CodY-dependent negative control. Although the sensor histidine kinase ComP is required for maximal expression of *srf* at both low and neutral pHs, it is not required for induction of *srf* in response to pH elevation. Interestingly, the known regulators that function in the *spo0K*-dependent pathway of *srf* transcriptional activation, RapC and CSF, do not significantly function in pH-dependent *srf* transcriptional regulation. This suggests that there exist other mechanisms that influence *srf* expression in a *spo0K*-dependent manner.

MATERIALS AND METHODS

Bacterial strains. All *B. subtilis* strains are listed in Table 1 and are derivatives of JH642. LAB452 was constructed by transforming JH642 with pXL5 (30), a plasmid containing a *lacZ* fusion to the 3.5-kb *Eco*RV-*Sal*I fragment bearing the 5′ end of the *srfA* gene and the *srf* promoter region, with selection for chloramphenicol resistance (Cm^r). LAB2583 was constructed by transforming LAB452 (30) with pJL62 (24) linearized with *Pst*I with selection for spectinomycin resistance (Spc^r) and screening for chloramphenicol sensitivity. LAB2690 and LAB2691 were produced by transforming LAB2583 with DNA from JMS750 and JMS751 (23) and selecting for Spc^r Cm^r and for Spc^r and macrolide, lincosamide, and streptogramin B resistance (MLS^r), respectively. LAB2692 was constructed by transforming LAB452 with DNA from JRL350 (24) and selecting for Cm^r and MLS^r. LAB2693 and LAB2694 were constructed by transforming LAB452 with DNA from LAB590 (Δ*comP sfp*) (31, 54) and PS37 (*codY*) (42) and selecting for Cm^r and neomycin resistance (Neo^r) and for Cm^r Spc^r, respectively.

Transformation. Competent *B. subtilis* cells were prepared as described previously (8, 42).

Culture medium. Difco sporulation medium (DSM) was made as described previously (29) and was routinely used for all *B. subtilis* strains. DSM-GG was made by adding sterile 50% glucose and 2.5% glutamine to final concentrations of 1.9 and 0.1%, respectively, to sterile DSM.

Culture growth and β-galactosidase assays. Inocula and DSM-GGTris (DSM-GG plus Tris-HCl) were prepared and sample collection for β-galactosidase assays was performed as described previously (4).

Construction of *B. subtilis* strain expressing epitope-tagged *srfB*. Plasmid pOH9 bearing the carboxy-terminal end of *srfB* fused to the DNA encoding the influenza virus hemagglutinin 1 (HA1) epitope was constructed. pOH9 also contains the *srf* promoter region upstream from the *srfB-HA* sequence so as to drive the transcription of the *srfC* gene upon integration of the plasmid into the *srf* operon locus. First, the *Bam*HI-*Pvu*II fragment of pMMN50 (28) containing the P*srf* promoter was inserted into *Bam*HI-*Stu*I-cleaved pBSK-HA (46). The resulting plasmid, pOH7, was then opened with *Eco*RI and ligated with the 1.8-kb fragment of pMMN7 (28) containing the *cat* gene of pC194 (19), yielding pOH8. The *Eco*RV fragment of p223-21K (28) containing the 3′ end of *srfB* was then inserted into the *Nru*I site of pOH8, creating pOH9 bearing the *srfB* coding sequence fused with the DNA encoding the HA epitope. Competent cells of *B. subtilis* OKB105 (29) were transformed with pOH9 with selection for Cm^r. A single Campbell recombination event was expected to result in integration of the

TABLE 1. *B. subtilis* strains

Strain	Genotype and antibiotic resistance phenotype	Reference or source
LAB452	*trpC2 pheA1 srfA-lacZ*::pXL5 Cm^r	30
LAB991	*trpC2 pheA1 comA*::Tn*917* SPβc2Δ2::Tn*917*::*srfA-lacZ*(pXL5) Spc^r	33
LAB2583	*trpC2 pheA1 srfA-lacZ*::pXL5 Spc^r	This study
LAB2690	*trpC2 pheA1 srfA-lacZ*::pXL5 Spc^r *rapC*::*cat* Spc^r Cm^r	This study
LAB2691	*trpC2 pheA1 srfA-lacZ*::pXL5 Δ*phrC* MLS^r Spc^r	This study
LAB2692	*trpC2 pheA1 srfA-lacZ*::pXL5 Δ*spo0K* Cm^r MLS^r	This study
LAB2693	*trpC2 pheA1 srfA-lacZ*::pXL5 Δ*comP* Cm^r Neo^r	This study
LAB2694	*trpC2 pheA1 srfA-lacZ*::pXL5 Δ*codY* Cm^r Spc^r	This study
JMS755	*trpC2 pheA1* Δ*phrC comQ*::*spc* Spc^r Erm^{r}[a]	
OKB105	*pheA1 sfp*	29
OKB192	*trpC2 pheA1* Δ*comQXPA*::Tn*917*	32

[a] Erm, erythromycin.

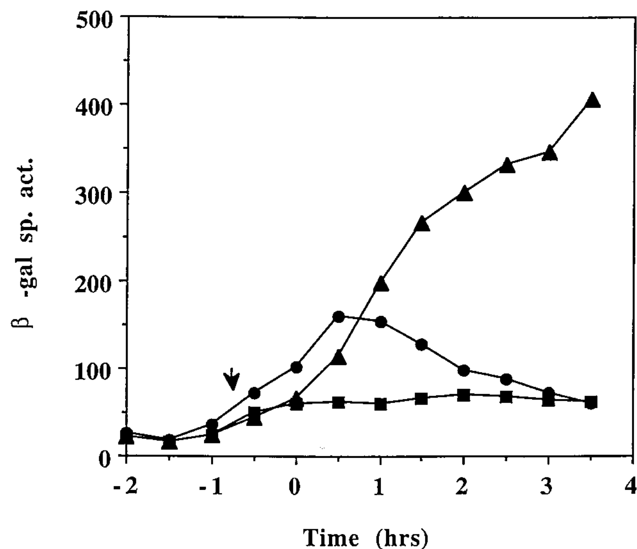

FIG. 2. Expression of *srfA-lacZ* in LAB452 grown in DSM-GG (squares), DSM-GGTris (triangles), and DSM without GG (circles). T_0 denotes the onset of stationary phase. The arrow indicates the time of Tris-HCl addition. β-Gal, β-galactosidase.

srfB-HA sequence into the 3′ end of the chromosomal *srfB* gene and positioning of the *srf* promoter upstream of *srfC*. Several transformants were observed to be Srf$^-$ despite the presence of the P*srf-srfC* fusion. The proper location of the integrated plasmid was confirmed by transformational linkage to *srfC*::Tn*917* of strain LAB223 (28). SrfB-HA protein from the Srf$^-$ transformants was detected by Western immunoblot analysis using monoclonal antibody 12CA5 (55) (see Fig. 3C). Western blot analysis was performed as previously described (4, 11).

Protein analysis. Cells from 10 ml of broth culture were suspended in 500 μl of 50 mM Tris-HCl, pH 7.8. After lysozyme treatment at 37°C for 15 min the cells were sonicated for 2 min. The cell debris was precipitated, and streptomycin sulfate was added to the crude extract to a final concentration of 1% (wt/vol). After 20 min of incubation on ice the nucleic acids were separated from the crude extract by centrifugation. Protein concentration was measured by using the procedure of Bradford (3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 5% polyacrylamide gels according to the method of Laemmli (22).

Surfactin production analysis. Surfactin was precipitated from 20 ml of culture supernatant by acidification at pH 2. The precipitate was extracted three times with methanol, and the collected extracts were evaporated prior to thin-layer chromatography (TLC) to increase surfactin concentration. A mixture of chloroform-methanol-H_2O (65:25:4) was used as the eluent. The TLC matrix was silica gel 60 from Fisher Scientific (Pittsburgh, Pa.). Surfactin was identified by its characteristic R_f of 0.64 (29) after spraying and charring with H_2SO_4.

Assay of *srfA*-directed β-galactosidase in cultures treated with conditioned medium. Stimulation of *srfA-lacZ* expression by cell-free supernatants was performed essentially as described by Solomon et al. (44). JH642, LAB2691 (Δ*phrC*), and OKB192 (Δ*comX*) cultures used for supernatant harvest were grown to T_2 (2 h after the end of exponential growth) in DSM-GG or DSM-GGTris at 37°C with shaking, which corresponded to an A_{595} of approximately 4.0 or 6.0, respectively. At that time, samples were collected and centrifuged at 5,000 rpm in a Sorvall SA600 rotor at 4°C, and the supernatants were filter sterilized and stored at −20°C. Before use in the assay the supernatants had their pHs adjusted to approximately 6.1 or 5.0 with NaOH or HCl, respectively. Control supernatants were collected from JH642 cultures grown in DSM-GG to an A_{595} of about 0.6 and were treated as described above. LAB452, LAB2692 (Δ*spo0K*), and LAB2693 (Δ*comP*) cultures for the *srfA-lacZ* assays were grown in DSM-GG at 37°C to an A_{595} of approximately 1.0 to 1.2 and then were mixed 1:1 with the pH-adjusted supernatants and reincubated at 37°C. Samples (1.0 ml) were then collected at 20-min intervals to test for β-galactosidase activity as described above.

RESULTS

Elevating the pH in DSM-GG cultures results in increased *srfA-lacZ* expression and surfactin production. We had previously reported the inhibitory effect of high external glucose and glutamine concentrations on sporulation gene expression (4). A similar repressive effect was observed when *srf-lacZ* expression was examined in cells growing in glucose-glutamine medium (11), indicating that other late-growth processes were suppressed when the preferred carbon and nitrogen sources were present in excess. But rather than this being a form of catabolite control, we have found that the repression observed might be due to the reduced culture pH, resulting, presumably, from the accumulation of acidic glycolytic end products as the glucose-glutamine-supplemented culture reached the end of exponential growth. Simply raising the pH of the culture by the addition of Tris-HCl or MOPS (morpholinepropanesulfonic acid) buffer resulted in dramatic derepression of late-growth genes, particularly those requiring the RNA polymerase sigma subunit σ^H for their transcription. As the expression of *srf* is also activated in late-growth cultures and also depends in part on *spo0H* (encoding σ^H), we examined the effect of raising the pH of the cultures grown in DSM-GG on the expression of a *srf-lacZ* operon fusion. In the DSM-GG-grown culture the pH drops to 5.0 near the end of the exponential phase of growth (4). The culture medium pH was raised to about 6.5 when Tris-HCl was added and did not fall below 5.5 during the period when samples were collected for assay of *srf*-directed β-galactosidase activity. Figure 2 shows that the presence of glucose and glutamine in DSM suppresses *srfA-lacZ* expression, but adjustment of culture pH with Tris-HCl increased the expression approximately fivefold above that observed in the untreated culture. The accumulation of the *srf* gene products (SrfA, SrfB, and SrfC [50]) as well as the production of surfactin also increased following adjustment of the DSM-GG pH. Figure 3 shows the relative levels of surfactin, as determined by TLC, immediately prior to Tris-HCl addition (T = 0) to a culture of OKB105 (Srf$^+$) cells in DSM-GG and after Tris-HCl addition (T = 3.5) to the DSM-GG-grown culture and a mock-treated DSM-GG culture. The amounts of surfactin synthetase proteins produced at the above times were also examined in cells of DSM-GG and Tris-HCl-treated DSM-GG cultures. More SrfA, -B, and -C proteins were produced in the Tris-treated culture than in the untreated DSM-GG culture medium (data not shown). Cells of strain LAB2426 containing an influenza virus HA epitope DNA-tagged allele of *srfB* were also tested for pH-dependent expression of *srf*. A higher level of SrfB-HA, as indicated by the presence of protein reacting with the 12CA5 monoclonal antibody (55), in the Tris-HCl-

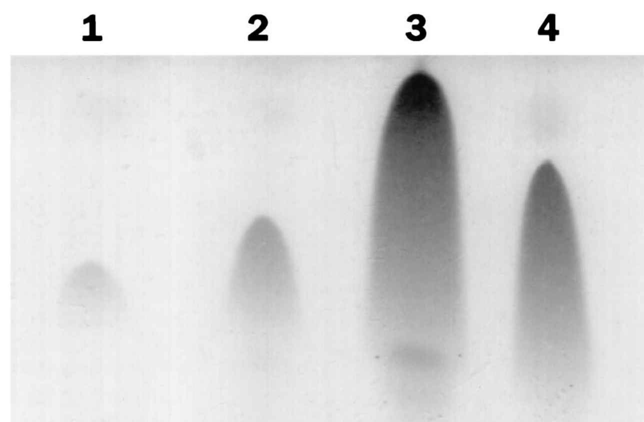

FIG. 3. Effect of Tris-HCl addition on surfactin production during *B. subtilis* growth. A TLC analysis showing the amounts of surfactin initially in the medium (lane 1), in the negative control at 3 h after addition of water (lane 2), and at 3 h after addition of Tris-HCl (lane 3) is presented. Lane 4 contains a surfactin standard. Sample volumes analyzed were specific to the total amount of cell protein in each sample.

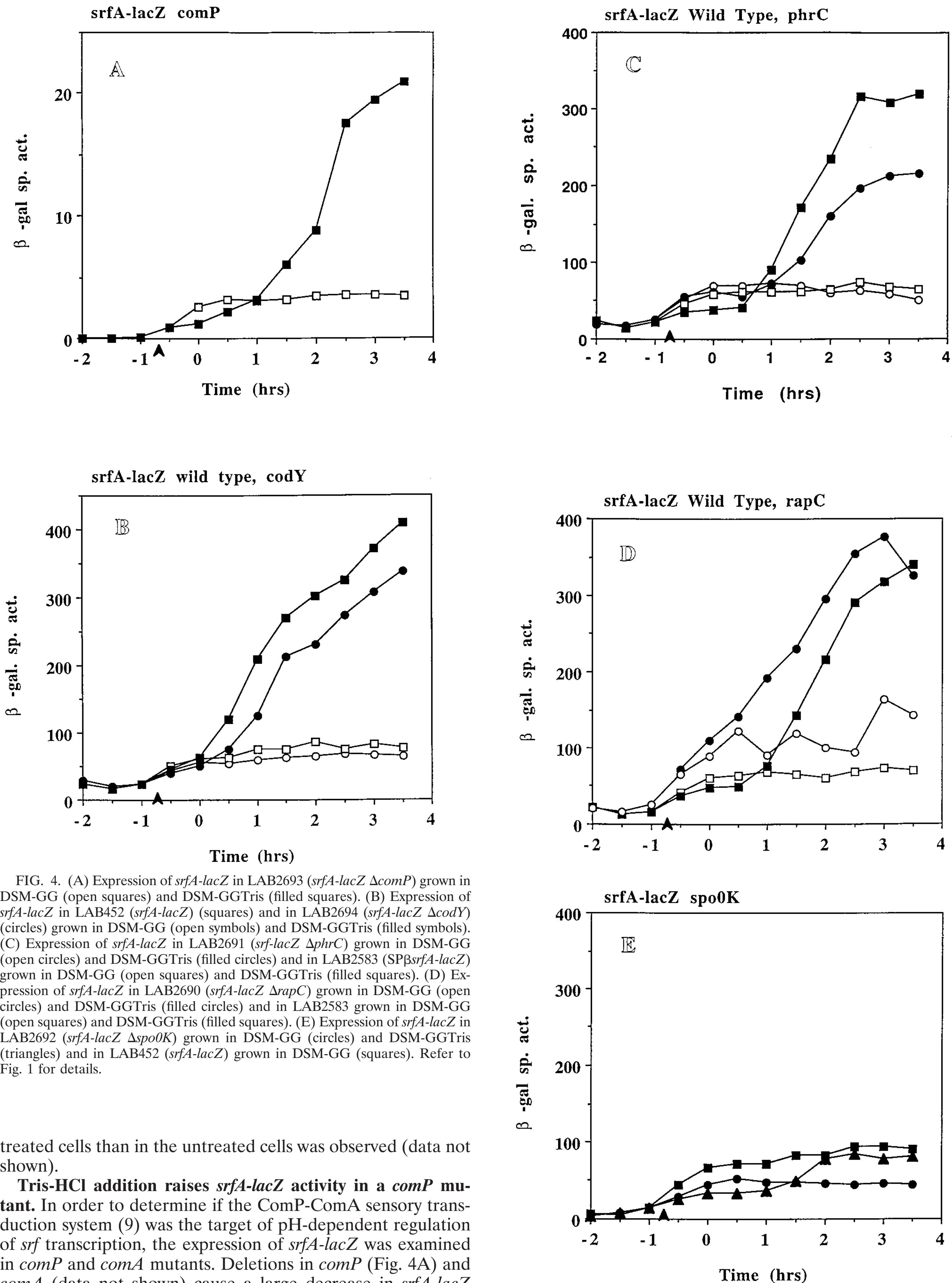

FIG. 4. (A) Expression of *srfA-lacZ* in LAB2693 (*srfA-lacZ* Δ*comP*) grown in DSM-GG (open squares) and DSM-GGTris (filled squares). (B) Expression of *srfA-lacZ* in LAB452 (*srfA-lacZ*) (squares) and in LAB2694 (*srfA-lacZ* Δ*codY*) (circles) grown in DSM-GG (open symbols) and DSM-GGTris (filled symbols). (C) Expression of *srfA-lacZ* in LAB2691 (*srf-lacZ* Δ*phrC*) grown in DSM-GG (open circles) and DSM-GGTris (filled circles) and in LAB2583 (SPβ*srfA-lacZ*) grown in DSM-GG (open squares) and DSM-GGTris (filled squares). (D) Expression of *srfA-lacZ* in LAB2690 (*srfA-lacZ* Δ*rapC*) grown in DSM-GG (open circles) and DSM-GGTris (filled circles) and in LAB2583 grown in DSM-GG (open squares) and DSM-GGTris (filled squares). (E) Expression of *srfA-lacZ* in LAB2692 (*srfA-lacZ* Δ*spo0K*) grown in DSM-GG (circles) and DSM-GGTris (triangles) and in LAB452 (*srfA-lacZ*) grown in DSM-GG (squares). Refer to Fig. 1 for details.

treated cells than in the untreated cells was observed (data not shown).

Tris-HCl addition raises *srfA-lacZ* activity in a *comP* mutant. In order to determine if the ComP-ComA sensory transduction system (9) was the target of pH-dependent regulation of *srf* transcription, the expression of *srfA-lacZ* was examined in *comP* and *comA* mutants. Deletions in *comP* (Fig. 4A) and *comA* (data not shown) cause a large decrease in *srfA-lacZ*

activity as has been previously observed. However, increasing the culture pH produces a fourfold increase in *srfA-lacZ* activity in the *comP* mutant compared to that in the cultures grown in DSM-GG without pH adjustment (Fig. 4A). A slight increase in *srfA-lacZ* activity was seen in a *comA* mutant (LAB991) when Tris-HCl was added to the culture medium, but the activity was too low in this strain for reliable calculation. These results indicate that optimum expression of *srf* after pH adjustment requires the ComP-ComA system, but since induction is still observed, the sensor kinase, ComP, is likely not the target of the pH-dependent effect.

A deletion in *codY* does not relieve the pH-dependent reduction of *srfA-lacZ* activity. It was necessary to determine if the repression of *srf* observed at low pH was due to the known transcriptional repressor of *srf* transcription, CodY (42). Hence, *srfA-lacZ* expression was examined in a *codY* mutant. No relief of repression was observed for a *codY* mutant in a low-pH culture (Fig. 4B), indicating that CodY was not functioning in pH-dependent *srf* repression. Figure 4B shows that a deletion in *codY* causes a slight but reproducible decrease in *srfA-lacZ* activity when DSM-GG cultures are treated with Tris-HCl. It is possible that the *codY* product may also have an indirect role in *srfA* expression when *B. subtilis* is grown in DSM-GGTris.

Mutation in *spo0K* severely impairs pH-dependent induction of *srfA-lacZ*. Neither ComP nor CodY appeared to be targets of the apparent pH-dependent control of *srf*. The role of the CSF/Spo0K/RapC system (23, 44) in *srf* regulation was next investigated. Figure 4C to E shows *srfA-lacZ* expression in DSM-GG and DSM-GGTris cultures of strains containing lesions in *phrC*, the gene which encodes CSF, *rapC*, which encodes the putative target of CSF, i.e., the Rap phosphatase that inactivates ComA, and *spo0K*, the oligopeptide permease gene, respectively. Expression of *srfA-lacZ* in LAB 2691 (Δ*phrC*) in DSM-GG (Fig. 4C) is comparable to that of the wild-type parent but shows a fourfold induction following Tris-HCl addition and about 65% of the induced activity observed in the wild-type cells. LAB2690 (Δ*rapC*) shows a slight increase in *srfA-lacZ* expression above that of LAB2583 (RapC$^+$) in DSM-GG (Fig. 4D) but a five- to sixfold induction of *srfA-lacZ* expression after Tris-HCl addition, comparable to that observed in wild-type cells. In DSM-GGTris, *srfA-lacZ* expression in LAB2690 begins about 1.0 to 1.5 h earlier, and overall expression is approximately 20% greater than that in LAB2583. Both LAB2690 and LAB2691 showed cell yields and culture pH profiles similar to those of LAB2583 (wild type) in both media (data not shown). Unlike what was found for strains LAB2690 and -2691, Tris-HCl addition to DSM-GG cultures of LAB2692 (Δ*spo0K*) failed to increase *srfA-lacZ* expression above that of the wild type in DSM-GG (Fig. 4E). A less than twofold induction was observed in the *spo0K* mutant when Tris-HCl was added to the culture. The fold increases in *srfA-lacZ* expression upon pH elevation were determined for all of the mutant strains tested along with those of the wild-type parent cultures run in parallel (Table 2). All strains showed a three- to eightfold increase in *srfA-lacZ* expression except for the *spo0K* mutant, which exhibited a less than twofold increase in expression upon pH adjustment. This suggests that the Spo0K peptide is involved in the pH-dependent induction of *srf* expression.

***srfA-lacZ*-inducing substance in late-growth culture supernatant has low level of activity at low pH.** The involvement of Spo0K oligopeptide permease in the pH-dependent induction of *srf*, along with the modest effects the *phrC* and *rapC* mutations had on the observed pH-dependent increase of *srfA-lacZ*, suggested that there exists another substance, apart from CSF and ComX, in conditioned medium of late-growth cultures that could stimulate *srf* transcription. To examine this possibility, the *srf*-inducing activities of conditioned culture media were tested by diluting early-growth cultures one to one in cell-free medium supernatant obtained from late-growth DSM-GG and DSM-GGTris cultures. Supernatant samples were collected from the following centrifuged cultures: (i) late-growth DSM-GG at a pH of ~5.0, (ii) DSM-GGTris at a pH of ~6.6, and (iii) early-growth cultures in DSM-GG. Each of the three supernatant samples was split, and one half was subjected to pH adjustment. Thus, half of the first supernatant was treated with NaOH to raise the pH to 6.1. Half of the second supernatant was treated with HCl to reduce the pH to 5.0. Finally, the early-growth supernatant was divided into high-pH (6.1) and low-pH fractions (5.0). *srf*-activating pheromones had been shown to accumulate in high cell density late in culture growth and to be in low concentration in early-growth cultures (16, 25). Therefore, the early-growth supernatants were included as negative controls.

TABLE 2. Fold increase of *srfA-lacZ* expression in DSM-GG by pH adjustment with Tris-HCl[a]

Type of strain	Expt	Fold increase (SD) in *srfA-lacZ* expression in strain containing:				
		comP	*rapC*	*phrC*	*spo0K*	*codY*
Mutant	1	8.1 (1.8)	2.8 (0.9)	3.7 (0.6)	1.5 (0.1)	4.7 (0.6)
	2	5.4 (0.6)	3.2 (0.4)	3.5 (0.3)	1.8 (0.1)	5.6 (0.6)
Wild type	1	5.0 (0.6)	4.5 (0.4)	4.6 (0.3)	3.4 (0.4)	4.7 (0.4)
	2	2.8 (0.1)	4.4 (0.5)	5.2 (0.6)	3.6 (0.5)	5.6 (0.8)

[a] Ratios of *srfA-lacZ* expression (Miller units) in DSM-GGTris cultures to that in DSM-GG cultures were calculated for samples of each strain collected at $T_{2.5}$, T_3, and $T_{3.5}$. Data are averages from two sets of experiments. As controls, wild-type *srf-lacZ* strains were analyzed in parallel with each mutant.

Addition of cell-free supernatant from late-growth cultures grown in DSM-GGTris to low-density cultures resulted in the induction of *srfA-lacZ*, but this induction was inhibited if the pH of the conditioned medium was reduced to a pH of 5 (Fig. 5A). The low-pH supernatant of a late-growth DSM-GG culture did not stimulate *srfA-lacZ*, but induction of the fusion was observed if the pH of this supernatant fluid was first adjusted to a pH of 6.1 with NaOH. This suggests that the capacity to extracellularly induce *srf* transcription is present in the DSM-GG medium but the low pH inhibits this activity. Neither of the control supernatants, from early-growth cultures with either a low or a high pH, stimulated *srfA-lacZ* to the same extent as either of the late-growth supernatants (Fig. 5A).

The induction of *srfA-lacZ* by the addition of high-pH-conditioned medium was found to depend on Spo0K, as expected from the experiments described above and from previous studies (39, 44, 45). Supernatant samples from late-growth, high-pH cultures of wild-type and Δ*phrC* cells were found to stimulate *srfA-lacZ* when added to *spo0K* cells of a low-density DSM-GG culture (Fig. 5B). The stimulation of *srfA-lacZ* caused by the Δ*phrC* supernatant was likely due to the presence of ComX pheromone and the sensory transduction system of ComP-ComA. Indeed, when supernatant was obtained from a late-growth culture of a Δ*comX* strain and tested for *srfA-lacZ*-stimulating activity in a *spo0K* mutant, none was observed. This might suggest that the *spo0K*-dependent induction of *srf* at neutral pH requires an extracellular substance other than CSF.

That this substance is not CSF (PhrC) was shown by adding

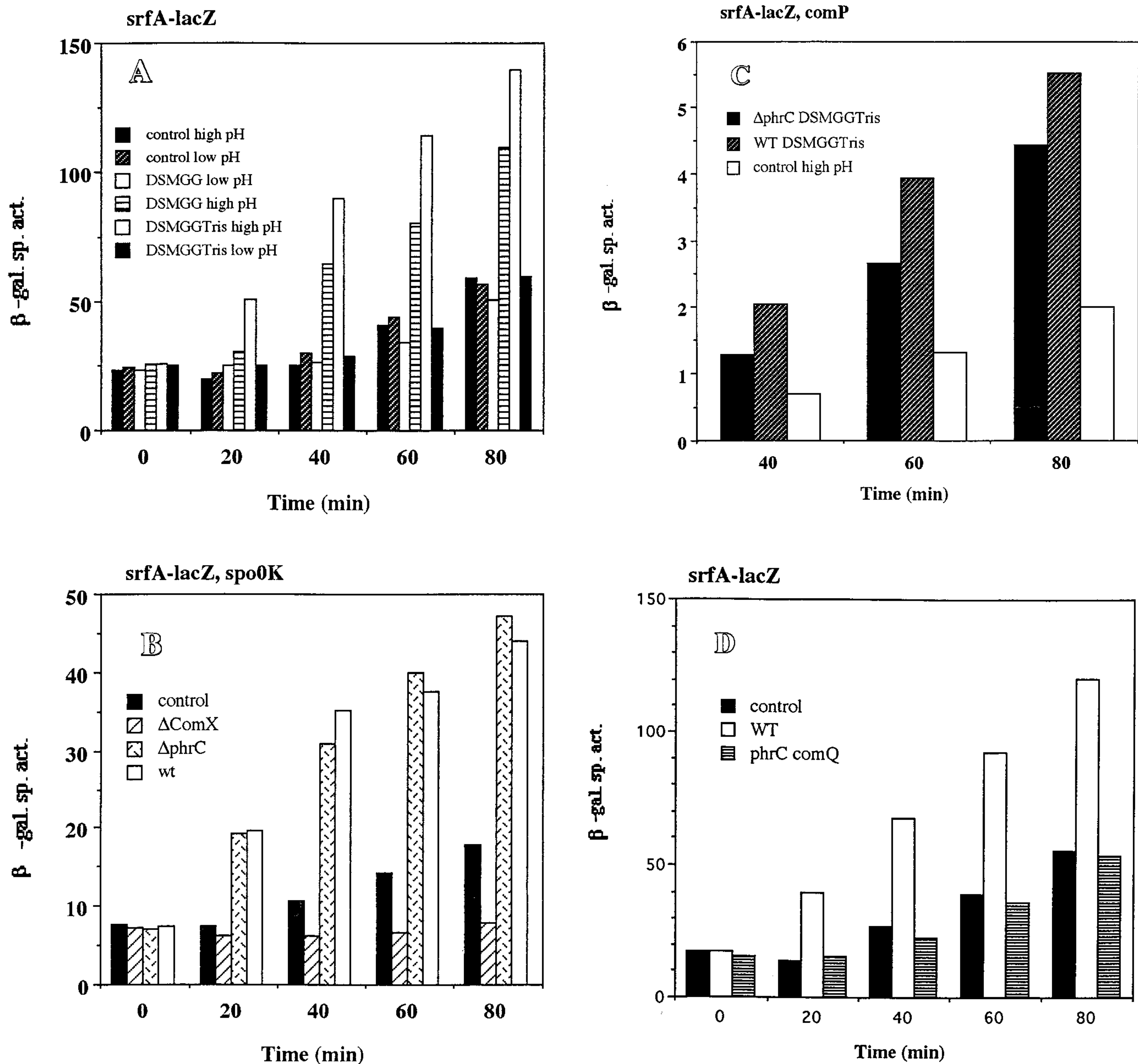

FIG. 5. Effects of DSM-GG and DSM-GGTris culture supernatants on expression of *srfA-lacZ*. LAB452 (*srfA-lacZ*), LAB2692 (*srfA-lacZ ΔspoOK*), and LAB2693 (*srfA-lacZ ΔcomP*) were grown in DSM-GG to a low density and then mixed 1:1 with pH-adjusted supernatants as described in Materials and Methods. Samples were collected every 20 min for β-galactosidase activity assays. Figures are representative of at least two experiments run under identical conditions. (A) Recipient cultures contained cells of strain LAB452 (*srfA-lacZ*). (B) Culture medium supernatants were collected from late-growth cultures of strains OKB192 (*ΔcomX*), LAB2691 (*ΔphrC*), and LAB452 (wild type). All supernatants were adjusted to pH 6.1. Recipient cultures contained cells of strain LAB2692 (*srfA-lacZ ΔspoOK*). (C) All supernatants were adjusted to pH 6.1. Recipient cultures contained cells of strain LAB2693 (*srfA-lacZ ΔcomP*). (D) Supernatants of late-growth JMS377 (*ΔcomQ ΔphrC*) culture, JH642 (wild-type parent) late-growth culture, and JH642 early-growth culture adjusted to pH 6.1. Recipient early-growth cultures contained cells of LAB452 (*srfA-lacZ*).

a high-pH, late-growth supernatant of a Δ*phrC* culture to cells of a *srfA-lacZ comP* strain, which should not respond to ComX. The level of *srfA-lacZ* after treatment of low-density culture with supernatant from a wild-type or Δ*phrC* culture was higher than that observed with supernatant from a wild-type low-density culture (Fig. 5C), suggesting the presence of an additional extracellular factor controlling *srf* transcription.

ComQ is required for extracellular induction of *srfA-lacZ* at neutral pH. Although the data above indicate the need for Spo0K, and not ComP, for the pH-dependent stimulation of *srf* expression, they do not rule out the possibility that ComX might somehow function in a Spo0K-dependent induction process. A late-growth culture supernatant from double mutant JMS755 (Δ*phrC comQ*::*spc*, a gift from B. Lazazzera) was added to early-growth cultures of LAB452 (*srfA-lacZ*). As shown in Fig. 5D, the supernatant from the double mutant showed no *srfA-lacZ*-stimulating activity, while the culture supernatant of the wild type activated *srf* expression. In a wild-type strain, both Spo0K and ComQ (and very likely ComX) are required for the extracellular activation of *srf* at neutral pH.

DISCUSSION

The expression of a *srfA-lacZ* operon fusion is affected by culture medium pH, as shown by an examination of *srf*-directed β-galactosidase activity in cells grown in nutrient broth sporulation medium supplemented with glucose and glutamine. Under these conditions, the pH of the culture drops to below 5.0 near the end of the exponential phase and *srfA-lacZ* expression, normally increasing at this point in the growth curve, remains low and approximately at the levels observed in early and mid-log phase. The addition of a pH stabilizer to raise the pH causes sharp increases in *srfA-lacZ* expression and surfactin production (Fig. 2 and 3). The maximum expression of *srf* at both low and neutral pH requires the ComP-ComA signal transduction system and presumably ComX, the peptide signal that is believed to mediate the cell-density-dependent activation of ComP (25). The pH-dependent induction of *srf* requires *spo0K*, which is known to function in the CSF-mediated activation of *srf* transcription (44, 45). Interestingly, the elimination of CSF by deletion of the *phrC* gene does not impair induction of *srf* expression in response to pH elevation, although expression levels do not reach those of wild-type *srfA-lacZ*-bearing cells. The negative regulators of *srf* transcription, RapC and CodY, also appear not to participate in pH-dependent control of *srf*, as evidenced by the absence of *srf* derepression in *rapC* and *codY* cells under low-pH culture conditions.

Experiments with cell-free culture supernatants added to early-growth cultures of *srf-lacZ*-bearing cells show that the extracellular factor necessary for induction of *srf* does not function at low pH. The low pH of the DSM-GG-grown culture could have several effects on the expression of *srfA-lacZ*. It is possible that the *spo0K*-encoded oligopeptide permease is not functional when the pH of the external environment is low. It is also possible that the peptide pheromone activating *srf* transcription through a Spo0K-dependent mechanism is not in the proper ionic state at low pH. Because the culture supernatant of a low-pH culture can be made active with respect to *srf* stimulation when its pH is raised, it is possible that the peptide is present in the low-pH DSMGG culture but is not able to activate *srf* induction.

The requirement for ComQ, and hence ComX, for the extracellular, *spo0K*-dependent induction of *srf* at neutral pH (Fig. 5D) might suggest that ComX also acts through a mechanism involving the Spo0K oligopeptide permease. This is supported by the data shown in Fig. 4A and 5C which show that ComP, the reported target of ComX, is not required for pH-dependent, extracellular induction of *srf* expression. However, previous studies from the Grossman laboratory provide evidence that is inconsistent with the hypothesis that ComX functions to induce *srf* through a Spo0K-dependent mechanism (25, 44, 45). A reasonable explanation of our results is that ComP functions both positively and negatively in the regulation of *srf* transcription. ComX has two functions, stimulation of ComP autokinase activity, rendering ComP a phosphate donor for ComA, and inhibition of a ComP phosphatase which can dephosphorylate ComA-P (Fig. 1). Histidine protein kinases of the sensor class of two-component regulatory proteins can possess both phosphate-donating and phosphate-removing activities that are affected by ligand binding (20, 21). In a *comP* mutant, *srf* expression is low but is still inducible by a pH- and Spo0K-dependent activity. If ComX is absent, due to a mutation of *comQ*, the product of which functions in ComX processing and secretion, the ComP phosphatase is active and dephosphorylates ComA. Thus, even in the presence of Spo0K and the extracellular factor governing pH-dependent *srf* induction, ComP phosphatase reduces ComA activity. However, if *comP* is eliminated (Fig. 4A and 5C) extracellular pH-dependent induction is observed, even though the level of expression of *srfA-lacZ*, overall, is low. The *comP*-independent induction of *srf* expression does not involve CSF (PhrC), as shown in Fig. 5C. An appropriate course of study, based on these results, is to attempt characterization of the extracellular *srf* induction mechanism in a *comP* mutant background.

The requirement for Spo0K in *srf* induction upon pH elevation suggests the involvement of the Phr/Rap system, but Spo0K could mediate the uptake of other peptide factors which might elicite an entirely different response. The oligopeptide permease is not specific for Phr-like factors but has also been implicated in the uptake of other peptides including the peptide antibiotic bialophos (36). Mutations in *spo0K* render *B. subtilis* cells bialophos resistant (36). Hence, it is possible that the *spo0K*-mediated response to pH elevation is a consequence of multiple extracellular peptide factors that activate separate, distinct regulatory response pathways.

ACKNOWLEDGMENTS

We thank A. Grossman and members of his lab as well as A. L. Sonenshein for providing strains used in this study.

The research reported herein was supported by grant GM54898 from the National Institutes of Health and by funds provided by USDA/ARS project no. 6435-41000-062-015.

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