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Superoxide Stress Decreases Expression of *srfA* through Inhibition of Transcription of the *comQXP* Quorum-Sensing Locus in *Bacillus subtilis*

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During the course of screening for competence-deficient mutants in the mutant collection constructed by the Japan Consortium of Bacillus Functional Genomics, a disruption mutant of *sodA* encoding superoxide dismutase was identified as a mutant with decreased transformation efficiency. In fact, in the *sodA* mutant we observed a severe decrease in the expression of *srfA* required for the development of genetic competence. Northern and primer extension analyses revealed inhibition of the transcription of the *comQXP* quorum-sensing locus in the *sodA* mutant, thereby preventing *srfA* expression. Furthermore, an excess amount of superoxide anion induced by the addition of paraquat also resulted in a decrease in *comQXP* transcription. Thus, it was concluded that high levels of superoxide are able to inhibit specifically the transcription of the *comQXP* operon. In support of this conclusion, the effect of added paraquat was significantly alleviated in a *comX*-independent *srfA* expression system.

Key words: oxidative stress, quorum sensing, *sodA*, *srfA*.

Abbreviations: SOD, superoxide dismutase; ROS, reactive oxygen species; Pm, phleomycin; Em, erythromycin; Amp, ampicillin.

Bacillus subtilis undergoes several adaptive responses to nutrient depletion and high cell density, including the development of genetic competence and the initiation of sporulation (*1*, *2*). In competence development, cell density signals trigger the phosphorylation of ComA, the response regulator of the ComP-ComA two-component system, resulting in the expression of the *srfA* operon (*3*, *4*). The activation of ComK requires ComS to be encoded within the *srfA* mRNA as another ORF (*1*). ComS releases ComK from the MecA-ClpCP-ComK complex, leading to the autoactivation of *comK*. In addition, several activators such as ComK itself and DegU, and repressors including CodY, AbrB and Rok, are involved in the activation of ComK (*1*, *2*). Finally, ComK activates the transcription of many genes, including late competence operons encoding the protein machinery components needed for the uptake and processing of foreign DNA (*5*). During the transition from the growing phase to stationary phase, Spo0A is activated by the phosphorelay system composed of histidine kinases, Spo0F and Spo0B, resulting in both transcriptional repression and the activation of many target genes (*2*). Spo0A is needed for *comK* transcription through the repression of *abrB*.

The *srfA* operon encodes the biosynthetic genes for a biosurfactant, surfactin, which has been reported to be important for swarming motility and fruiting body formation in the natural isolates of *B. subtilis* (*6–10*). Expression of *srfA* is under the control of two systems that control extracellular activity, ComQXP and RapC-CSF (*1*, *11–13*). The ComX pheromone is secreted into the medium and modified with an isoprenoid unit dependent on a membrane protein, ComQ, and then the accumulated ComX is sensed by the receptor ComP kinase, resulting in phosphorylation of the cognate response regulator ComA (*14*, *15*). The nucleotide sequences of the *comQXP* locus exhibit polymorphisms in natural strains, resulting in the strain-specific activation of ComA by the ComX pheromone (*16*, *17*). RapC and RapF are inhibitors of the DNA-binding of ComA-P, which activates both *rapC* and *rapF* transcription, thereby constituting a regulatory loop (*18–20*). PhrC and PhrF, extracellular pentapeptides, inhibit RapC and RapF activities, respectively, after incorporation into the cytoplasm, leading to the activation of ComA-P (*12*, *20*). Finally, ComA-P binds to the *srfA* promoter and then *srfA* expression is fully activated. Furthermore, Spx inhibits the interaction between ComA-P and the α-subunit of RNA polymerase, and then the ClpXP protease degrades Spx, resulting in the activation of the ComA regulon (*21*). In addition, the expression of *spx* is induced by diamide stress (*22*).

In this paper we report a decrease in transformation efficiency caused by the inhibition of *srfA* transcription in a *sodA* mutant deficient in SOD activity. Further analysis revealed that transcription of the *comQXP* operon was decreased by either an excess amount of superoxide anion in the *sodA* mutant or by the addition of paraquat.

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EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Media—All the *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* and *Escherichia coli* cells were grown in Modified Competence (MC) medium and Luria-Bertani (LB) medium, respectively (*23*). Concentrations of antibiotics used were described previously (*24*).

Materials—Synthetic oligonucleotides were prepared commercially by Tsukuba Oligo Service (Ibaraki, Japan) and are listed in Table 2. Sequencing was carried out using a 377 DNA Sequencer (Perkin-Elmer) and a Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

Screening for Deficient-Mutants in Competence Development—Disruption mutants were precultured in Antibiotic medium III (Difco) containing erythromycin overnight at 37°C, and inoculated into MC medium. Two hours after the end of the logarithmic growth phase, the kanamycin-resistant pLC1 plasmid was added to the culture (*25*). A four-fold volume of Antibiotic medium III was added 30 min after the addition of DNA. Cultivation was continued for further 2 h, and 100 μl of the culture was plated onto an LB agar plate containing 10 μg/ml kanamycin and 0.5 μg/ml erythromycin. The number of transformant cells was counted after overnight incubation at 37°C. In every set of experiments, transformation of *B. subtilis* 168 strain was carried out as a control.

Plasmid Construction—All the plasmids used in this study are listed in Table 3. To construct pMutin-sodA, the PCR product amplified by using oligonucleotide primer pairs SodA-F and SodA-R was treated with *Hin*dIII and *Bam*HI and cloned between the *Hin*dIII and *Bam*HI sites of pMutinIII (*26*). To construct pPhl-sodA and pPhl-sodF, the PCR products amplified by using sodA-PF and sodA-PR, and sodF-PF and sodF-PR as oligonucleotide primers were treated with *Eco*RI and *Sal*I, and cloned into pPhl-2 (*24*) treated with the same enzymes.

β-*Galactosidase Assay*—Samples were withdrawn at hourly intervals for the measurement of β-galactosidase activities as described previously (*25*). Conditioned medium was prepared by removing the cells by centrifugation and filter-sterilizing the supernatant. Cells were grown in MC medium to mid-log phase and divided into two equal volumes. To each half, either conditioned or fresh medium was added, and then samples were withdrawn.

RNA Isolation, Primer Extension Analysis and Northern Hybridization—Total RNA was isolated from the cells as described previously (*27*). A primer extension analysis of *comQXP* transcripts was performed as described previously (*28*). For the detection of *comQXP* transcripts, comQXP-Biotin was used as a primer. Detection of biotinylated DNA was performed using a non-radioisotope DNA detection kit (Chemiluminescence; Toyobo Co.). For Northern analysis digoxigenin-labeled probe DNA was prepared by PCR amplification with primers comQXP-1 and comQXP-2 (Table 2) and a PCR DIG Probe Synthesis Kit (Boehringer Mannheim, Germany). RNA was denatured by formamide and blotted onto a Nylon membrane (Boehringer Mannheim, Germany), and the hybridized bands were detected with a DIG Luminescent Detection Kit (Boehringer Mannheim, Germany).

Table 1. ***Bacillus subtilis*** **strains used in this study.**

Strain	Relevant phenotype and description	Reference or source
168	*trpC2*	Laboratory stock
ORB3834	*trpC2 pheA1 spx*::Km^r	*21*
SODd	*trpC2 sodA*::pMutin-sodA (Em^r)	This study
OAM230	*trpC2 sodA*::Pm^r	This study
OAM231	*trpC2 sodF*::Pm^r	This study
OSM103	*trpC2* Δ*comQXPA* (Em^r)	*35*
OSM102	*trpC2 srfA-lacZ* (Em^r Cm^r) Δ*comQXPA* (Em^r)	*35*
OAM186	*trpC2 srfA-lacZ* (Em^r Cm^r)	*42*
OAM232	*trpC2 srfA-lacZ* (Em^r Cm^r) *sodA*::Pm^r	This study
OAM245	*trpC2 srfA-lacZ* (Em^r Cm^r) *rapC*::Em^r (*lacZ*::Tc′)	*42*
OAM276	*trpC2 srfA-lacZ* (Em^r Cm^r) *rapC*::Em^r (*lacZ*::Tc′) *sodA*::Pm^r	This study
OAM233	*trpC2 srfA-lacZ* (Em^r Cm^r) *spx*::Km^r	*42*
OAM234	*trpC2 srfA-lacZ* (Em^r Cm^r) *spx*::Km^r *sodA*::Pm^r	This study
8G33	*trpC2 comK-lacZ* (Km^r)	*5*
OAM235	*trpC2 comK-lacZ* (Km^r) *sodA*::Pm^r	This study
OAM236	*trpC2 comG-lacZ*(Cm^r)	*24*
OAM237	*trpC2 comG-lacZ*(Cm^r) *sodA*::Pm^r	This study
BD4244	*hisA1 leu8 metB5 srfA-lacZ* (Em^r Cm^r) *amyE*::Δ*Labc comP*(Em^r) *comXP*::Sp^r	*15*
OAM238	*trpC2 srfA-lacZ* (Em^r Cm^r) *amyE*::Δ*Labc comP* (Em^r) *comXP*::Sp^r	This study
OAM239	*trpC2 srfA-lacZ* (Em^r Cm^r) *amyE*::Δ*Labc comP* (Em^r) *comXP*::Sp^r *sodA*::Pm^r	This study
BFS1211	*trpC2 rapF-lacZ* (Em^r)	*35*

Table 2. **Oligonucleotides used in this study.**

Name	Sequence
sodA-F	5′-GCCGAAGCTTGTTGTATTGGCTGTTGA-3′
sodA-R	5′-CGCGGATCCAAAGCCATAGCCAACTC-3′
sodA-PF	5′-ATGGAATTCATTACCTTATGCGTACGATG-3′
sodA-PR	5′-ATGGTCGACTGTCAAAGGTTCCAAATACA-3′
sodF-PF	5′-ATGGAATTCATCTTATCAAGCGGAGATGT-3′
sodF-PR	5′-ATGGTCGACTTCAGGCCATCGACATAGC-3′
comQXP-Biotin	5′-biotin-GAGGATTCAGCAAAGGAGAAT-3′
comQXP1	5′-ATGAAGGAGATTGTGGAGCA-3′
comQXP2	5′-CACCCCATTGACGGGTTATT-3′

RESULTS

Disruption of sodA *Decreases Transformation Efficiency and the Transcription of the* srfA *and* com *Genes*—During the course of screening for competence-deficient mutants in the mutant collection constructed by the Japan

Table 3. **Plasmids used in this study.**

Plasmid	Description	Reference or source
pMutinIII	Insertional Em^r Amp^r plasmid carrying promoterless *lacZ*	*26*
pMutin-sodA	pMutinIII carrying a part of *sodA*	This study
pPhl-2	pUC19-based Pm^r plasmid	*24*
pPhl-sodA	pPhl-2 carrying a part of *sodA*	This study
pPhl-sodF	pPhl-2 carrying a part of *sodF*	This study

Table 4. **Transformation efficiency of various cells.**[a]

Strain	Relevant genotype	No. of viable cells (cells/ml)	No. of transformants (cells/ml)	Transformation efficiency (%)	Ratio (%)
168		2.30×10^8	2.79×10^4	1.21×10^{-2}	100
SODd	*sodA*	1.25×10^8	0.021×10^4	0.017×10^{-2}	1.4
OAM231	*sodF*	2.44×10^8	2.23×10^4	0.91×10^{-2}	75

[a]Total DNA containing a kanamycin resistance marker was added to the cell culture at T2. After the transformation cells were subjected to serial dilutions. Each fraction was plated onto an LB agar plate with kanamycin to count numbers of transformed cells. To count numbers of viable cells the diluted fraction was plated onto an LB agar plate without kanamycin.

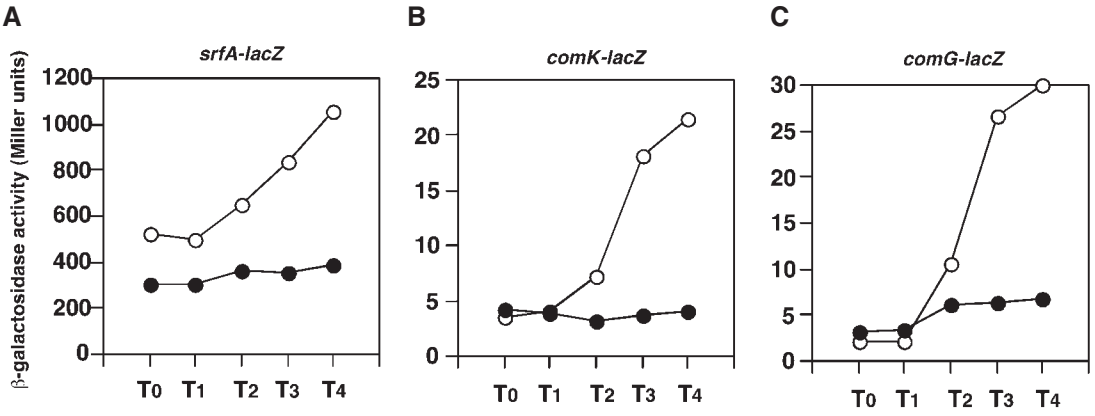

Fig. 1. **Expression of *srfA-lacZ*, *comK-lacZ* and *comG-lacZ* in *sodA* mutant cells.** Cells were grown in MC medium, and β-galactosidase activities were determined described under "EXPERIMENTAL PROCEDURES." Numbers on x axis represent the duration of culture in hours relative to the end of the vegetative growth (T0). (A) Open circles, OAM186 (WT); closed circles, OAM232 (*sodA*). (B) Open circles, 8G33 (WT); closed circles, OAM235 (*sodA*). (C) Open circles, OAM236 (WT); closed circles, OAM237 (*sodA*).

Consortium of Bacillus Functional Genomics with a transformation assay (*29*, *30*), a disruption mutant of *sodA* encoding a manganese-associated type of SOD was found to exhibit decreased transformation efficiency (Table 4). In the genome of *B. subtilis*, a paralogous gene of *sodA*, *sodF* is located at another locus. To examine the possibility that disruption of *sodF* results in a decrease in transformation efficiency, a *sodF* mutant was constructed and its transformation efficiency was determined. As shown in Table 4, the *sodF* disruptant exhibited transformability similar to the wild-type. The SOD activities in the wild-type, *sodA* and *sodF* strains were examined and a >90% decrease and a similar level of activity were observed in *sodA* and *sodF* cells, respectively, compared to that in the wild-type (data not shown). These results indicate that only *sodA* is responsible for cellular SOD activity. This is consistent with previous results (*31*, *32*).

It was expected that a decrease in the expression of certain competence-related genes might bring about reduced transformability in the *sodA* mutant. To examine the possible influence of the *sodA* mutation on the expression of *srfA*, *comK* and *comG*, a late competence operon, *lacZ* was used in fusion with these genes. The introduction of the *sodA* mutation into strains carrying the *lacZ* fusions and examination of the β-galactosidase activities of these strains in MC medium were carried out. As shown in Fig. 1, the expression of *srfA-lacZ* was significantly decreased in the *sodA* mutant. Since sufficient *srfA* expression is required for the activation of *comK*, the expression of *comK-lacZ* and *comG-lacZ* was also predicted to be decreased in the *sodA* mutant. Assays of the β-galactosidase activities of both fusions in the wild-type and the *sodA* strains confirmed this prediction (Fig. 1).

The RapC-CSF and ClpXP-Spx Systems Are Not Involved in the sodA *Effect on* srfA *Expression*—The expression of *srfA* is regulated by several distinct pathways. To test which regulatory pathway is involved in the *sodA* effect on *srfA* expression, it was necessary to determine whether *sodA* disruption still abolishes *srfA* expression in the *rapC* or *spx* mutant. RapC is an inhibitory molecule that interacts directly with ComA-P in the RapC-PhrC system, and Spx destabilizes the interaction of ComA-P with RNA polymerase; *i.e.*, both are negative regulators of ComA-P (*18*, *21*). In the *rapC* mutant, the expression of *srfA* was increased as reported previously (*12*), while *srfA* expression was still decreased by the *sodA* disruption in the *rapC* mutant, indicating that the *sodA* mutation acts on *srfA* expression independently of the RapC-CSF system (Fig. 2A). RapF-PhrF is a redundant regulating system for the expression of *srfA* in *B. subtilis* cells. We also examined the involvement of *rapF* in the effect of *sodA* on *srfA* expression, and observed no bypass of the *sodA* effect by the *rapF* mutation (data not shown). Thus, we concluded that the *sodA* effect does not involve the RapF function. Next we examined *srfA* expression in the *spx* mutant, and observed a slight decrease, but not an increase, in *srfA* expression. This is consistent with the fact that the repressor activity of Spx has been observed only in the *clpP* and *clpX* mutants (*22*), and the slight decrease in *srfA-lacZ* expression might be due to some secondary effect of the *spx* mutation, because Spx is a highly pleiotropic regulator (*21*, *22*). The *sodA* effect on *srfA* expression was

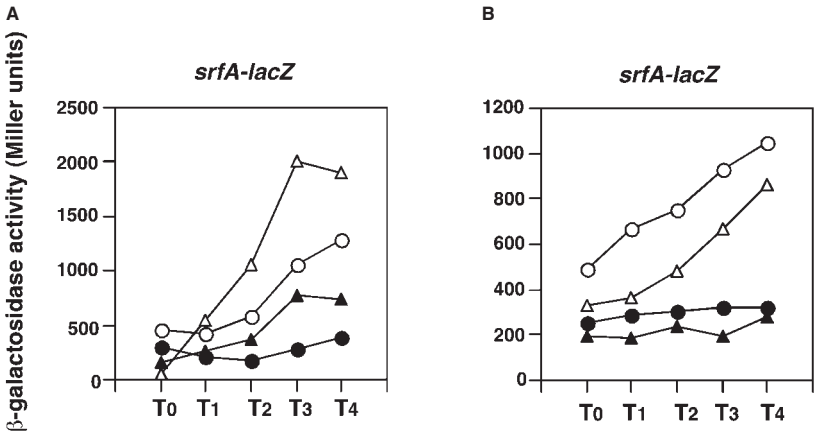

Fig. 2. **Effect of the *sodA* mutation on the expression of *srfA-lacZ* in *rapC* and *spx* mutant cells.** Cells were grown in MC medium, and β-galactosidase activities were determined as described under "EXPERIMENTAL PROCEDURES." Activities of β-galactosidase are shown. Numbers on the *x* axis indicate the duration of culture in hours relative to the end of the vegetative growth (T0). A. Open circles, OAM186 (WT); open triangles, OAM245 (*rapC*); closed circles, OAM232 (*sodA*); closed triangles, OAM276 (*rapC sodA*). B. Open circles, OAM186 (WT); open triangles, OAM233 (*spxA*); closed circles, OAM232 (*sodA*); closed triangles, OAM234 (*spx sodA*).

observed even with the *spx* mutation (Fig. 2B). This demonstrates that the ClpXP-Spx pathway does not overlap the pathway in which high concentrations of superoxide anion affect the regulation of *srfA* expression.

The Transcription of comQXP *Is Downregulated by the* sodA *Mutation*—The ComQXP quorum-sensing system is the major regulatory factor for *srfA* expression. The addition of conditioned medium prepared from the culture supernatant of wild-type cells in stationary phase to wild-type cells in early logarithmic phase has been shown to induce the immediate expression of *srfA* (*11*). To examine the production of the ComX pheromone in *sodA* cells, this feeding assay was performed. As expected, the conditioned medium from wild-type cells induced early *srfA* expression, whereas that from *comX* cells did not (Fig. 3). Conditioned medium from *sodA* cells failed to induce *srfA* expression, suggesting that the ComX pheromone is not produced or secreted for some reason in *sodA* cells. Therefore, we investigated the transcription of *comQXP* itself in *sodA* cells. The amount of mRNA in both wild-type and *sodA* cells was quantified by primer extension analysis (Fig. 4A). Using the total RNA from wild-type cells, a single cDNA of the *comQXP* operon was detected. The transcription initiation site of the *comQXP* operon had been determined previously and we confirmed essentially the same result, i.e., that transcription starts one nucleotide upstream from the formerly determined site of *comQXP* (*33*). In contrast, a faint transcript was detected using total RNA from *sodA* cells. Further, no transcripts were observed when total RNA of cells treated with paraquat, a superoxide-generating agent, were used. Based on these results, the transcription of the *comQXP* operon is observed to have been severely decreased because of a high concentration of superoxide, and thus *srfA* expression also decreased.

As shown in Fig. 4B, from the total RNA of wild-type cells, three transcripts were detected by Northern blot analysis. The readthrough transcripts covering *comA* from the upstream promoter were predicted by a previous analysis of *comA* expression (*13*). Thus, the 5.4 kb, 4.1 kb and 3.5 kb mRNAs correspond to *comQXPA* plus downstream *yuxO*, *comQXPA* and *comQXP*, respectively. On the other hand, from the total RNA of the *sodA* cells, only a single barely detectable band was observed, confirming that transcription of the *comQXP* locus is significantly decreased. A slightly delayed expression of *comA*

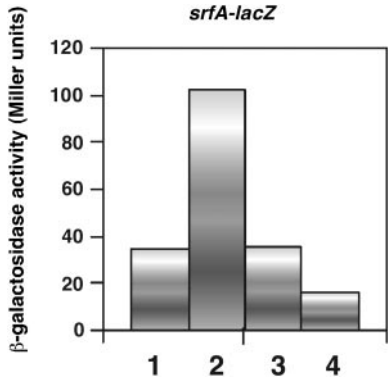

Fig. 3. **Detection of ComX activity.** Conditioned medium was added to the wild-type cell culture carrying the *srfA-lacZ* fusion (OAM186) grown in MC medium at early logarithmic phase. The conditioned media were prepared as described in "EXPERIMENTAL PROCEDURES." Lane 1, MC medium; 2, OAM186; 3, OAM232 (*sodA*); 4, OSM102 (*comX*).

was observed with a Campbell-type *comA-lacZ* fusion in *sodA* cells as compared to that in the wild-type cells under these conditions (data not shown). This observation indicates that *comA* transcription is not significantly affected by the *sodA* mutation. This is consistent with the fact that *comA* constitutes a monocistronic operon (*13*).

Paraquat Decreases srfA *Expression but Not ComX-Independent* srfA *Expression*—Since the expression of *srfA* is sensitive to high concentrations of the superoxide anion, it was expected that the addition of paraquat would result in a decrease in *srfA* expression. As shown in Fig. 5E, a dose-dependent repression of *srfA* expression was observed with increasing concentrations of the added paraquat. This observation is consistent with a recent DNA microarray study of paraquat-treated cells (supplementary data in Ref. *34*). Further, since the ComQXP system is the target of superoxide stress in the regulation of *srfA* expression, *comX*-independent *srfA* expression would not be influenced by paraquat addition. To confirm this, we used a strain carrying an isopropyl-β-D-thiogalactoside (IPTG)–driven mutant *comP* gene, which renders ComP to be constitutively active without ComX, at the *amyE* locus. This strain showed resistance to superoxide, although a slight decrease in *srfA* expression was observed at high concentrations of paraquat (Fig. 5F).

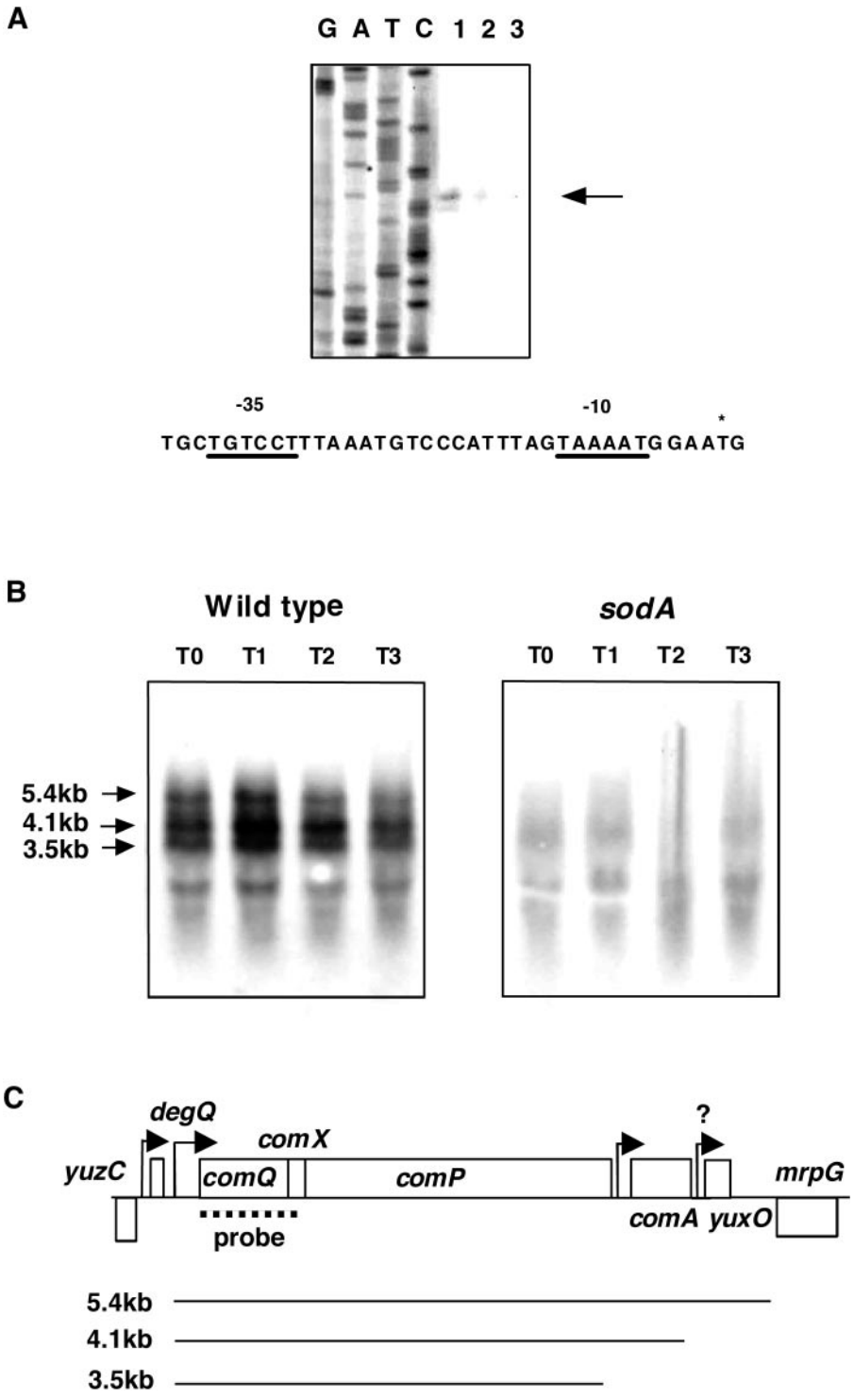

Fig. 4. ***comQXP* expression is downregulated in the *sodA* mutant.** (A) Detection of *comQXP* transcripts by primer extension analysis. Cells were grown in MC medium and harvested at T2. The reaction was done with RNA isolated from the following strains: 1, 168; 2, OAM230 (*sodA*); 3, 168 treated with 30 μM paraquat. Sequence ladders of the non-coding strand were obtained with the same primer as that used for primer extension. The arrow indicates *comQ* transcripts. The nucleotide sequence of the *comQ* promoter region is shown below the panel. The underlined nucleotides show the –35 and –10 regions; the asterisk indicates the transcription initiation site. (B) Northern analysis of the *comQXP* operon was performed using the indicated probe, which was prepared by PCR using the oligonucleotide pair comQXP-1 and comQXP-2 (Table 2). Cells were grown in MC medium and harvested at the indicated times relative to the end of vegetative growth stage (T0). RNA was isolated from both 168 and OAM230 at the indicated times, and 20 μg of total RNA from each sample was applied per lane. The arrows indicate the detected mRNAs. (C) Schematic representation of the genome structure around *comQXP* is shown. The arrows and boxes on the map show promoters and open reading frames, respectively. The dotted and straight lines indicate the probe used for Northern hybridization and detected mRNAs, respectively.

This observation reinforces the notion that superoxide stress primarily acts on the transcription of *comQXP* in the *srfA* regulation system. Next, we examined whether the effect of the *sodA* mutation was bypassed in this *comX*-independent expression of *srfA*. In the absence of IPTG, no *srfA-lacZ* expression was observed, as expected. A partial suppression of the effect of the *sodA* mutation was observed as shown in Fig. 5G. The observed retardation of *srfA* expression in *sodA* cells might be due to a possible polar effect of the disruption of *comXP* on *comA* expression. Alternatively, there could be another target of superoxide in the regulatory pathway of *srfA* expression (see "DISCUSSION"). Since the production of ComX is reduced by the addition of paraquat, the expression of genes in the ComA regulon would be expected to have decreased. To test this, we chose *rapF*, a typical gene of the ComA regulon (*35*), and examined *rapF* expression with increased concentrations of paraquat. As a result, *rapF* expression

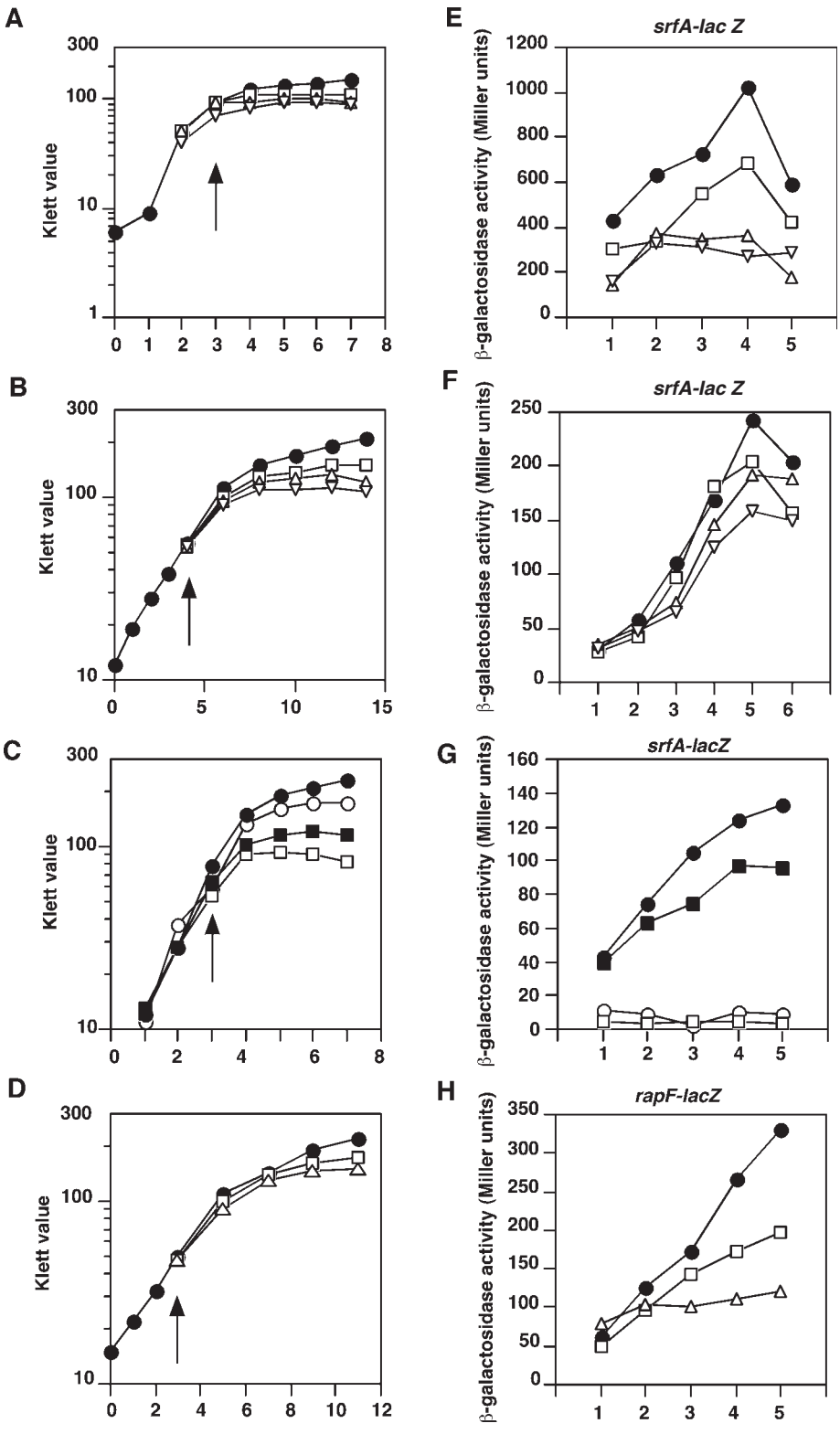

Fig. 5. **Superoxide acts on the expression of the ComA regulon in a *comQXP*-dependent manner.** Cells were grown in MC medium, and β-galactosidase activities were determined as described in "EXPERIMENTAL PROCEDURES." Cell growth was monitored with a Klett optical density meter, and the activities of β-galactosidase are shown in panels A–H. Numbers on the x axes represent the growth time in hours in A–D. Arrows in A–D indicate the initiation of sampling. Numbers on the *x* axes in E–H indicate time in hours. (A, B, E and F) Closed circles, no addition of paraquat; squares, triangles and reverse triangles, show cultures with 3 μM, 10 μM and 30 μM paraquat, respectively. Paraquat was added 30 min prior to the beginning of sampling and 1 mM IPTG was initially added. (A and E), OAM186 (*srfA-lacZ*). (B and F), OAM238 (carrying the ComX-independent *srfA* expression system). (C and G) Circles, OAM238; squares, OAM239 (*sodA*). Closed symbols, addition of 1 mM IPTG. Open symbols, no addition of IPTG. (D and H) BFS1211 (*rapF-lacZ*). Closed circles, no addition of paraquat; squares and triangles show cultures with 5 μM and 20 μM paraquat, respectively.

was found to decrease in reponse to the addition of paraquat in a dose-dependent manner (Fig. 5H). Furthermore, the expression of another member of the ComA regulon, *degQ*, was also inhibited by the addition of paraquat, (*36*, data not shown). These results confirm the susceptibility of *comQXP* transcription to superoxide stress.

DISCUSSION

The aerobic growth of organisms inevitably produces several reactive oxygen species (ROS) because of the activity of the respiratory chain (*37*). At high concentrations, superoxide anion reacts with a [4Fe-4S] cluster, resulting in the

generation of reduced iron ions and hydrogen peroxide, resulting in damage to enzymes containing the [4Fe-4S] cluster, such as fumarase and aconitase (*38*). To avoid this, the superoxide anion is dismutated by SOD to hydrogen peroxide. To protect cells from hydrogen peroxide, most organisms have a catalase, which catalyzes the reduction of hydrogen peroxide to water and oxygen. Further, the reaction between reduced iron ions and hydrogen peroxide generates hydroxy radicals, a species of ROS particularly harmful to DNA, lipids and proteins (*37*, *38*). In the *sodA* mutant, constitutively high concentrations of superoxide anions are achieved, which damage DNA indirectly. A deficiency of SOD activity is known to cause a variety of specific phenomena in bacteria. In *Salmonella typhimurium* and *Shigella flexneri*, virulence has been ascribed to the detoxification of oxidative attack by the hosts. The presence of a *sodA* muation in these bacteria has been reported to yield cells that are less virulent due to SOD-deficiency (*39*, *40*). In this study, the transcription of the quorum-sensing system gene *comQXP* has been identified as one of the cellular targets of excess superoxide anion. It is possible that some unknown transcription factor might be involved in the inhibition of *comQXP* transcription, although the identity of such a factor remains unknown. The inhibition of *comQXP* transcription in *sodA* cells might be a checkpoint in the expression system of the ComA regulon, including *srfA*, since the production of the surfactin needed for swarming motility and fruiting body formation would be repressed under conditions of oxidative stress via the inhibition of the ComX-dependent quorum-sensing system (*6*, *8–10*).

In a ComX-independent *srfA* expression system, the effect of *sodA* disruption was significantly recovered, but a delay in expression was observed. This suggests that there might be another target for superoxide stress in the regulation of *srfA* expression. We have demonstrated that PerR, which is a repressor inactivated by hydrogen peroxide (*41*), directly regulates *srfA* expression as an activator (*42*). The introduction of the *sodA* mutation would result in an elevated cellular concentration of superoxide. [4Fe-4S] enzymes are inactivated in an SOD-deficient mutant of *E. coli*, indicating that more superoxide reacts with the [4Fe-4S] cluster and generates hydrogen peroxide (*38*). The possible increase in cellular hydrogen peroxide concentration might lead to a partial inactivation of PerR. As a consequence, perhaps, slight decreases in *comX*-independent *srfA* expression were observed in *sodA* cells.

Spo0A and DegU are response regulators governing the initiation of sporulation and exoenzyme production, respectively (*2*, *23*). The expression of *srfA* is known to be influenced by Spo0A-P and DegU-P; *i.e.*, disruption of *spo0A* or the introduction of the *degU32* mutation, which renders DegU-P resistant to dephosphorylation, decreases *srfA* expression (*23*, *43*). The *sodA* mutation would not affect the function of Spo0A-P, because in *sodA* cells, the initiation process of sporulation has been known to be unimpaired (*31*, and unpublished result). Moreover, the *sodA* mutation did not influence exoprotease production according to the results of a plate assay (unpublished results), suggesting that DegU-P is not hyperactivated in *sodA* cells, unlike the DegU32 mutant protein. Consequently, it is unlikely that the *sodA* mutation affects *srfA* expression through influence on both regulators. Moreover, CodY is known to repress *srfA* expression under amino acid–rich conditions (*44*). We used MC medium containing casamino acids and *srfA* expression was fully induced, indicating that CodY has no role in the regulation of *srfA* expression under these conditions.

In the *sodA* disruptant, no induction of *srfA* expression in the early stationary phase was observed, although a basal level of *srfA-lacZ* expression was detected (Fig. 1). As shown in Fig. 5H, an increase in the concentration of paraquat added to the cells (i.e., cellular concentrations of superoxide anion) induced a dose-dependent decrease in the expression of the ComA regulon, including *rapF* and *rapC*, thereby activating the DNA-binding of ComA-P through the inhibition of these negative regulators. This regulatory loop would account for the basal level of *srfA* expression in the *sodA* disruptant.

Here we show that superoxide stress results in a decrease in the transcription of the *comQXP* operon independently of Spx (Fig. 6). Future studies to elucidate these findings in greater detail should contribute to understanding of the cellular response of *B. subtilis* to oxidative stress.

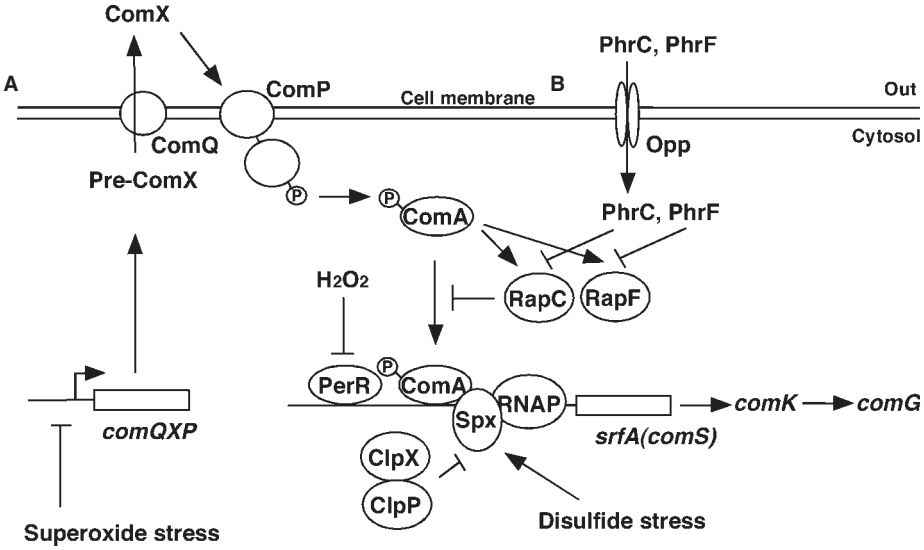

Fig. 6. **Schematic representation of the regulatory network for *srfA* expression.** Arrows indicate activation, protein processing, incorporation of pheromone, transfer of phosphoryl group or expression of genes. T-bars indicate inhibition of gene expression, DNA-binding or protein interaction, or protein degradation. Boxes and circles show open reading frames and proteins, respectively. ComP is composed of two domains, a membrane-embedded sensor and a cytosolic autokinase. Spx is not a DNA-binding protein but an inhibitor of the interaction of a regulator protein with the α-subunit of RNAP. We observed that PerR binds to the upstream region of the *srfA* promoter and activates *srfA* expression (*41*). Opp, oligopeptidepermiase; RNAP, RNA polymerase

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