JOURNAL OF BACTERIOLOGY, Oct. 2005, p. 6659–6667 Vol. 187, No. 19
0021-9193/05/$08.00+0 doi:10.1128/JB.187.19.6659–6667.2005

The H_2O_2 Stress-Responsive Regulator PerR Positively Regulates *srfA* Expression in *Bacillus subtilis*

Kentaro Hayashi,[1]† Taku Ohsawa,[1]† Kazuo Kobayashi,[2] Naotake Ogasawara,[2] and Mitsuo Ogura[1]*

Department of Marine Science, School of Marine Science and Technology, Tokai University, 3-20-1 Orido, Shimizu, Shizuoka 424-8610, Japan,[1] and Graduate School of Information Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-019, Japan[2]

Received 3 June 2005/Accepted 22 July 2005

***srfA* is an operon required for the synthesis of surfactin and the development of genetic competence in *Bacillus subtilis*. We observed that the expression of *srfA* is downregulated upon treatment with H_2O_2. Thus, we examined the involvement of several oxidative stress-responsive transcription factors in *srfA* expression. Our DNA microarray analysis revealed that the H_2O_2 stress-responsive regulator PerR is required for *srfA* expression. This was confirmed by *lacZ* fusion analysis. A ComX feeding assay and epistatic analyses revealed that the role of PerR in *srfA* expression is independent of other known regulators of *srfA* expression, namely, *comQXP*, *rapC*, and *spx*. Gel mobility shift and footprint assays revealed that PerR binds directly to two tandemly arranged noncanonical PerR boxes located in the upstream promoter region of *srfA*. A transcriptional *srfA-lacZ* fusion lacking both PerR boxes showed diminished and PerR-independent expression, indicating that the PerR boxes we identified function as positive *cis* elements for *srfA* transcription.**

The *Bacillus subtilis srfA* operon encodes the biosynthetic genes for surfactin, a biosurfactant that has been reported to be important for the swarming motility and fruiting body formation of natural isolates of *B. subtilis* (3, 7, 18–20). In addition, the *srfA* operon is involved in the development of genetic competence, since the *srfA* operon encodes another gene, *comS*, that is required for *comK* activation (9). The *comK* gene encodes the competence transcription factor required for the expression of late competence operons, including *comE* (42). *srfA* expression is dependent on two extracellular controlling systems, namely, ComQXP and RapC-CSF (competence and sporulation factor) (9, 22, 38). When the ComX pheromone is secreted into the medium, the increase in its concentration is sensed by the ComP receptor, which activates the cognate response regulator ComA by phosphorylation (22, 36), resulting in ComA-P. RapC inhibits the DNA binding of ComA-P, while the extracellular CSF peptide inhibits RapC after the CSF peptide is incorporated into the cytoplasm (6, 38). Thus, RapC and CSF constitute a positive regulatory device for ComA-P, which leads to the binding of ComA-P to the *srfA* promoter. Another regulatory molecule that affects ComA-P-mediated *srfA* transcription is Spx, which inhibits the interaction between ComA-P and RNA polymerase; when the ClpXP protease degrades Spx, activation of the ComA regulon results (29–30). In addition, the expression of the *spx* gene is induced by diamide stress (30).

B. subtilis has three Fur (ferric uptake regulator) homologues, one of which is PerR, a repressor of several members of the peroxide stress regulon, such as *ahpCF*, *katA*, *zosA*, and *mrgA* (24). The PerR protein has two divalent ion-binding sites, one for the zinc cation and the other for various regulatory cations. For example, Mn(II)-, Ni(II)-, or Fe(II)-bound PerR is active as a repressor (17). It has been shown that H_2O_2 inactivates the DNA-binding activity of PerR (17, 24).

srfA expression has been reported to decrease upon H_2O_2 treatment (see supplementary data in reference 25). We also observed a similar phenomenon. These hint at another mechanism that regulates the *srfA* operon, namely, one that is linked to the oxidative stress response. To characterize this mechanism, we tested the effect of disrupting transcription factor genes that regulate the expression of oxidative stress-responsive genes on the expression of *srfA* and other genes by using DNA microarray analysis. This analysis revealed that the disruption of *perR* blocks *srfA* expression. When we analyzed the mechanism by which PerR regulates *srfA*, we found that PerR binds directly to the *srfA* promoter region. Furthermore, *srfA* promoter deletion analysis using *lacZ* fusions confirmed that two tandemly oriented PerR-binding sites function as positive *cis* elements for *srfA* transcription.

MATERIALS AND METHODS

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) (21) medium and Luria-Bertani (LB) medium, respectively. The concentrations of antibiotics used have been described previously (33).

Materials. Synthetic oligonucleotides were commercially prepared by Tsukuba Oligo Service (Ibaraki, Japan) and are shown in Table 2.

Construction of plasmids and strains. A chloramphenicol-resistant *perR* disruptant was constructed by a PCR-based method without cloning of DNA into *E. coli* as described previously (31). For this, six primers were used, namely, perR-F1, perR-R1, perR-F2, perR-R2, pUC-1, and pUC-2. All the plasmids used in this study are listed in Table 3. To construct pHis-perR, a PCR product generated by using the oligonucleotide primer pairs perR-HisB and perR-HisH was treated with BamHI and HindIII and cloned into pQE8 (QIAGEN, Hilden, Germany) treated with the same restriction enzymes. This added the histidine tag to the N terminus of PerR. To construct pMutin-rapC, a PCR product amplified by using rapC-F and rapC-R was treated with HindIII and BglII and cloned between the HindIII and BamHI sites of pMutinIII (41). pMutin-rapC was transformed into *B. subtilis* 168, and the *lacZ* gene in the resultant strain was

* Corresponding author. Mailing address: Department of Marine Science, School of Marine Science and Technology, Tokai University, 3-20-1 Orido, Shimizu, Shizuoka 424-8610, Japan. Phone: 543-34-0411 (ext. 2934). Fax: 543-34-9834. E-mail: oguram@scc.u-tokai.ac.jp.
† These authors contributed equally to this work.

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

Strain	Relevant phenotype and description	Reference or source
168	*trpC2*	Laboratory stock
OAM240	*trpC2 perR*::Cm^r	This study
LAB358	*trpC2 pheA SP*β *c2del2*::Tn*917*::*srfA-lacZ* (Em^r Cm^r)	27
YBCPd	*trpC2 ybcP*::pMutInIII (Em^r *skfB-lacZ*)	43
OAM186	*trpC2 SP*β *c2del2*::Tn*917*::*srfA-lacZ* (Em^r Cm^r)	This study
OSM102	*trpC2 SP*β *c2del2*::Tn*917*::*srfA-lacZ* (Em^r Cm^r) Δ*comQXPA* (Em^r)	34
OAM241	*trpC2 SP*β *c2del2*::Tn*917*::*srfA-lacZ* (Em^r Cm^r) *perR*::Cm^r	This study
OAM245	*trpC2 SP*β *c2del2*::Tn*917*::*srfA-lacZ* (Em^r Cm^r) *rapC*::pMutInIII (Em^r *lacZ*::Tc^r)	This study
OAM246	*trpC2 SP*β *c2del2*::Tn*917*::*srfA-lacZ* (Em^r Cm^r) *rapC*::pMutInIII (Em^r *lacZ*::Tc^r) *perR*::Cm^r	This study
RAPCd	*trpC2 rapC*::pMutInIII (Em^r *rapC-lacZ*)	This study
OAM262	*trpC2 rapC*::pMutInIII (Em^r *rapC-lacZ*) *perR*::Cm^r	This study
ORB3834	*trpC2 pheA1 spx*::Km^r	29
OAM233	*trpC2 SP*β *c2del2*::*Tn917*::*srfA-lacZ* (Em^r Cm^r) *spx*::Km^r	This study
OAM247	*trpC2 SP*β *c2del2*::*Tn917*::*srfA-lacZ* (Em^r Cm^r) *spx*::Km^r *perR*::Cm^r	This study
8G33	*trpC2 comK-lacZ* (Km^r)	42
OAM242	*trpC2 comK-lacZ* (Km^r) *perR*::Cm^r	This study
OGM113	*trpC2 leuC7 comE-lacZ* (Em^r)	32
OAM243	*trpC2 comE-lacZ* (Em^r)	This study
OAM244	*trpC2 comE-lacZ* (Em^r) *perR*::Cm^r	This study
OAM248	*trpC2 amyE*::*srfA1-lacZ* (Cm^r::Tc^r)	This study
OAM249	*trpC2 amyE*::*srfA1-lacZ* (Cm^r::Tc^r) *perR*::Cm^r	This study
OAM250	*trpC2 amyE*::*srfA2-lacZ* (Cm^r::Tc^r)	This study
OAM251	*trpC2 amyE*::*srfA2-lacZ* (Cm^r::Tc^r) *perR*::Cm^r	This study
OAM252	*trpC2 amyE*::*srfA3-lacZ* (Cm^r::Tc^r)	This study
OAM253	*trpC2 amyE*::*srfA3-lacZ* (Cm^r::Tc^r) *perR*::Cm^r	This study

inactivated by transformation of placZ::Tc. To construct pIS-srfA1, pIS-srfA2, and pIS-srfA3, PCR products amplified by using srfA-B1 and srfA-H1, srfA-B2 and srfA-H1, and srfA-B3 and srfA-H1, respectively, were treated with BamHI and HindIII and cloned into pIS284 treated with the same enzymes. After linearization with PstI, each plasmid was transformed into *B. subtilis* 168, after which the chloramphenicol resistance markers of the resultant strains were replaced with tetracycline markers by transformation with plasmid ECE75. Sequencing of the cloned PCR fragments was performed with a 377 DNA sequencer (Perkin-Elmer) and a Dye Terminator cycle sequencing kit (Applied Biosystems).

β-Galactosidase assays. For β-galactosidase assays, samples were withdrawn at hourly intervals and the β-galactosidase activities were measured as described previously (33). Conditioned medium was prepared by removing the cells by centrifugation and filter sterilizing the supernatant. Cells were grown in MC medium to early log phase and divided into two equal volumes. To each half, either conditioned or fresh medium was added, and cultivation was continued, after which samples were withdrawn. In all assays using strains carrying the *perR* disruption, the strains were used immediately after construction to avoid the accumulation of suppressors.

Production and purification of His-tagged PerR. The protein was produced in *E. coli* and purified as described previously (35). His-tagged PerR was produced as a soluble protein in *E. coli*, and thus, purification was performed by stepwise elution with imidazole from a Ni-affinity column. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the fractions, the purified protein was dialyzed against TEDG buffer (26).

DNA microarray analysis. For DNA microarray analysis, both the control strain 168 and its chloramphenicol-resistant derivative bearing the *perR* disruption were grown in LB liquid medium and harvested at an optical density of 0.4. The procedures used to isolate RNA and perform DNA microarray analysis have been described previously (34, 45).

Gel mobility shift and DNase I footprint assays. For the gel shift assay, we employed a procedure using biotinylated DNA probes as described previously (35). The footprint assay was performed as follows. Probe DNAs were prepared by PCR using srfA-4 and srfA-2 or srfA-4a and srfA-2a as primers. The 60-μl reaction mixtures contained 100 ng DNA, 1 mg bovine serum albumin, 20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 0.2% Tween 20 (wt/vol), 30 mM KCl, an appropriate amount of the protein solution in TEDG buffer, and 4 units of DNase I (Roche, Indianapolis). The reaction mixture was left at room temperature for 5 min and then subjected to phenol extraction and subsequent ethanol precipitation after the addition of stop solution (0.1% SDS, 20 mM EDTA, 200 mM NaCl, 40 μg/ml tRNA). After the addition of a loading dye, the samples were applied onto a 6% polyacrylamide gel. After electrophoresis, the DNA was transferred to a positively charged nylon membrane. Biotinylated DNA was detected as described previously (35). A sequence ladder was generated by using a cycle sequencing kit (Toyobo Co.) employing the biotinylated srfA-2a or srfA-4 primer.

RESULTS

Expression of *srfA* is susceptible to H_2O_2 stress. We observed that *srfA* expression was downregulated upon H_2O_2 treatment in a dose-dependent manner (Fig. 1A). It has been known that *E. coli* β-galactosidase is unstable under stress conditions, including heat shock (1); hence, it was possible that this decrease might not be due to an actual change in *srfA* expression. To rule out this, we chose *skfB* encoding a protein involved in the cell killing factor production and examined its expression in the same condition (43). The expression of the *skfB-lacZ* fusion was not influenced by the addition of H_2O_2 (Fig. 1B), supporting the observation with respect to *srfA* expression.

DNA microarray analysis of the effect of disrupting *perR*. The observation that the expression of *srfA* is susceptible to H_2O_2 stress suggested that PerR, which is a repressor of several members of the peroxide stress regulon, may be involved in regulating *srfA* expression. To test this notion, we disrupted the *perR* gene in *B. subtilis* and tested the effect of this on the expression of *srfA* and other genes by DNA microarray analysis. Microarray analysis was performed twice using a glass plate on which pairs of the arrays were printed. In other words, four sets of hybridization data were obtained. Genes whose expression level in the *perR* disruptant differed from that in the parental strain by threefold in every data set were considered to be genes that could be regulated by PerR. We detected many such genes, since 88 genes were upregulated by >3-fold in the

TABLE 2. Oligonucleotides used in this study

Name	Sequence
perR-R1	5′-GTAATGACTATAGAAATCGGAC-3′
perR-R2	5′-GTTATCCGCTCACAATTCTGTGCAGCCATCCGTCATGC-3′
perR-F1	5′-CGTCGTGACTGGGAAAACGCGTCTGCCAAGAGTGTTCG-3′
perR-F2	5′-CCATCACTATTCCTCCAATGC-3′
pUC1	5′-GTTTTCCCAGTCACGACG-3′
pUC2	5′-GAATTGTGAGCGGATAAC-3′
rapC-F	5′-GCGCAAGCTTGTAATTCCTTCTTCAGCGGT-3′
rapC-R	5′-GCCGAGATCTCCTGGTTGCTGTCAATGTTT-3′
perR-HisB	5′-ATTGGATCCATGGCTGCACATGAACTAAA-3′
perR-HisH	5′-GCTAAGCTTTCAATGATTTTCTTTTTTCGAACAC-3′
srfA-1	5′-biotin-GCGCGGTACACATAGTCATGTAAA-3′
srfA-2	5′-TTATCTTTCTACCGTTCAGT-3′
srfA-2a	5′-biotin-TTATCTTTCTACCGTTCAGT-3′
srfA-3	5′-TAGTGGAAATGATTGCGGCA-3′
srfA-4	5′-biotin-GTTGTAAGACGCTCTTCGCA-3′
srfA-4a	5′-GTTGTAAGACGCTCTTCGCA-3′
katA-1	5′-biotin-AGCTGTTACAACAAGGTTT-3′
katA-2	5′-TTTGATTATCTCCAACCGGA-3′
rapG-1	5′-biotin-ATCATCTCTCCTTCATATA-3′
rapG-2	5′-CATGTTTCTTGATGGCAAGG-3′
srfA-B1	5′-CATGGATCCAGTTTGGTTTAAAAATTTTT-3′
srfA-B2	5′-CATGGATCCTGTAAATAATGTTTAGTGG-3′
srfA-B3	5′-CATGGATCCATGATTGCGGCATCCCGC-3′
srfA-H1	5′-CATAAGCTTCCGCTATTAAAGCAGGCT-3′

perR disruptant while 111 genes were downregulated by >3-fold (Hayashi et al. in list of experimental data available at Kegg expression database [http://www.genome.jp/kegg/expression/]). These included genes whose expression was not been previously shown to be regulated by PerR. We detected all of the known PerR-regulated genes, namely, *ahpCF*, *hemAXCDBL*, *katA*, *zosA*, *fur*, and *mrgA*, with the exception of *perR* itself, in addition to several of the function-unknown "y genes" detected by the DNA microarray analysis conducted by Helmann et al. (16). As shown in Table 4, transcription of the anaerobic and respiratory genes controlled by the ResE-ResD two-component system (e.g., the *cydABC*, *nasCDEF*, and *resABCDE* operons) was highly induced by the deletion of *perR* (28, 44). It is possible that the stress caused by the *perR* mutation may directly or indirectly activate *resDE* transcription, thereby upregulating the ResD regulon. Alternatively, these genes may be induced by the stress caused by the *perR* disruption, which acts independently of the ResD activity. Moreover, the expression of *spx* (*yjbD*) was induced in the *perR* disruptant.

TABLE 3. Plasmids used in this study

Plasmid	Description	Reference or source
pQE8	pUC19-based Amp^r plasmid	Qiagen
pHis-perR	pQE8 carrying *perR*	This study
pMutInIII	Amp^r Em^r	41
pIS284	Amp^r Cm^r *lacZ*	I. Smith
ECE75	Amp^r Cm^r::Tc^r	Bacillus Stock Center
placZ	Amp^r *lacZ*::Tc^r	35
pIS-srfA-1	pIS284 carrying the *srfA* promoter region (−170 to +134)	This study
pIS-srfA-2	pIS284 carrying the *srfA* promoter region (−144 to +134)	This study
pIS-srfA-3	pIS284 carrying the *srfA* promoter region (−122 to +134)	This study

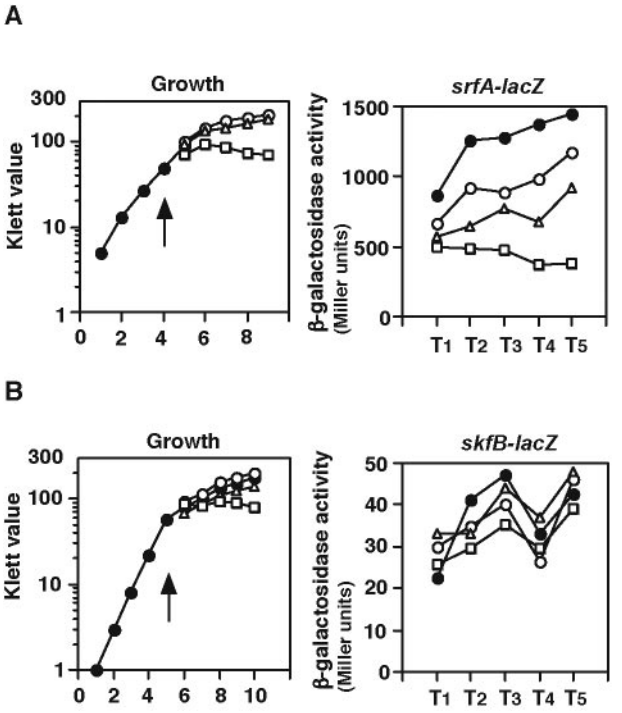

FIG. 1. Decrease in *srfA* expression by H_2O_2 treatment. Cells were grown in modified competence medium, and their β-galactosidase activities were determined as described in Materials and Methods. Growth of the cells monitored with an Klett optical density meter and activities of β-galactosidase are shown in the left and right panels, respectively. The *x* axis represents the growth time in hours (left panels) and the duration of culture in hours relative to the end of vegetative growth (right panels). Several experiments were performed, and typical results are shown. Arrows in the left panels indicate the time when the indicated concentration of H_2O_2 was added. Closed circles, no addition of H_2O_2; open circles, 0.2 mM H_2O_2; open triangles, 1 mM H_2O_2; open squares, 5 mM H_2O_2. (A) and (B) show the experiments using *srfA-lacZ* (OAM186) and *skfB-lacZ* (YBCPd), respectively.

Notably, regarding the genes that were downregulated by *perR* disruption, these include the *srfA* operon and chemotaxis-related genes. This suggests that PerR positively regulates the expression of these genes.

***srfA* expression is decreased in *perR* disruptant cells.** We further characterized the positive regulation of *srfA* expression by PerR. First, we confirmed that the disruption of *perR* downregulated *srfA* in competence medium (Fig. 2A). Since the effects of the *perR* mutation on expression of genes in the cell are highly pleiotropic, a control experiment was needed to demonstrate a specific effect of the *perR* mutation on *srfA* expression. We examined the expression of *rapC-lacZ* in the *perR* cells and did not observe any fluctuation of *rapC-lacZ* expression by the *perR* mutation (data not shown). Thus, it was concluded that *srfA* is indeed downregulated by the *perR* mu-

TABLE 4. Genes regulated by PerR[a]

Gene	Function	Transcriptional ratio (*perR*/WT)[b]	Known regulator (reference)
ahpCF	Alkyl hydroperoxide reductase	23.6, 16.4	PerR (24)
alsD	Alpha-acetolactate decarboxylase	14.7	ResD (44)
alsS	Alpha-acetolactate synthase	16.6	ResD (44)
ctaA	Cytochrome *caa*3 oxidase	6.5	ResD (44)
cydABC	Cytochrome *bd* ubiquinol oxidase	239.6, 195.0, 101.8	ResD (44)
fnr	Transcriptional regulator of anaerobic genes	9.2	ResD (44)
fur	Iron uptake repressor	5.1	PerR (24)
glpD	Glycerol-3-phosphate dehydrogenase	7.7	ResD (44)
hemEHY	Heme synthesis	7.2, 6.1, 4.5	ResD (44)
hemAXCDBL	Heme synthesis	21.9, 12.8, 9.1, 9.4, 6.6, 4.5	PerR (24)
hmp	Flavohemoglobin	32.6	ResD (44)
katA	Catalase	32.9	PerR (24)
lctEP	L-lactate dehydrogenase and permease	104.5, 40.1	ResD (44)
mrgA	DNA-binding stress protein	24.1	PerR (24)
nasCDEF	Assimilatory nitrate reductase	4.7, 21.3, 7.4, 4.6	ResD (44)
resABC	Cytochrome *c* biogenesis protein	3.1, 3.7, 3.5	ResD (44)
resDE	Two-component system	3.2, 3.2	ResD (44)
spx	Inhibitor of interaction between ComA and RNAP	9.8	SigM (39)
zosA	Zinc-transporting ATPase,	41.7	PerR (24)
hag	Flagellin	0.17	SigD (15)
srfAA, *AB*, *AC*, *AD*	Surfactin synthetase	0.27, 0.29, 0.041, 0.26	ComA (9)
mcpB	Methyl-accepting chemotaxis protein	0.29	SigD (15)

[a] Genes that constitute an operon are shown in a single line. Ratios of increases or decreases in gene expression are shown according to gene order.
[b] Averages of the four data sets. WT, wild type.

tation. Any decrease in *srfA* expression would be expected to reduce the expression of *comK* and the late *com* operons and reduce the transformation efficiency of the bacterium. Indeed, the expression of both *comK* and the late *com* operon *comE* was almost abolished by the disruption of *perR* (Fig. 2B and C). Moreover, the *perR* disruptant exhibited a low efficiency of transformation (Table 5), which is consistent with the decrease in *comK* expression. Notably, this phenotype was highly unstable, probably because of the occurrence of a suppressor mutation distinct from that restoring the slow-growth phenotype (see Discussion).

The ComQXP system is not involved in the reduction of *srfA* expression by the disruption of *perR*. The ComQXP quorum-sensing system is a major regulator of *srfA* expression. Previously, it has been shown that adding conditioned medium prepared from a wild-type cell culture in stationary phase to wild-type cells in early logarithmic phase immediately induces *srfA* expression (22). To test the possibility that the *perR* disruptant cells may produce less ComX pheromone, this feeding assay was performed. As expected, the conditioned medium from wild-type cells induced early *srfA* expression by wild-type cells, whereas that from *comX*Δ cells did not (Fig. 3). However, the addition of the conditioned medium from *perR* disruptant cells induced levels of *srfA* expression by wild-type cells similar to those with the medium conditioned by wild-type cells. Thus, similar levels of ComX are present in the culture supernatants of the *perR* disruptant and wild-type cells.

We also speculated on whether the decreased expression of *srfA* in *perR* disruptant cells was due to the impaired ability of the ComP receptor to bind ComX. However, this was not the case, since the transcription of *pel*, *rapC*, and *rapF*, which belong to the ComA regulon and require the functional ComP

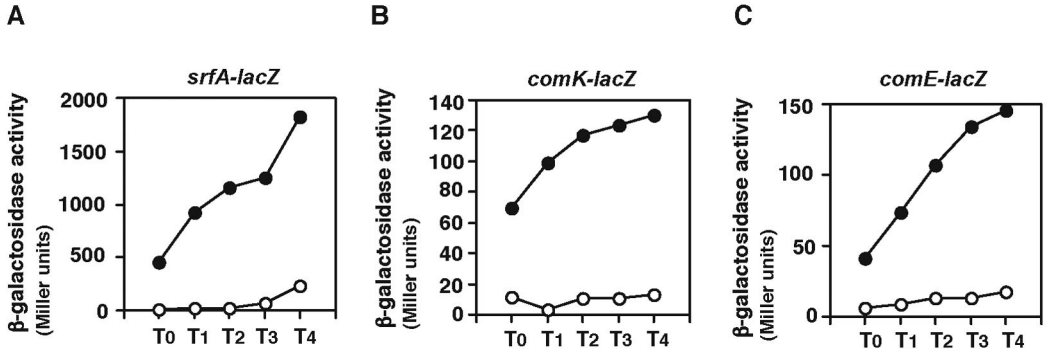

FIG. 2. Expression of *srfA-lacZ*, *comK-lacZ*, and *comE-lacZ* in *perR* disruptant cells. Cells were grown in modified competence medium, and their β-galactosidase activities were determined as described in Materials and Methods. The *x* axis represents the duration of culture in hours relative to the end of vegetative growth (T0). Several experiments were performed, and typical results are shown. (A) Closed circles, wild-type OAM186; open circles, *perR* disruptant OAM241. (B) Closed circles, wild-type 8G33; open circles, *perR* disruptant OAM242. (C) Closed circles, wild-type OAM243; open circles, *perR* disruptant OAM244.

TABLE 5. Transformation efficiency of *perR* cells

Strain	Relevant genotype	No. of viable cells (cells/ml)	No. of transformants (cells/ml)	Transformation efficiency (%)[a]	Ratio (%)
168		2.43×10^8	6.5×10^3	0.26×10^{-2}	100
OAM240	*perR*	0.30×10^8	0.05×10^3	0.016×10^{-2}	6.1

[a] Total DNA containing a kanamycin resistance marker was added to the cell culture at T_2. After the transformation, cells were subjected to serial dilutions. Each fraction was plated onto an LB agar plate with or without kanamycin to count numbers of transformed cells and viable cells, respectively.

receptor for their expression (34), was not affected by the disruption of *perR*, as shown by our microarray data and the *lacZ* fusion analysis for *rapC*. These observations together suggest that the ComQXP regulatory system is functional in *perR* disruptant cells.

The RapC-CSF and ClpXP-Spx systems are also not involved in reduction of *srfA* expression by disruption of *perR*. As described in the introduction, the expression of *srfA* is regulated by several distinct pathways. One involves RapC, an inhibitory molecule in the RapC-CSF system that directly interacts with ComA-P, and another is Spx, which destabilizes the interaction of ComA-P with RNA polymerase. Thus, both RapC and Spx are negative regulators of ComA-P (6, 29). To test whether the RapC pathway is involved in the decreased *srfA* expression induced by the *perR* disruption, an epistatic analysis of the *perR* mutation in the *rapC* mutant was performed. As shown in Fig. 4A, *srfA* expression was still decreased by the *perR* disruption in the *rapC* mutant, indicating that PerR does not affect *srfA* expression through the RapC-CSF system.

Since the expression of *spx* was induced in the *perR* disruptant, we speculated that higher levels of Spx may decrease the expression of *srfA*. If so, the introduction of the *spx* mutation into *perR* disruptant cells could suppress the effect of the *perR* disruption on *srfA* expression. To test this, we examined *srfA* expression in an *spx perR* double mutant. However, the negative effect of *perR* disruption on *srfA* expression was still observed, indicating that Spx has no role in repression of *srfA* in the *perR* cells (Fig. 4B). The result is consistent with the fact that the repressor activity of Spx has been observed only in the *clpP* or *clpX* mutant (30). Thus, we concluded that PerR positively regulates *srfA* expression independently of the RapC-CSF and ClpXP-Spx regulatory systems.

PerR binds to the *srfA* promoter. The above observations led us to hypothesize that PerR may directly regulate *srfA* expression by binding to the *srfA* promoter. To examine this, we produced a His-tagged PerR protein in *E. coli* and purified it from the cleared lysate of the cells. We used gel mobility shift analysis to first test whether this PerR protein could bind to the *katA* promoter and found it could do so in the absence of manganese (Fig. 5A, lane 2); moreover, the addition of manganese did not change the DNA-binding activity of the protein (data not shown). We speculated that the apparent manganese insensitivity of this PerR protein may be because it has already bound a metal ion to activate the protein on its second metal-binding site during its production and purification. An activated PerR protein in the production or purification process has been observed previously (17). Next, we tested whether PerR can bind directly to the *srfA* promoter region. As shown in Fig. 5A (lane 4), the addition of PerR to the reaction mixture containing the *srfA* promoter resulted in the formation of a DNA-protein complex. In contrast, PerR failed to form DNA-protein complexes with promoters of genes that PerR is

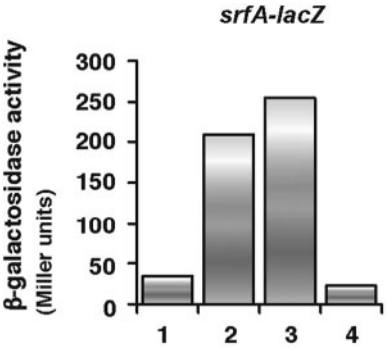

FIG. 3. Amount of ComX in *PerR* disruptant cells. Various conditioned media were added to wild-type cell cultures carrying the *srfA-lacZ* fusion (OAM186) grown in modified competence medium at the early logarithmic phase. The conditioned media were prepared as described in Materials and Methods from the wild-type strain OAM186 (lane 2), the *perR* disruptant OAM241 (lane 3), and the *comX*Δ mutant OSM102 (lane 4). The effect of adding unconditioned modified competence medium is shown in lane 1. The β-galactosidase activities shown were those measured half an hour after the conditioned medium was added.

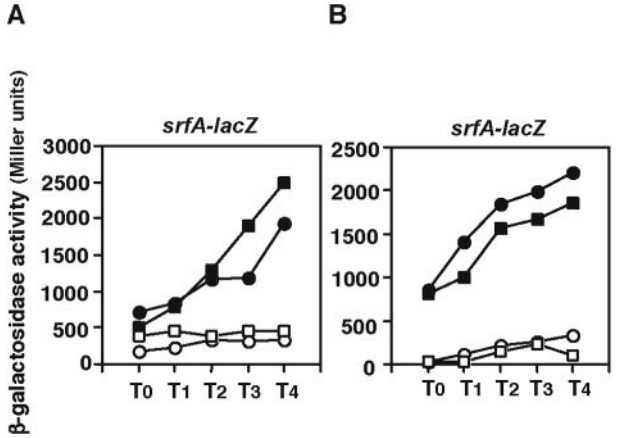

FIG. 4. Effect of the *perR* disruption on the expression of *srfA-lacZ* in *rapC* and *spx* mutant cells. The cells were grown in modified competence medium, and their β-galactosidase activities were determined as described in Materials and Methods. The numbers on the *x* axis represent the duration of culture in hours relative to the end of the vegetative growth (T0). Several experiments were performed, and typical results are shown. (A) Closed circles, wild-type strain OAM186; closed squares, *rapC* disruptant OAM245; open circles, *perR* disruptant OAM241; open squares, *rapC perR* double disruptant OAM246. (B) Closed circles, wild-type strain OAM186; closed squares, *spx* disruptant OAM233; open circles, *perR* disruptant OAM241; open squares, *spx perR* double disruptant OAM247.

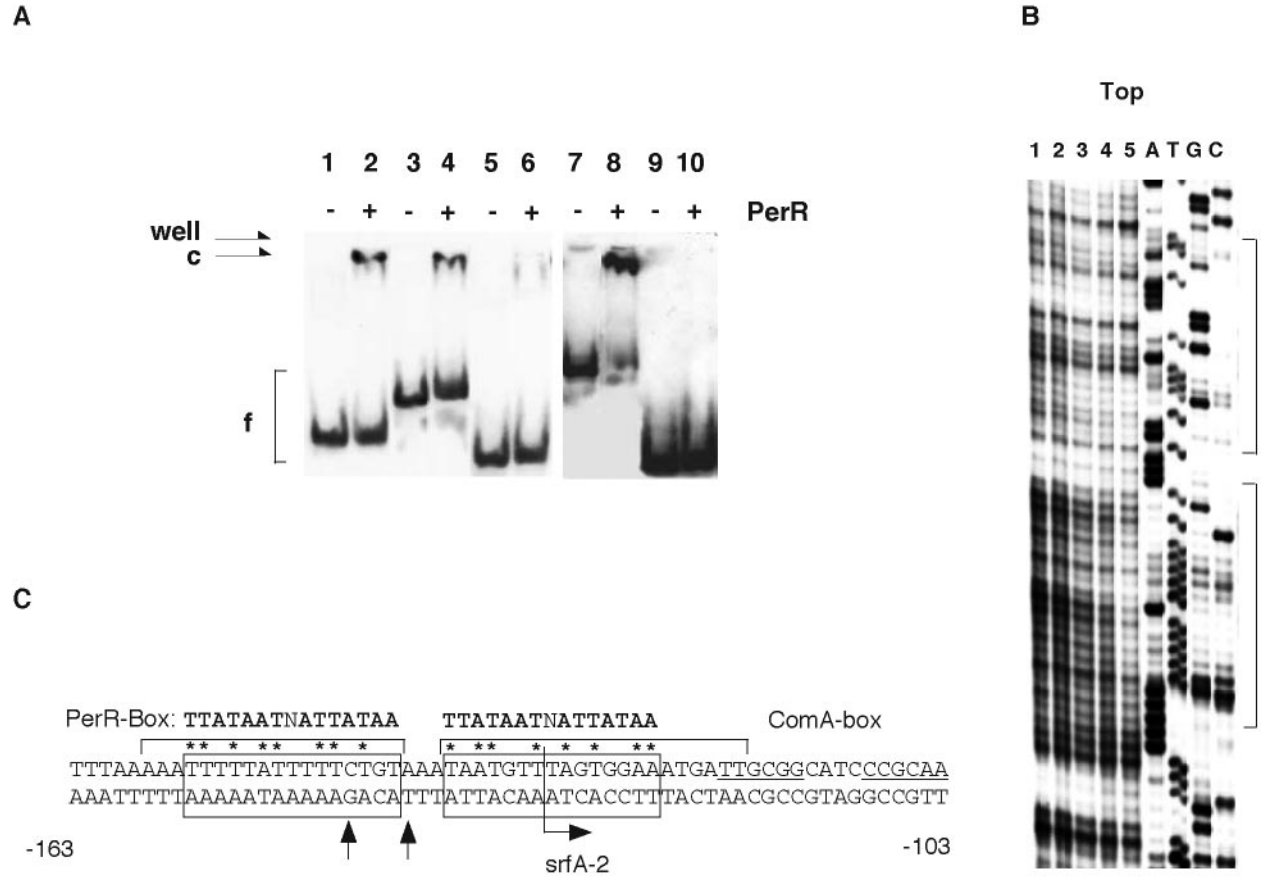

FIG. 5. Gel mobility shift and footprint assays of the *srfA* promoter using PerR. The gel shift assay was performed as described in Materials and Methods. A 6% native polyacrylamide gel was used. (A) The *katA* and *srfA* probe DNAs span positions −131 to +65 and −237 to +10 relative to the transcription start site, respectively. The *rapG* probe spans the 180-bp-long promoter region of *rapG*. These probes were prepared by PCR using the katA-1 and katA-2, srfA-1 and srfA-2, and rapG-1 and rapG-2 primers. The *srfA*-2 probe DNA spans position −130 to +10 relative to the transcription start site and was amplified by PCR using srfA-3 and srfA-2a. c and f indicate the protein-DNA complex and free probe, respectively. "well" means the start point of the electrophoresis. Reactions contained poly(dI-dC) (0.1 μg/25 μl). The 2 nM probes were incubated with 200 nM His-tagged PerR. − and +, reactions without and with PerR, respectively. Left panel, lanes 1 and 2, *katA*; lanes 3 and 4, *srfA*; lanes 5 and 6, *rapG*. Right panel, lanes 7 and 8, *srfA*; lanes 9 and 10, *srfA*-2. (B) The *srfA* promoter prepared by PCR using srfA-4 and srfA-2 (40 nM) was incubated in separate reactions with the increased amount of His-tagged PerR and subjected to DNase I cleavage. Brackets along the gel indicate the protected regions. Lane 1 shows no PerR, while lanes 2, 3, 4, and 5 show the effect of 0.1 μM, 0.2 μM, 0.4 μM, and 0.6 μM of PerR, respectively. (C) The nucleotide sequence of the *srfA* promoter region is shown. Brackets over the nucleotide sequence and arrowheads indicate the protected regions and nucleotides, respectively. The numbers at either side of the nucleotide sequence show the nucleotide positions relative to the transcription start site. The PerR and ComA boxes are boxed and underlined, respectively. The bent arrow indicates the 5′ terminus of the *srfA*-2 probe. The asterisks show the nucleotides matching the consensus sequence of the PerR box (10).

known not to regulate, e.g., *rapG* (Fig. 5A, lane 6). To localize the PerR-bound region in the *srfA* promoter, we performed a gel shift assay using a *srfA* probe bearing a deletion. While PerR bound to the −237 to +10 region, it did not to the −130 to +10 region, indicating that the −237 to −130 region contains a PerR-bound sequence(s) (right panel in Fig. 5A). These results show clearly that PerR binds specifically to the *srfA* promoter.

To further characterize the sequence(s) bound by PerR in the *srfA* promoter, we performed DNase I footprinting analysis. On the top strand, protection was observed in the regions spanning −158 to −141 and −137 to −117 relative to the transcription start site (Fig. 5B). As shown in Fig. 5C, each protected region contains a sequence that weakly resembles the consensus sequence for PerR recognition, i.e., the −155 to −141 and −137 to −123 regions (8 matches of 14 bases). Both sequences are located immediately upstream from the ComA box in the *srfA* promoter (−118 to −103). With respect to the bottom strand, although the region that is protected from DNase I cleavage was not clearly observed due to the intrinsic insensitivity to DNase I of this region, the intensities of signals from −140T and −144G were nevertheless clearly weakened (data not shown).

Confirmation that the PerR boxes in the *srfA* promoter region act as positive *cis* elements. To confirm that the two PerR boxes in the *srfA* promoter region that we identified are positive *cis* elements, strains carrying transcriptional *lacZ* fusions using three different promoter regions of *srfA* at the *amyE* locus were constructed. The *perR* disruption was introduced into each strain, and the β-galactosidase activities of the strains were examined. The expression of *srfA1-lacZ* (−170 to +134) was decreased by the introduction of the *perR* disrup-

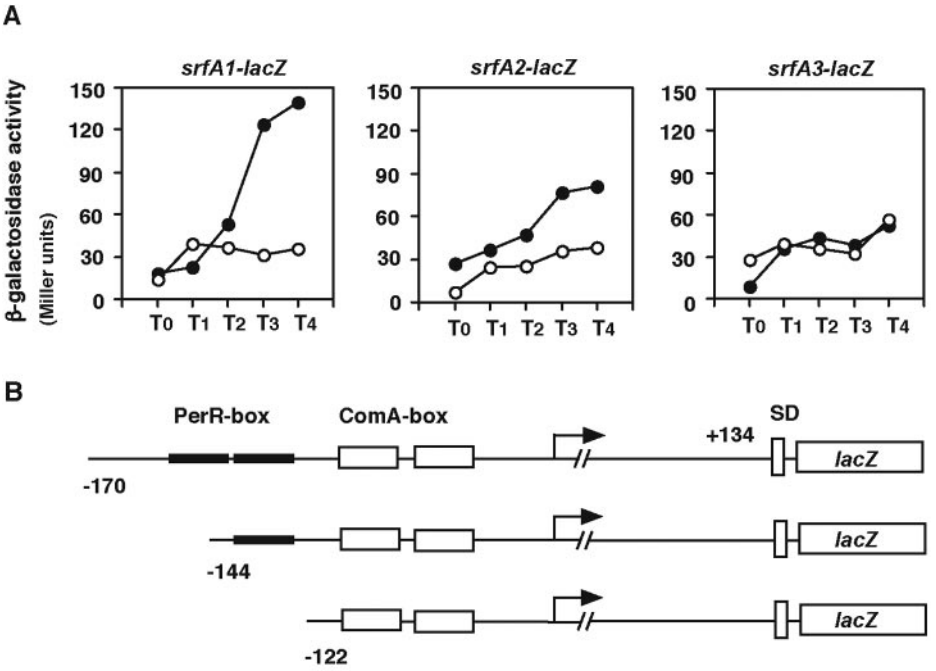

FIG. 6. Confirmation that the Per boxes function as *cis* elements in *srfA* expression. (A) Various cells were grown in modified competence medium, and sampling was initiated at late logarithmic phase. The β-galactosidase activities from the *srfA-lacZ* fusions were determined as described in Materials and Methods. The numbers on the *x* axis represent the duration of culture in hours relative to the end of vegetative growth (T0). The closed and open symbols indicate the wild-type and *perR* disruptant strains, respectively. Several experiments were performed, and typical results are shown. (B) Schematic representation of the *srfA-lacZ* fusions. The closed and open boxes indicate the PerR (−153 to −141 and −137 to −123) and ComA (−118 to −103 and −74 to −59) boxes, respectively. The 5′ and 3′ ends of the fusions are indicated as nucleotide positions relative to the transcription start point. The bent arrows show the promoter sequence of *srfA*. SD indicates the Shine-Dalgarno sequence of *lacZ*.

tion (Fig. 6A). This was expected, because this fusion carries both of the PerR boxes and the ComA boxes. The expression of *srfA2-lacZ* (−144 to +134), which carries only one of the PerR boxes, was decreased to about 65% of that of *srfA1-lacZ*. This decrease can be ascribed to the disruption of the upstream PerR box. The introduction of the *perR* disruption further decreased the expression of *srfA2-lacZ*, indicating that the downstream PerR box functions for the expression of the fusion. The expression of *srfA3-lacZ* (−122 to +134), which lacks both PerR boxes, decreased to levels similar to that of *srfA1-lacZ* on the *perR* disruption background. Thus, the *srfA* upstream region that lacks both PerR boxes cannot fully induce the expression of the *lacZ* fusion. Moreover, the expression of *srfA3-lacZ* was similar in wild-type and *perR* disruptant cells, indicating the PerR-independent expression of the fusion. This reinforced the functionality of the PerR boxes that we identified. Therefore, we concluded that these PerR boxes indeed function as positive *cis* elements for the expression of *srfA*.

DISCUSSION

Here we showed for the first time that the *B. subtilis* molecule PerR, which represses members of the H_2O_2 stress regulon, can selectively activate gene expression by direct DNA binding. This is supported by a recent report showing that Fur (PerR is a *B. subtilis* homologue of Fur) functions as an activator by directly binding to several promoters in *Neisseria meningitidis* (8). In addition, the *Streptococcus pyogenes* PerR homologue positively regulates *csp*, a cold-shock protein gene, probably through DNA binding to the upstream region of *csp* (4). Moreover, *the Borrelia* oxidative stress response regulator BosR, which has 51% similarity to PerR, positively regulates *napA* encoding an oxidative-stress-related Dps/Dpr homologue by DNA binding to the promoter (2). In contrast, although several genes are known to be positively regulated by Fur in *E. coli* (40), the mechanism for this positive regulation has been elucidated as being indirect, at least with respect to genes involved in iron metabolism (23). Thus, it is not clear at this point whether all the genes that were positively regulated by PerR in *B. subtilis*, as determined by our microarray analysis, are direct targets of PerR binding or are downregulated only as indirect effects of the *perR* mutation.

Tandemly arranged PerR boxes were detected in the promoters of *B. subtilis ahpC*, *hemA*, and *zosA*, as well as in the *srfA* promoter (11, 17). In the gel shift assay using the *katA* or *srfA* probe shown in Fig. 5A, bands showing similar mobility were observed. This might be inconsistent with the fact that the *katA* and *srfA* probes carry one and two PerR boxes, respectively. In the footprint assay, His-tagged PerR only weakly protected the PerR box regions from DNase I cleavage. This suggests that PerR binds with a weak affinity to the PerR boxes in the *srfA* promoter. Thus, it seems that in the gel shift assay with the *srfA* promoter, a DNA-protein complex containing only one PerR dimer might be generated.

In the *srfA* promoter, the two PerR boxes are located in the promoter-distal region, whereas these boxes are located at the

promoter-proximal region of genes known to be repressed by PerR (10, 17). This promoter-distal location of the PerR box in the *srfA* promoter is consistent with the fact that the positive and negative *cis*-acting sites of Fur are located in promoter-distal and -proximal regions, respectively (8). The mechanism by which PerR activates *srfA* remains unclear. The proximal location of the PerR boxes relative to the ComA box in the *srfA* promoter suggests that PerR may interact with ComA and that this is needed to induce the full activation of *srfA* expression by ComA. Alternatively, PerR may activate *srfA* expression independently of ComA.

The profile of the PerR regulon that our DNA microarray analyses revealed differs to some extent from the profile identified by Helmann et al. (16). These disparities may be due to the different media and harvesting times used (14). The *perR* mutant is known to grow slowly and to tend to accumulate suppressors that lead to rapid growth, like that seen with wild-type cells (5). It should be noted that the cell culture from which the RNA fraction used for the microarray analysis was obtained retained the slow-growth phenotype (data not shown).

Spo0A and DegU are response regulators that govern the initiation of sporulation and exoenzyme production, respectively (12, 21). The expression of *srfA* is known to be influenced by Spo0A-P and DegU-P, since disruption of *spo0A* or introduction of the *degU32* mutation (which renders DegU-P resistant to dephosphorylation) decreases *srfA* expression (13, 21). The *perR* mutation would not affect the function of Spo0A-P, since in *perR* disruptant cells, the initiation of sporulation appears to be unimpaired (5; unpublished results). Moreover, we found by a plate assay that the *perR* disruption does not influence exoprotease production (unpublished results), which suggests that DegU-P is unlikely to be hyperactivated in *perR* disruptant cells, unlike when the DegU32 mutant protein is expressed. Consequently, it is unlikely that PerR affects *srfA* expression by influencing either regulator. Notably, CodY has also been known to repress *srfA* expression under an amino acid-rich condition (37). However, we used MC medium containing Casamino Acids and found *srfA* expression was fully induced. This indicates that CodY does not participate in the regulation of *srfA* expression under our experimental conditions.

In summary, we have shown here that *B. subtilis* PerR activates the expression of *srfA* by direct promoter DNA binding. It is known that H_2O_2 stress inactivates the DNA-binding activity of PerR (17, 24). Since PerR is required for activation of *srfA*, it seems that H_2O_2 stress inactivates PerR, thereby inhibiting *srfA* expression, as shown in Fig. 1A. Thus, PerR is involved in the regulatory system for expression of *srfA*, checking oxidative stress conditions in the cells.

ACKNOWLEDGMENTS

We thank P. Zuber and M. M. Nakano for ORB3834.

This work was supported by the Research and Study Program of Tokai University Educational System General Research Organization to M.O. and by a Grant-in-aid for Scientific Research on Priority Areas (C) "Genome Biology" to N.O.

REFERENCES

1. **Benson, A. K., and W. G. Haldenwang.** 1993. The sigma B-dependent promoter of the *Bacillus subtilis sigB* operon is induced by heat shock. J. Bacteriol. **175:**1929–1935.
2. **Boylan, J. A., J. E. Posey, and F. C. Gherardini.** 2003. *Borrelia* oxidative stress response regulator, BosR: a distinctive Zn-dependent transcriptional activator. Proc. Natl. Acad. Sci. USA **100:**11684–11689.
3. **Branda, S. S., J. E. Gonzalez-Pastor, S. Ben-Yehuda, R. Losick, and R. Kolter.** 2001. Fruiting body formation by *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **98:**11621–11626.
4. **Brenot, A., K. Y. King, and M. G. Caparon.** 2005. The PerR regulon in peroxide resistance and virulence of *Strteptococcus pyogenes*. Mol. Microbiol. **55:**221–234.
5. **Casillas-Martinez, L., A. Driks, B. Setlow, and P. Setlow.** 2000. Lack of a significant role for the PerR regulator in *Bacillus subtilis* spore resistance. FEMS Microbiol. Lett. **188:**203–208.
6. **Core, L., and M. Perego.** 2003. TPR-mediated interaction of RapC with ComA inhibits response regulator-DNA binding for competence development in *Bacillus subtilis*. Mol. Microbiol. **49:**1509–1522.
7. **Cosmina, P., F. Rodriguez, F. de Ferra, G. Grandi, M. Perego, G. Venema, and D. van Sinderen.** 1993. Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. Mol. Microbiol. **8:**821–831.
8. **Delany, I., R. Rappuoli, and V. Scarlato.** 2004. Fur functions as an activator and as a repressor of putative virulence genes in *Neisseria meningitidis*. Mol. Microbiol. **52:**1081–1090.
9. **Dubnau, D., and C. M. Lovett, Jr.** 2002. Transformation and recombination, p. 453–472. *In* A. L. Sonenshine, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. American Society for Microbiology, Washington, D.C.
10. **Fuangthong, M., and J. D. Helmann.** 2003. Recognition of DNA by three ferric uptake regulator (Fur) homologs in *Bacillus subtilis*. J. Bacteriol. **185:**6348–6357.
11. **Gaballa, A., and J. D. Helmann.** 2002. A peroxide-induced zinc uptake system plays an important role in protection against oxidative stress in *Bacillus subtilis*. Mol. Microbiol. **45:**997–1005.
12. **Grossman, A. D.** 1995. Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. Annu. Rev. Genet. **29:**477–508.
13. **Hahn, J., and D. Dubnau.** 1991. Growth stage signal transduction and the requirements for *srfA* induction in development of competence. J. Bacteriol. **173:**7275–7282.
14. **Hatfield, G. W., S. P. Hung, and P. Baldi.** 2003. Differential analysis of DNA microarray gene expression data. Mol. Microbiol. **47:**871–877.
15. **Helmann, J. D., and C. P. Moran, Jr.** 2002. RNA polymerases and sigma factors, p. 289–312. *In* A. L. Sonenshine, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. American Society for Microbiology, Washington, D.C.
16. **Helmann, J. D., M. F. Wu, A. Gaballa, P. A. Kobel, M. M. Morshedi, P. Fawcett, and C. Paddon.** 2003. The global transcriptional response of *Bacillus subtilis* to peroxide stress is coordinated by three transcription factors. J. Bacteriol. **185:**243–253.
17. **Herbig, A. F., and J. D. Helmann.** 2001. Roles of metal ions and hydrogen peroxide in modulating the interaction of the *Bacillus subtilis* PerR peroxide regulon repressor with operator DNA. Mol. Microbiol. **41:**849–859.
18. **Kearns, D. B., and R. Losick.** 2003. Swarming motility in undomesticated *Bacillus subtilis*. Mol. Microbiol. **49:**581–590.
19. **Kearns, D. B., F. Chu, R. Rudner, and R. Losick.** 2004. Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. Mol. Microbiol. **52:**357–369.
20. **Kinsinger, R. F., M. C. Shirk, and R. Fall.** 2003. Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. J. Bacteriol. **185:**5627–5631.
21. **Kunst, F., T. Msadek, and G. Rapoport.** 1994. Signal transduction network controlling degradative enzyme synthesis and competence in *Bacillus subtilis*, p. 1–20. *In* P. J. Piggot, C. P. Moran, Jr., and P. Youngman (ed.), Regulation of bacterial differentiation. American Society for Microbiology, Washington, D.C.
22. **Magnuson, R., J. Solomon, and A. D. Grossman.** 1994. Biochemical and genetic characterization of a competence pheromone from *B. subtilis*. Cell **77:**207–216.
23. **Masse, E., and S. Gottesman.** 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **99:**4620–4625.
24. **Mongkolsuk, S., and J. D. Helmann.** 2002. Regulation of inducible peroxide stress responses. Mol. Microbiol. **45:**9–15.
25. **Mostertz, J., C. Scharf, M. Hecker, and G. Homuth.** 2004. Transcriptome and proteome analysis of *Bacillus subtilis* gene expression in response to superoxide and peroxide stress. Microbiology **150:**497–512.
26. **Mukai, K., M. Kawata, and T. Tanaka.** 1990. Isolation and phosphorylation of the *Bacillus subtilis degS* and *degU* gene products. J. Biol. Chem. **265:**20000–20006.
27. **Nakano, M. M., L. Xia, and P. Zuber.** 1991. Transcription initiation region of the *srfA* operon, which is controlled by the *comP-comA* signal transduction system in *Bacillus subtilis*. J. Bacteriol. **1732:**5487–5493.
28. **Nakano, M. M., and P. Zuber.** 2002. Anaerobiosis, p. 393–404. *In* A. L.

Sonenshine, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and its closest relatives: from genes to cells. American Society for Microbiology, Washington, D.C.

29. **Nakano, S., M. M. Nakano, Y. Zhang, M. Leelakriangsak, and P. Zuber.** 2003. A regulatory protein that interferes with activator-stimulated transcription in bacteria. Proc. Natl. Acad. Sci. USA **100:**4233–4238.
30. **Nakano, S., E. Kuster-Schock, A. D. Grossman, and P. Zuber.** 2003. Spx-dependent global transcriptional control is induced by thiol-specific oxidative stress in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **100:**13603–13608.
31. **Nanamiya, H., G Akanuma, Y. Natori, R. Murayama, S. Kosono, T. Kudo, K. Kobayashi, N. Ogasawara, S. M. Park, K. Ochi, and F. Kawamura.** 2004. Zinc is a key factor in controlling alternation of two types of L31 protein in the *Bacillus subtilis* ribosome. Mol. Microbiol. **52:**273–283.
32. **Ogura, M., and T. Tanaka.** 2000. *Bacillus subtilis comZ* (*yjzA*) negatively affects expression of *comG* but not *comK*. J. Bacteriol. **182:**4992–4994.
33. **Ogura, M., Y. Ohshiro, S. Hirao, and T. Tanaka.** 1997. A new *Bacillus subtilis gene*, *med*, encodes a positive regulator of *comK*. J. Bacteriol. **179:**6244–6253.
34. **Ogura, M., H. Yamaguchi, K. Yoshida, Y. Fujita, and T. Tanaka.** 2001. DNA microarray analysis of *Bacillus subtilis* DegU, ComA and PhoP regulons: an approach to comprehensive analysis of *B. subtilis* two-component regulatory systems. Nucleic Acids Res. **29:**3804–3813.
35. **Ogura, M. K. Shimane, K. Asai, N. Ogasawara, and T. Tanaka.** 2003. Binding of response regulator DegU to the *aprE* promoter is inhibited by RapG, which is counteracted by extracellular PhrG in *Bacillus subtilis*. Mol. Microbiol. **49:**1685–1697.
36. **Piazza, F., P. Tortosa, and D. Dubnau.** 1999. Mutational analysis and membrane topology of ComP, a quorum-sensing histidine kinase of *Bacillus subtilis* controlling competence development. J. Bacteriol. **181:**4540–4548.
37. **Serror, P., and A. L. Sonenshein.** 1996. CodY is required for nutritional repression of *Bacillus subtilis* genetic competence. J. Bacteriol. **178:**5910–5915.
38. **Solomon, J. M., B. A. Lazazzera, and A. D. Grossman.** 1996. Purification and characterization of an extracellular peptide factor that affects two different developmental pathways in *Bacillus subtilis*. Genes Dev. **10:**2014–2024.
39. **Thackray, P. D., and A. Moir.** 2003. SigM, an extracytoplasmic function sigma factor of *Bacillus subtilis*, is activated in response to cell wall antibiotics, ethanol, heat, acid, and superoxide stress. J. Bacteriol. **185:**3491–3498.
40. **Touati, D.** 2000. Iron and oxidative stress in bacteria. Arch. Biochem. Biophys. **373:**1–6.
41. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis*. Microbiology **144:**3097–3104.
42. **van Sinderen, D., A. ten Berge, B. J. Hayama, L. Hamoen, and G. Venema.** 1994. Molecular cloning and sequence of *comK*, a gene required for genetic competence in *Bacillus subtilis*. Mol. Microbiol. **11:**695–703.
43. **Westers, H., P. G. Braun, L. Westers, H. Antelmann, M. Hecker, J. D. Jongbloed, H. Yoshikawa, T. Tanaka, J. M. van Dijl, and W. J. Quax.** 2005. Genes involved in SkfA killing factor production protect a *Bacillus subtilis* lipase against proteolysis. Appl. Environ. Microbiol. **71:**1899–1908.
44. **Ye, R. W., W. Tao, L. Bedzyk, T. Young, M. Chen, and L. Li.** 2000. Global gene expression profiles of *Bacillus subtilis* grown under anaerobic conditions. J. Bacteriol. **182:**4458–4465.
45. **Yoshida, K.-I., K. Kobayashi, Y. Miwa, C.-M. Kang, M. Matsunaga, H. Yamaguchi, S. Tojo, M. Yamamoto, R. Nishi, N. Ogasawara, T. Nakayama, and Y. Fujita.** 2001. Combined transcriptome and proteome analysis as a powerful approach to study genes under glucose repression in *Bacillus subtilis*. Nucleic Acids Res. **29:**683–692.