JOURNAL OF BACTERIOLOGY, May 2004, p. 3056–3064 Vol. 186, No. 10
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.10.3056–3064.2004

Bacillus subtilis SalA (YbaL) Negatively Regulates Expression of *scoC*, Which Encodes the Repressor for the Alkaline Exoprotease Gene, *aprE*

Mitsuo Ogura,[1] Atsushi Matsuzawa,[1] Hirofumi Yoshikawa,[2] and Teruo Tanaka[1]*

Department of Marine Science, School of Marine Science and Technology, Tokai University, Shizuoka 424-8610,[1] and Department of Biosciences, Tokyo University of Agriculture, Setagaya-Ku, Sakuraoka, Tokyo 156-8502,[2] Japan

Received 20 October 2003/Accepted 28 January 2004

During the course of screening for exoprotease-deficient mutants among *Bacillus subtilis* gene disruptants, a strain showing such a phenotype was identified. The locus responsible for this phenotype was the previously unknown gene *ybaL*, which we renamed *salA*. The predicted gene product encoded by *salA* belongs to the Mrp family, which is widely conserved among archaea, prokaryotes, and eukaryotes. Disruption of *salA* resulted in a decrease in the expression of a *lacZ* fusion of the *aprE* gene encoding the major extracellular alkaline protease. The decrease was recovered by the cloned *salA* gene on a plasmid, demonstrating that the gene is involved in *aprE* expression. Determination of the *cis*-acting region of SalA on the upstream region of *aprE*, together with epistatic analyses with *scoC*, *abrB*, and *spo0A* mutations that also affect *aprE* expression, suggested that *salA* deficiency affects *aprE-lacZ* expression through the negative regulator ScoC. Northern and reverse transcription-PCR analyses revealed enhanced levels of *scoC* transcripts in the *salA* mutant cells in the transition and early stationary phases. Concomitant with these observations, larger amounts of the ScoC protein were detected in the mutant cells by Western analysis. From these results we conclude that SalA negatively regulates *scoC* expression. It was also found that the expression of a *salA-lacZ* fusion was increased by *salA* deficiency, suggesting that *salA* is autoregulated.

Bacillus subtilis aprE, which encodes the major extracellular alkaline protease, is one of the genes whose expression is induced postexponentially in response to stationary-phase stresses, and the transcription of *aprE* is strictly regulated by complex and redundant systems (43). Spo0A-AbrB and DegS-DegU are the major factors that control *aprE* expression. Spo0A is a master regulator of early developmental responses, such as sporulation initiation, and regulates transcription of many genes negatively and positively (11, 43). During transition from growing to stationary phase, Spo0A is activated by phosphorylation, causing the transcriptional repression of *abrB*, which encodes a global transcription factor regulating many target genes (44, 45). This event is critical for a *B. subtilis* cell to adapt to stationary-phase stresses. For example, the repression of *abrB* by phosphorylated Spo0A results in the activation of *spo0E*, *spo0H*, *spoVG*, and *aprE* expression (5, 6, 41, 45) and the inactivation of *scoC* and *rbs* operon expression (32, 42).

ScoC (Hpr) was identified as a repressor for the expression of *aprE* and *nprE*, the gene encoding the major extracellular neutral protease (4, 14, 33). It was further demonstrated that ScoC regulates the expression of *sinI* and two oligopeptide transporter operons (20, 36), and a consensus DNA sequence, 5′-RATAnTATY-3′, has been proposed for the binding of the ScoC protein (16). The *sinI* gene, located directly upstream of *sinR*, encodes a protein that binds SinR and thereby inhibits its regulatory activity (1, 7, 9). SinR binds the upstream regions of the target genes, including *aprE*, *amyE*, *sacB*, *spoIIA*, *spoIIG*, *spoIIE*, and *spo0A* (7, 8, 9, 23, 24, 25). The *sinI* and *sinR* genes are, however, in different transcription units and subject to different regulation. Thus, the expression of *sinI* but not *sinR* is regulated by Spo0A, AbrB, and ScoC (Fig. 1A) (1, 9, 36). Consequently, ScoC and SinR form a regulatory unit repressing *aprE* expression, in which SinR is activated through the inhibition of *sinI* transcription by ScoC (Fig. 1A). Furthermore, SinR and Spo0A are in a transcriptional circuit, i.e., Spo0A activates *sinI* expression, SinI inhibits SinR and SinR inhibits *spo0A* expression (24, 25, 36). Recently, it was shown by DNA microarray analysis that many genes are under the control of ScoC (2).

Expression of *aprE* is also subject to positive regulation. DegU, the response regulator of the DegS-DegU two-component system, serves as a positive factor when it is phosphorylated (21). In addition, five more positive factors (DegQ, DegR, SenS, TenA, and ProB) are known to enhance *aprE* expression (30, 39).

These elaborate and redundant regulatory factors influencing the expression of *aprE* presumably constitute a network that responds to and integrates both internal and external signals and leads to the fine-tuning of *aprE* expression to cope with changing environments.

In this paper, we report a new locus, *salA*, whose disruption resulted in a reduction of *aprE* expression. It was shown that *salA* encodes a negative regulator of *scoC*, which explains the phenotype of the *salA* disruption. It was also suggested that *salA* was under negative autoregulation.

MATERIALS AND METHODS

Bacterial strains and culture media. All the *B. subtilis* strains used in this study are listed in Table 1. The DNA sequences upstream of the *aprE*-coding regions in constructs SG35.18, SG 35.21, SG 35.20, SG35.8Δ34, and SG35.8Δ6 (13) were confirmed by sequence determination. *B. subtilis* and *Escherichia coli* cells were

* Corresponding author. Mailing address: Department of Marine Science, School of Marine Science and Technology, Tokai University, 3-20-1 Shimizuorido, Shizuoka 424-8610, Japan. Phone: 543-34-0411, ext. 2933. Fax: 543-34-9834. E-mail: teruot@scc.u-tokai.ac.jp.

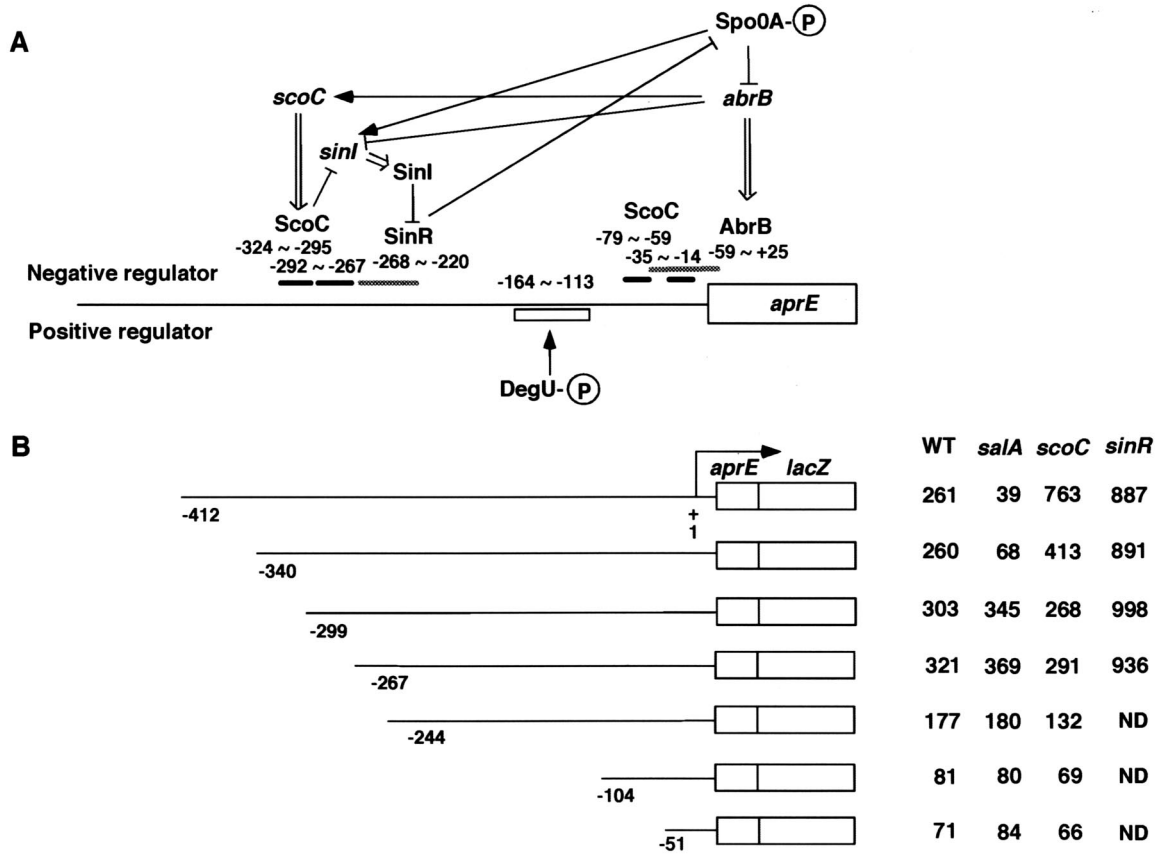

FIG. 1. Regulation of *aprE* by multiple regulators, and determination of the *cis*-acting region of SalA on the control region of *aprE*. (A) Schematic representation of *aprE* regulation by *trans*-acting regulators. The horizontal line indicates the DNA region upstream of *aprE*. The thick and hatched bars above the horizontal line show the regions protected by binding of ScoC, SinR, and AbrB (7, 16, 45). The box below the horizontal line shows the *cis*-acting site for DegU as deduced by analyses using deletions of the upstream region and the *degU32*(Hy) mutation (13). The numbers indicate the nucleotides relative to the transcriptional start site of *aprE*. The arrows and T bars indicate stimulation and inhibition, respectively, and the double-lined arrows show translation of the gene. The P's in the circles depict the phosphate group incorporated in DegU and Spo0A. (B) The lines indicate the regions containing the control region of *aprE*, and the boxes denote translational fusions between the 8th codon of *aprE* and the15th codon of *lacZ* (13). The numbers below the lines indicate the nucleotide numbers relative to the transcription start site of *aprE*, and those in the four columns show β-galactosidase activities expressed in Miller units. ND, not determined. Strains OAM145, OAM146, OAM147, OAM218, OAM148, OAM149, and OAM150 were used as wild-type strains (WT); OAM151, OAM152, OAM153, OAM 219, OAM154, OAM155, and OAM156 were used as *salA* mutants; OAM157, OAM158, OAM159, OAM 220, OAM160, OAM161, and OAM162 were used as *scoC* mutants; and OAM221, OAM222, OAM223, and OAM224 were used as *sinR* mutants. Cells were grown in Schaeffer's sporulation medium, and β-galactosidase activities were determined as described in Materials and Methods. Three independent experiments were done in which samples were withdrawn at hourly intervals in stationary phase (T1 through T5). The highest values observed between T2 and T3 from the three experiments were averaged and shown. The standard deviations were within 15% in all the experiments.

grown in Schaeffer's sporulation and 2× SG (52) media and in Luria-Bertani (LB) medium, respectively. LBCG is a modified version of LB medium in which casein and gelatin were added (46). The concentrations of the antibiotics added to the medium were 1 μg/ml for erythromycin, 10 μg/ml for kanamycin and tetracycline, 100 μg/ml for spectinomycin and ampicillin, and 5 μg/ml for chloramphenicol and phleomycin.

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

Screening for mutants in exoprotease production. The strains used for screening were the gene disruption mutants constructed by the insertion of pMutin-derived plasmids (18, 49) into *B. subtilis* genes, which were obtained from Japanese Consortium of Bacillus Functional Genomics. Into the mutants we introduced a multicopy pUBH1 derivative, pLC1, which carries *proB* and enhances exoprotease production in a DegS-DegU-dependent manner (17, 29). We used this strategy so that the disruption mutants show higher exoprotease activities, and thus any phenotype defective in exoprotease production was easily detected on LBCG plates. The mutant strains carrying pLC1 were spread on LB plates containing erythromycin and kanamycin, and after overnight incubation at 37°C, the colonies formed were transferred by toothpicks onto the LBCG plates containing kanamycin. The halos around the colonies were compared with that formed by *B. subtilis* 168 carrying pLC1 after more than 24 h.

Plasmid construction. All the plasmids used in this study are listed in Table 3. To create pMutin-salA and pMutin-scoC, the PCR products amplified by using the oligonucleotide primer pairs Sal-d1-F and Sal-d1-R, and ScoC-F and ScoC-R, respectively, were treated with HindIII and BamHI and cloned between the HindIII and BamHI sites of pMutinIII (49). To construct pUKM-salA, the PCR product amplified by using Sal-d2-F and Sal-d2-R as oligonucleotide primers was treated with BamHI and HindIII and cloned into pUKM504 (28) treated with the same enzymes. To make pPHL2-sinR, the PCR product amplified by using SinR-F and SinR-R as primers was treated with HindIII and EcoRI and cloned into plasmid pPHL2 (31) treated with the same enzymes. To construct ptrp-salA the PCR product amplified by using the primers Sal-f-F and Sal-f-R was treated with EcoRI and HindIII and cloned into ptrpBG1 (38) that had been treated with the same enzymes. Plasmid pTHP15 was constructed by the insertion between the EcoRI and ClaI sites in ptrpBG1 of a PCR fragment, which was obtained by using the primers HPRCON and LACZ3 and DNA from strain BG4224 as a template. This procedure resulted in the same construct as SG35.18 except that the new construct contains the sequence up to −299 with respect to

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

Strain	Genotype[a]	Reference or source
CU741	*trpC2 leuC7*	S. A. Zhaler
OM210	*trpC2 leuC7 salA*::pMutin-salA (Em^r)	pMutin-salA × CU741
OM211	*trpC2 leuC7 salA*::pMutin-salA (Em^r) (*lacZ*::Tc^r)	pLacZ::Tc × OM210
OM212	*trpC2 leuC7 salA*::pUKM-salA (Km^r)	pUKM-salA × CU741
OM213	*trpC2 leuC7 scoC*::pMutin-scoC (Em^r)	pMutin-socC × CU741
OM214	*trpC2 leuC7 scoC*::pMutin-scoC (Em^r) (*lacZ*::Tc^r)	pLacZ::Tc × OM213
OM215	*trpC2 leuC7 sinR*::Pm^r	pPHL2-sinR × CU741
KK101	*trpC2 pheA1 spo0A*::Sp^r	18
TT734	*trpC2 leuC7 abrB*::Km^r	pGCB2 × CU741
BG4224	*trpC2 his-1 thr-5 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r)	13
BG4226	*trpC2 his-1 thr-5 amyE*::*aprE-lacZ*(−340 [SG35.21], Cm^r)	13
BG4225	*trpC2 his-1 thr-5 amyE*::*aprE-lacZ*(−244 [SG35.20], Cm^r)	13
BG4202	*trpC2 his-1 thr-5 amyE*::*aprE-lacZ*(−104 [SG35.8Δ34], Cm^r)	13
BG4160	*trpC2 his-1 thr-5 amyE*::*aprE-lacZ*(−51 [SG35.8Δ6], Cm^r)	13
OAM145	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r)	BG4224 DNA × CU741
OAM146	*trpC2 leuC7 amyE*::*aprE-lacZ*(−340 [SG35.21], Cm^r)	BG4226 DNA × CU741
OAM147	*trpC2 leuC7 amyE*::*aprE-lacZ*(−299, Cm^r)	pHTP15 × CU741
OAM218	*trpC2 leuC7 amyE*::*aprE-lacZ*(−267, Cm^r)	pTSN35 × CU741
OAM148	*trpC2 leuC7 amyE*::*aprE-lacZ*(−244[SG35.20], Cm^r)	BG4225 DNA × CU741
OAM149	*trpC2 leuC7 amyE*::*aprE-lacZ*(−104 [SG35.8Δ34], Cm^r)	BG4202 DNA × CU741
OAM150	*trpC2 leuC7 amyE*::*aprE-lacZ*(−51 [SG35.8Δ6], Cm^r)	BG4160 DNA × CU741
OAM151	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *salA*::Em^r (*lacZ*::Tc^r)	OM211 DNA × OAM145
OAM152	*trpC2 leuC7 amyE*::*aprE-lacZ*(−340 [SG35.21], Cm^r) *salA*::Em^r (*lacZ*::Tc^r)	OM211 DNA × OAM146
OAM153	*trpC2 leuC7 amyE*::*aprE-lacZ*(−299, Cm^r) *salA*::Em^r (*lacZ*::Tc^r)	OM211 DNA × OAM147
OAM219	*trpC2 leuC7 amyE*::*aprE-lacZ*(−267, Cm^r) *salA*::Em^r (*lacZ*::Tc^r)	OM211 DNA × OAM218
OAM154	*trpC2 leuC7 amyE*::*aprE-lacZ*(−244 [SG35.20], Cm^r) *salA*::Em^r (*lacZ*::Tc^r)	OM211 DNA × OAM148
OAM155	*trpC2 leuC7 amyE*::*aprE-lacZ*(−104 [SG35.8Δ34], Cm^r) *salA*::Em^r (*lacZ*::Tc^r)	OM211 DNA × OAM149
OAM156	*trpC2 leuC7 amyE*::*aprE-lacZ*(−51 [SG35.8Δ6], Cm^r) *salA*::Em^r (*lacZ*::Tc^r)	OM211 DNA × OAM150
OAM157	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *scoC*::Em^r (*lacZ*::Tc^r)	OM214 DNA × OAM145
OAM158	*trpC2 leuC7 amyE*::*aprE-lacZ*(−340 [SG35.21], Cm^r) *scoC*::Em^r (*lacZ*::Tc^r)	OM214 DNA × OAM146
OAM159	*trpC2 leuC7 amyE*::*aprE-lacZ*(−299, Cm^r) *scoC*::Em^r (*lacZ*::Tc^r)	OM214 DNA × OAM147
OAM220	*trpC2 leuC7 amyE*::*aprE-lacZ*(−267, Cm^r) *scoC*::Em^r (*lacZ*::Tc^r)	OM214 DNA × OAM218
OAM160	*trpC2 leuC7 amyE*::*aprE-lacZ*(−244 [SG35.20], Cm^r) *scoC*::Em^r (*lacZ*::Tc^r)	OM214 DNA × OAM148
OAM161	*trpC2 leuC7 amyE*::*aprE-lacZ*(−104 [SG35.8Δ34], Cm^r) *scoC*::Em^r (*lacZ*::Tc^r)	OM214 DNA × OAM149
OAM162	*trpC2 leuC7 amyE*::*aprE-lacZ*(−51 [SG35.8Δ6], Cm^r) *scoC*::Em^r (*lacZ*::Tc^r)	OM214 DNA × OAM150
OAM221	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *sinR*::Pm^r	OM215 DNA × OAM145
OAM222	*trpC2 leuC7 amyE*::*aprE-lacZ*(−340 [SG35.21], Cm^r) *sinR*::Pm^r	OM215 DNA × OAM146
OAM223	*trpC2 leuC7 amyE*::*aprE-lacZ*(−299, Cm^r) *sinR*::Pm^r	OM215 DNA × OAM147
OAM224	*trpC2 leuC7 amyE*::*aprE-lacZ*(−267, Cm^r) *sinR*::Pm^r	OM215 DNA × OAM218
OAM163	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *salA*::Km^r	OM212 DNA × OAM145
OAM164	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *salA*::Km^r *scoC*::Em^r (*lacZ*::Tc^r)	OM214 DNA × OAM163
OAM167	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *spo0A*::Sp^r	KK101 DNA × OAM145
OAM168	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *abrB*::Km^r	TT734 DNA × OAM145
OAM169	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *spo0A*::Sp^r *abrB*::Km^r	OAM167 DNA × OAM145 OAM168 DNA
OAM170	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *salA*::Em^r(*lacZ*::Tc^r) *spo0A*::Sp^r	OAM167 DNA × OAM151
OAM171	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *salA*::Em^r(*lacZ*::Tc^r) *abrB*::Km^r	OAM168 DNA × OAM151
OAM180	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *salA*::Em^r(*lacZ*::Tc^r) *spo0A*::Sp^r *abrB*::Km^r	OAM169 DNA × OAM151

[a] The numbers in parentheses and the letters in brackets indicate the deletion end points upstream of the transcription start point of *aprE* and promoter constructs, respectively. Em^r, erythromycin resistance; Tc^r, tetracycline resistance; Pm^r, phleomycin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Sp^r, spectinomycin resistance.

the transcription start site of *aprE*. Plasmid pTSN35 was constructed in the same procedure as that used for pTHP15 except that primer SINCON was used instead of HPRCON. To make pDG-salA, the PCR product amplified by using Sal-op-F and Sal-op-R as primers was treated with HindIII and SalI and cloned into plasmid pDG148, a shuttle vector capable of replication in both *E. coli* and *B. subtilis* (40), which had been treated with the same enzymes. Sequences were confirmed for the entire, cloned DNA regions. To construct pGCB2, the kanamycin resistance gene cassette obtained by the SmaI digestion of pBEST508 (15) was inserted into the blunt-ended BstXI site in the *abrB* gene cloned on a pBR322-derived plasmid, pGC516R (34).

Site-directed mutagenesis. To introduce amino acid substitutions into SalA, site-directed mutagenesis of the cloned *salA* gene on pDG-salA was carried out with the oligonucleotide pairs Sal-m1 and Sal-m2 and a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.) according to the procedure described in the instructions. Mutations were confirmed by sequence determination of the entire *salA*-coding region on the plasmid constructed.

β-Galactosidase and sporulation assays. Samples were withdrawn at hourly intervals for the measurement of β-galactosidase activities as described previously (32). For a sporulation test, cells were grown in 2× SG medium at 37°C for 24 h (31), and the serial dilutions of the cell culture were plated on LB agar plates before and after heating at 80°C for 10 min.

RNA isolation and Northern blot analysis. Total RNA was isolated as described previously (53). A DNA probe for detecting *scoC* was prepared by PCR with a DIG Probe Synthesis Kit (Boehringer Mannheim, Mannheim, Germany) and the primers ScoC-1 and ScoC-2 (Table 2). RNA was denatured with formamide and blotted to a Nylon membrane (Boehringer Mannheim). mRNA for *scoC* was detected with the DNA probe and a DIG Luminescent Detection Kit (Boehringer Mannheim).

Real-time reverse transcription (RT)-PCR analysis. cDNA was synthesized and amplified using relevant PCR primers and a Real Time One Step RNA PCR Kit (Takara Biomedicals, Shiga, Japan) according to the procedure recommended by the supplier. The amplification step of DNA was monitored by the Smart Cycler System (Cepheid) with SYBR Green I, and the relative concentrations of mRNAs were estimated by the procedure provided by Cepheid. The PCR products synthesized by this procedure were verified by melting curve analysis and by agarose gel electrophoresis for size determination. The rRNA levels in the RNA samples were also determined to confirm that the same amounts of RNA were used for the PCR.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence
Sal-d1-F	5′-GGAAGCTTCGATTTGAAGAGCTGCC-3′
Sal-d1-R	5′-TTGGATCCAACCTTTTTCCCTAGACG-3′
Sal-d2-F	5′-CTAGGATCCGGGGCGCTTATAGAAGTCGG-3′
Sal-d2-R	5′-TGAAAGCTTATAAACAGACGGAGCAAATT-3′
Sal-op-F	5′-GATAAGCTTGTAAACGAATACACAAAAGGG-3′
Sal-op-R	5′-GATCTCGAGTTTTATACCTGCACTGACAT-3′
Sal-f-F	5′-ATGGAATTCAGCTTTTATTACGGGAAATAT-3′
Sal-f-R	5′-ATGAAGCTTTACTTCATCTTCTCTTATCA-3′
SinR-F	5′-GATAAGCTTATTGGCCAGCGTATTAAA-3′
SinR-R	5′-GATGAATTCTCATGTTTCTCATCGAGCA-3′
SinR-R2	5′-AAAATCCCAAAAAGAGGAGTAG-3′
HPRCON	5′-CAGTGAATTCATCATGCTTTGAAAAAATATCACG-3′
SINCON	5′-CAGTGAATTCCATTGTTCTCACGGAAGCAC-3′
LACZ3	5′-CTGACGCAGTCGGCATAACC-3′
ScoC-F	5′-AGTAAGCTTGAGTGGAACCGCCCTATGA-3′
ScoC-R	5′-ATCGGATCCGCCGTTTGGAGAACCTTAAA-3′
Sal-m1	5′-AAAAGGCGGCGTAGATCTGTCAACTGTGTCAG-3′
Sal-m2	5′-CTGACACAGTTGACAGATCTACGCCGCCTTTT-3′
ScoC-1	5′-ATCGAGTGGAACCGCCCTATGA-3′
ScoC-2	5′-CAGCCGGTTCATCCGCCGCAA-3′

Western blot analysis. Western blotting and the subsequent detection of digoxigenin-labeled protein bands were done according to the procedure described previously (12). Polyclonal antibody against a synthetic 13-amino-acid peptide, NH_2-ADEPAEELEPVNS-COOH, derived from a C-terminal part of ScoC was raised in rabbits and prepared by Sawady technology Inc. (Tokyo, Japan).

RESULTS

Disruption of *salA* resulted in a decrease of *aprE-lacZ* expression. We screened for exoprotease-deficient strains among the *B. subtilis* gene disruption mutants by the halo assay (see Materials and Methods). As a result, we found a strain carrying a disruption at the *ybaL* locus to produce a significantly smaller halo around the colony (data not shown). We renamed *ybaL* *salA*, which stands for a regulator of *sco*C, *aprE*, and *ybaL* (see below). The *salA* gene was originally reported as a locus (*rec223*) that gave rise to a mitomycin C-sensitive phenotype and caused a defect in DNA-mediated transformation when present on a multicopy plasmid (10, 35). Although we compared the sensitivity of the wild-type CU741 and *salA* strains by inoculating them in liquid LB medium or on LB plates containing 1.6-fold serial dilutions of mitomycin C, we could not detect any difference in sensitivity between the strains, nor could we reproduce the multicopy effect of *salA* on transformation (data not shown).

The *salA* gene is predicted to encode a protein of the Mrp family that is conserved among eukaryotes, prokaryotes, and archaea and carries an ATP-binding motif (Fig. 2) (3, 50). It should be noted that the term Mrp was defined by its genetic location in *E. coli* (3) but not by the function. To confirm the phenotype of low exoprotease production, we examined the expression of an *aprE-lacZ* translational fusion placed at the *amyE* locus in a *salA* disruption mutant. Although the halo is formed mainly by the exocellular neutral protease, the *nprE* gene product (17), we used the *aprE-lacZ* fusion to study the effect of *salA* on *aprE* expression, since the regulation of *aprE* expression has been better studied than that of *nprE*. The region upstream of the *aprE*-coding sequence in the strain used extends as far as −412 relative to the transcriptional start site. As shown in Fig. 3, the β-galactosidase activity derived from *aprE-lacZ* in the mutant was about 1/10 the level at the peak value detected in the wild-type cells. In this experiment, we used the wild-type and *salA* strains carrying a multicopy plasmid, pDG148, so that they serve as the control strains for the next experiment. The presence or absence of the vector plasmid had no effect on *aprE* expression (data not shown).

When plasmid pDG-salA carrying the intact *salA* gene was introduced into the *salA* disruption mutant, the level of *aprE-lacZ* expression was recovered roughly to that found in the wild-type cells (Fig. 3), indicating that the decrease in *aprE-lacZ* expression in the mutant was caused by the *salA* disruption. In pDG-salA, the *salA* gene is located downstream of the Pspac promoter that is inducible by IPTG (isopropyl-1-thio-β-D-galactopyranoside), but the addition of IPTG was not necessary for complementation. This result shows that the Pspac promoter is leaky, producing enough SalA to stimulate *aprE* expression from the pDG-salA construct without the addition of IPTG. The level of *aprE-lacZ* expression in the wild-type strain carrying pDG-salA (Fig. 3) was similar to that found in the wild-type strain carrying the pDG148 vector (Fig. 3). Even in the presence of IPTG, the expression level of *aprE-lacZ* was similar (data not shown). This was unexpected, since pDG-salA in the wild-type strain would cause a further enhancement in *aprE-lacZ* expression. We found by RT-PCR that, in the wild-type cells carrying pDG-salA, the induction levels of *salA* mRNA synthesis by IPTG diminished after exponential growth (19.0-fold at T0 [the growth time in hours relative to the end of vegetative growth], and 7.1-fold at T1), and reached a similar level (1.4-fold) at T2 as that found in the cells grown without IPTG (data not shown). Since the expression of *aprE-lacZ*

TABLE 3. Plasmids used in this study

Plasmid	Description[a]	Source or reference
pUBH1	Multicopy *B. subtilis* plasmid; Km^r	17
pLC1	pUBH1 carrying *proB;* causes overexpression of *aprE*	29
pMutinIII	Insertional Em^r plasmid carrying promoterless *lacZ*	49
pMutin-salA	Em^r pMutinIII carrying transcriptional fusion *salA-lacZ*	This study
pMutin-scoC	Em^r pMutinIII carrying transcriptional fusion *scoC-lacZ*	This study
pUKM504	Insertional Km^r plasmid	28
pUKM-salA	Km^r pUKM504 carrying an internal part of *salA*	This study
pPHL2	pUC19-based Pm^r plasmid/31 pPHL2-sinR/pPHL2 carrying an internal part of *sinR*	This study
pLacZ::Tc	Amp^r Tc^r; carrying *lacZ*::Tc^r	32a
ptrpBG1	Insertional Amp^r Cm^r plasmid carrying *trpE-lacZ*	38
pTHP15	ptrpBG1 carrying the *aprE* promoter region up to −299	This study
pTSN35	ptrpBG1 carrying the *aprE* promoter region up to −267	This study
pDG148	Multicopy *B. subtilis* and *E. coli* plasmid; Amp^r Km^r	40
pDG-salA	Amp^r Km^r pDG148 carrying *salA*	This study
pDG-salA-m	Amp^r Km^r pDG148 carrying mutant *salA* (G119D and K120L)	This study
pBEST508	Amp^r Km^r	15
pGC516R	Amp^r; carrying *abrB*	34
PGCB2	Amp^r pGC516R carrying *abrB*::Km^r	This study

[a] Amp^r, ampicillin resistance. For other abbreviations see Table 1, footnote *a*.

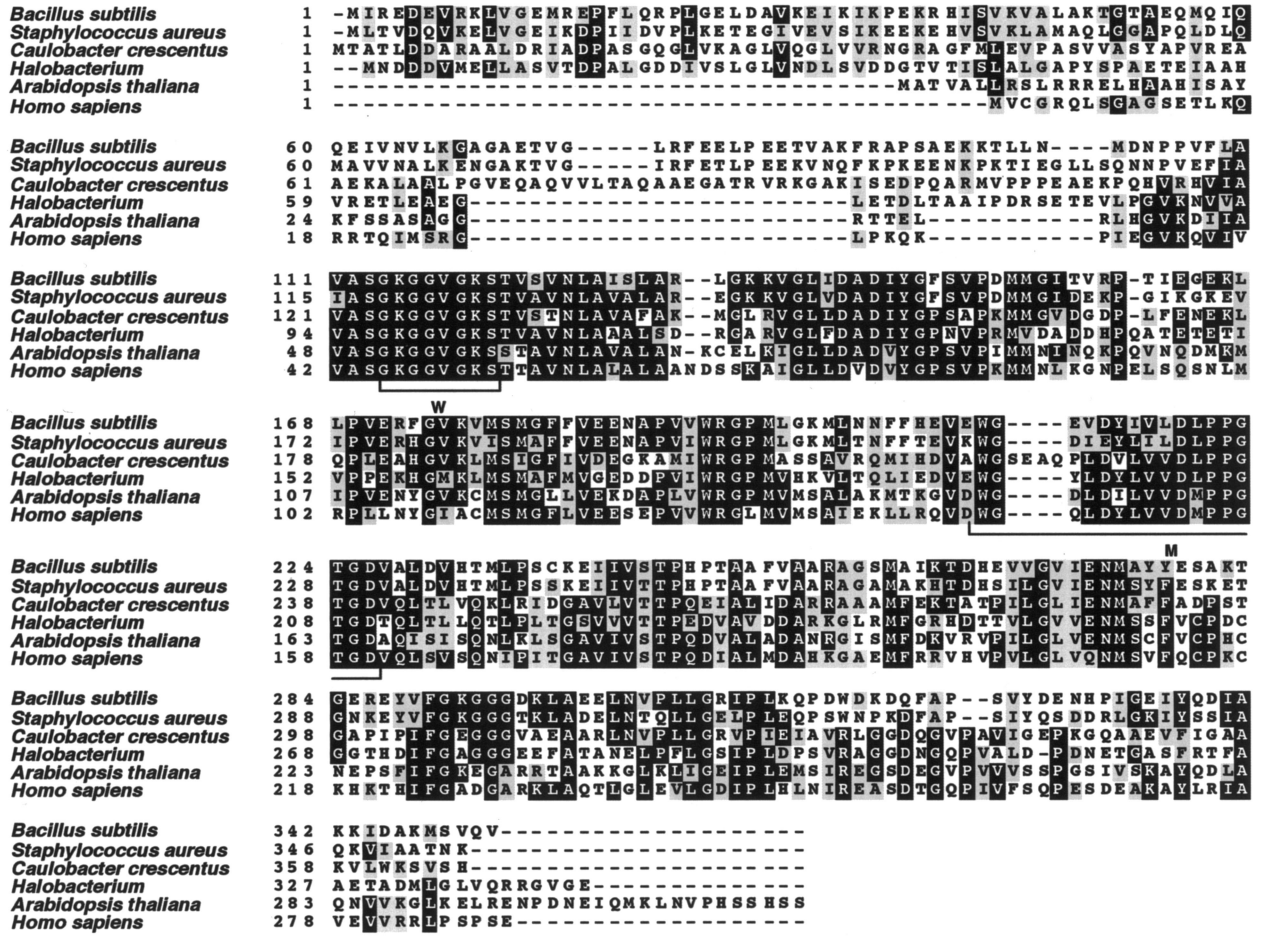

FIG. 2. Alignment of amino acids among Mrp family proteins. The numbers indicate the positions of amino acids starting from the N-terminal end of each protein. The amino acids marked by black and gray boxes indicate identical and similar amino acids, respectively. Accession numbers in PIR; *B. subtilis*, A69743; *Staphylococcus aureus*, A90012; *Caulobacter crescentus*, F87508; *Halobacterium*, H84268; *Arabidopsis thaliana*, T06147; *Homo sapiens*, AK022722. Multiple alignment was carried out by CLUSTAL W (47), and the figure was made by BOXSHADE. The brackets marked by W and M under the alignment indicate the conserved Walker-motif and the MRP signature, respectively (51; BSORF Web site [http://bacillus.genome.ad.jp]).

reaches the highest level after T2 in our experimental condition (Fig. 3 and 4), a possible enhancing effect of SalA amplified by the addition of IPTG on *aprE-lacZ* expression could not be seen in the experiments shown in Fig. 3. However, it may also be possible that the expression level of *aprE* does not respond linearly to the level of SalA and the enhancing effect is seen only below a certain level of SalA. A further study will be necessary to clarify the relationship between the SalA level and *aprE* expression.

Complementation of *salA* by pDG-salA was also observed in a strain bearing a translational *aprE-lacZ* fusion at the original *aprE* locus constructed by Campbell-type recombination (data not shown).

One characteristic of the Mrp group proteins is that they contain an ATP-binding motif (Fig. 2) (51). Since the SalA protein also carries such a motif, we examined whether it is involved in the regulation of *aprE-lacZ* expression. We introduced two amino acid substitutions on pDG-salA that are highly conserved among ATP-binding motifs by changing G119 and K120 to D and L, respectively. That an alteration of K in the motif has a dramatic effect on the protein activity has been demonstrated for *B. subtilis clpC* recently (48). The amino acid changes on plasmid pDG-Sal-m thus constructed, however, did not essentially affect the complementing activity of pDG148-salA in the *salA* mutant (Fig. 3). The variations in the β-galactosidase activities after T3 might be due to the difference in the stability between the *salA* mRNAs or the SalA proteins derived from the wild-type and the mutant *salA* cells. Consequently, we conclude that the putative ATP-binding motif in SalA is not involved in the regulation of *aprE* at least in a multicopy state.

Determination of the *cis*-acting region of SalA upstream of *aprE*. It has been demonstrated that ScoC, SinR, and AbrB exert their negative effects on *aprE* expression by binding to the *cis* elements located upstream of the *aprE*-coding sequence (Fig. 1A). The positive regulator DegU directed by the *degU32* (Hy) mutant gene has also been shown to exert a positive effect at a site between −164 and −113 relative to the transcriptional start site (Fig. 1A) (13). If SalA regulates *aprE* expression through its effect on any of these factors, determination of the *cis*-acting region of SalA should provide information on the target of SalA. We introduced the *salA* disruption mutation

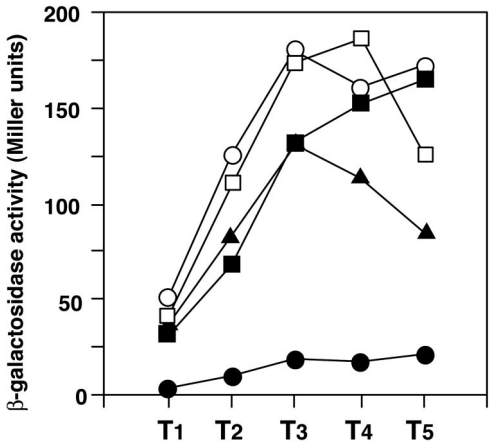

FIG. 3. Inhibition of *aprE-lac*Z expression by *salA* disruption, and recovery by intact or mutant *salA* on plasmid pDG148. Cells were grown in Schaeffer's sporulation medium, and β-galactosidase activities were determined as described in Materials and Methods. Data are from one of two experiments. Numbers on the *x* axis represent the growth time in hours relative to the end of the vegetative growth (T0). ○, OAM145 (WT) carrying pDG148; ●, OAM151 (*salA*) carrying pDG148; □, OAM145 (WT) carrying pDG-salA; ■, OAM151 (*salA*) carrying pDG-salA; ▲, OAM151 (*salA*) carrying pDG-salA-m.

into strains bearing *aprE-lacZ* fusions in which sequential deletions upstream of the *aprE* promoter had been introduced (reference 13 and this study). The β-galactosidase activities determined for these strains are shown in Fig. 1B. The *salA* mutation caused a decrease in the expression of the *aprE-lacZ* fusions with the sequences up to −412 and −340 of *aprE*, whereas it had no negative effect on the five fusions carrying deletions from −299 to −51. The DNA region downstream from −299 contains the SinR and AbrB binding sites, and also the target of DegU (7, 13, 41, 45), suggesting that these factors will not be the primary target of the *salA* mutation.

Gaur et al. (7) reported that the negative effect of multicopy *sinR* on *aprE* expression was seen in a construct carrying the region up to −340 but not −244. As shown in Fig. 1B, similar levels of stimulatory effect by *sinR* deficiency were observed in the constructs carrying deletions up to −412 through −267, indicating that the target of SinR resides in a region downstream from −267.

The stimulation of *aprE* expression by *scoC* deficiency was observed with the construct up to −340 but not with those up to −299 (Fig. 1). The results are in concert with the previous results in which ScoC binds to two regions from −324 to −267 (Fig. 1A) (16). On the other hand, the results are somewhat different from those observed previously in which a small but not a full ScoC effect was still observed with a fusion up to −244 (13). The discrepancy may have arisen by a strain difference. We note that *aprE-lacZ* expression was rather reduced in the fusions with only the proximal ScoC-binding site (Fig. 1B). Similar results were observed previously (13).

The deletion analyses shown here indicate that the target site of SalA is different from that of SinR and resides in a region around or upstream of −299 where the target site of ScoC is located.

Epistatic analyses of *salA* and other regulators, *spo0A*, *abrB*, and *scoC*. Spo0A activates *aprE* expression in two ways: one is through the repression of *abrB* and the other through the transcriptional activation of *sinI* (Fig. 1A). We examined whether the decrease in *aprE-lacZ* expression by *salA* deficiency was still observed in a strain lacking the Spo0A-AbrB pathway. As shown in Fig. 4A, the *salA* mutation caused a decrease in *aprE-lacZ* expression in both the *spo0A* and *spo0A abrB* backgrounds. In a separate experiment, we observed that the *salA* mutation did not affect the sporulation efficiency of the cells grown in 2× SG medium (data not shown). These results demonstrate that the negative effect of SalA on *aprE-lacZ* expression is not through the regulatory function of Spo0A.

Since the *salA* mutation resulted in a decrease of *aprE-lacZ*

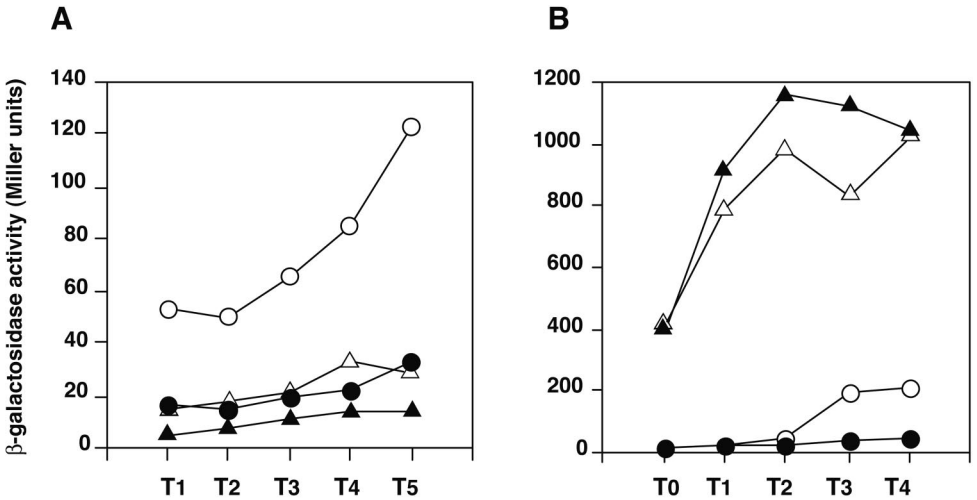

FIG. 4. Epistatic relationship between *salA* and *spo0A*, *abrB*, and *scoC* in *aprE-lacZ* expression. Cells were grown in Schaeffer's sporulation medium, and β-galactosidase activities were determined as described in Materials and Methods. Data are from one of two experiments. Numbers on the *x* axis represent the growth time in hours relative to the end of the vegetative growth (T0). (A) ○, OAM169 (*spo0A abrB*); ●, OAM180 (*spo0A abrB salA*); △, OAM167 (*spo0A*); ▲, OAM170 (*spo0A salA*). (B) ○, OAM145 (WT); ●, OAM151 (*salA*); △, OAM157 (*scoC*); ▲, OAM164 (*scoC salA*).

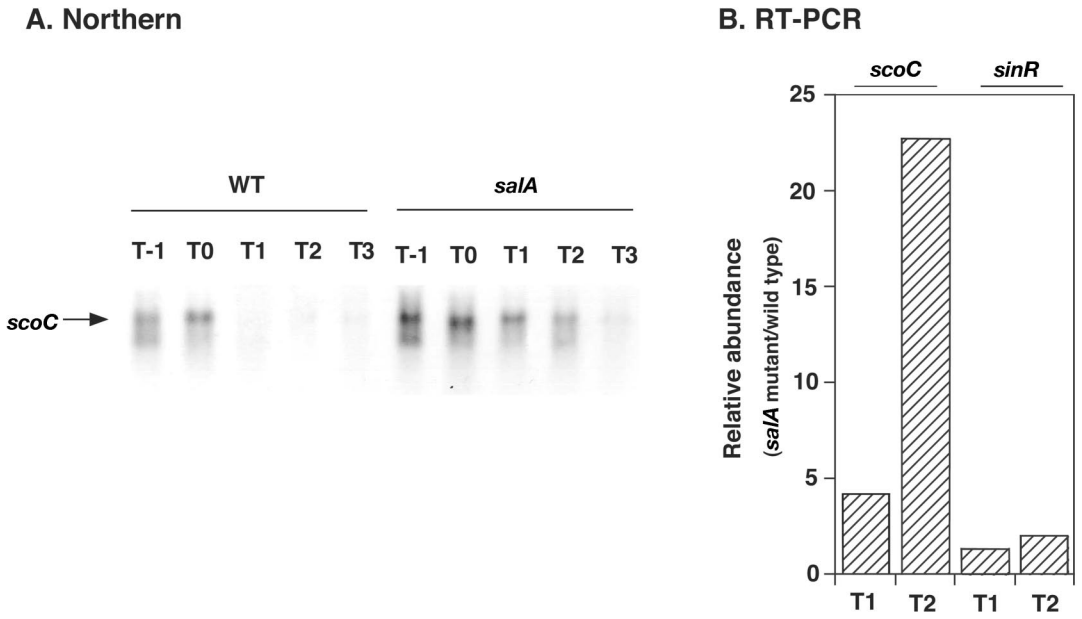

FIG. 5. Abundance of *scoC* transcripts in a *salA*-deficient mutant compared with that in the wild-type strain. Strains used were CU741 and OM210 for the wild-type (WT) and *salA* strains, respectively. The procedures for RNA isolation and Northern and RT-PCR analyses are described in Materials and Methods. The RNA samples for the Northern and RT-PCR analyses were prepared separately. (A) Northern analysis. Cells were grown in Schaeffer's sporulation medium, and samples (20 ml) were withdrawn from T−1 to T4. Twenty micrograms of the isolated RNAs was applied to each lane, and *scoC* transcripts were detected with a digoxigenin-labeled PCR probe. In a separate experiment we have confirmed that there was no detectable band corresponding to the *scoC* transcript in the RNA samples obtained from strain OM213 (*scoC*). (B) RT-PCR analysis. Levels of mRNAs for *scoC* and *sinR* were quantified at T1 and T2 by using 1 μg each of the RNA samples. The oligonucleotide pairs ScoC-1 and ScoC-2 and SinR-F and SinR-R2 were used to detect *sinR* and *scoC* mRNAs, respectively. Relative abundance of mRNA at each stage was determined by dividing the mRNA levels in the mutant cells by those estimated in the wild-type cells.

expression directed from a region upstream from −299, *scoC* was the next candidate of the target of SalA. If this is the case, then the expression levels of *aprE-lacZ* in *scoC* and *scoC salA* mutants would be similar. The results depicted in Fig. 4B show that the inhibition of *aprE-lacZ* expression by *salA* deficiency was overcome by *scoC* mutation. However, the small difference in the β-galactosidase levels in the *scoC* and *scoC salA* mutants was reproducible. These results suggest that the regulatory pathway of SalA in *aprE* expression overlaps but does not completely coincide with that of ScoC.

Enhanced and prolonged transcription of *scoC* in the *salA* mutant in early stationary phase. The results on the *cis*-acting region of SalA and the epistatic analyses may indicate that the *salA* disruption caused an increase in *scoC* expression or ScoC activity in the cell, resulting in repression of *aprE-lacZ*. To test this possibility, we first carried out Northern analysis on *scoC* using a *scoC*-specific probe and RNAs obtained from both the wild-type and *salA* strains. The results depicted in Fig. 5A show that the level of the *scoC* transcript in the wild-type strain was the highest during transition phase, followed by a decrease in early stationary phase. A similar profile of β-galactosidase activities from a *scoC-lacZ* transcriptional fusion in strain OM213 (*salA*$^+$) was observed (data not shown). It was shown previously that the expression of *scoC-lacZ* declined only slightly during stationary phase (33). The discrepancy may be due to the different stability of the transcripts derived from *scoC* itself and different *scoC-lacZ* fusions. In the *salA* mutant, the *scoC* transcript levels at T−1 and T0 were significantly higher compared with those in the wild-type cell (Fig. 5A), and the difference was more apparent at T1 and T2. These results show that *scoC* transcription in the *salA* mutant was at a higher level and prolonged.

We further quantified the observed differences in the mRNA levels by RT-PCR using RNAs prepared separately. In the *salA* cells, the *scoC* mRNA levels were 4- and 22-fold higher at T1 and T2, respectively, than those in the wild-type cells (Fig. 5B). These results are in agreement with those of the Northern analysis. The *sinR* mRNA contents determined by RT-PCR are also shown in Fig. 5B. The *sinR* transcript level in the *salA* mutant was no more than twofold compared to that in the wild-type strain at T2, which supports the notion that *sinR* is not the primary target of SalA. Similar results were obtained by Northern analysis on *sinR* mRNAs in the wild-type and *salA* mutant cells (data not shown).

We next examined the ScoC protein levels by Western analysis using an antibody raised against a C-terminal 13-amino-acid peptide of ScoC. As shown in Fig. 6, the ScoC antibody bound to a protein band with a molecular size corresponding to that of ScoC. The protein band was absent in a cell extract from the *scoC* mutant, indicating that the band detected is indeed that of ScoC. Unexpectedly, the ScoC levels in the wild-type cells were found to be similar among the cell extracts obtained from different stages of stationary phase (Fig. 6). This is in contrast to the results of the Northern experiments described above, and raised an interesting question of how the expression of *aprE* in the stationary-phase cells is enabled in the presence of the negative regulator, ScoC. In the *salA* strain, the ScoC levels were higher as expected from the results of the

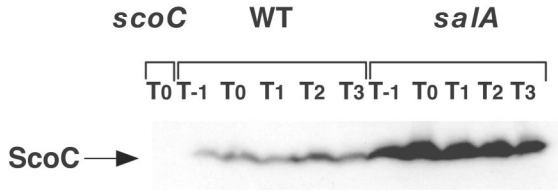

FIG. 6. Western analysis on ScoC proteins. Strains used were CU741, OM210, and OM213 for the wild-type (WT), *salA* mutant, and *scoC* mutant strains, respectively. Cells grown in Schaeffer's sporulation medium (50 ml) were withdrawn from T−1 to T3, washed and resuspended in 1 ml of TE buffer (10 mM Tris-HCl [pH 8.0], EDTA 1 mM), and disrupted by French press. Equal amounts of the protein were applied on a sodium dodecyl sulfate–14% polyacrylamide gel, and the ScoC protein was detected by ScoC antibody as described in Materials and Methods.

Northern blot analysis (Fig. 5A). On the basis of these results, we conclude that the elevated and prolonged transcription of *scoC* leads to the production of larger amounts of ScoC in the *salA* cells.

Expression of *salA*. To study the expression of *salA* itself, a *salA-lacZ* translational fusion was constructed at the *amyE* locus, and β-galactosidase activities were determined in the *salA* and wild-type cells. It was found that the expression of *salA-lacZ* was constitutive, and the β-galactosidase activities at the transition state were twofold higher in the *salA* disruptant (data not shown), suggesting that *salA* expression is autoregulated.

DISCUSSION

We have shown in this paper that a new locus, *salA* (*ybaL*), negatively regulates *scoC* expression. It is a monocistronic operon gene located at 13.4° on the *B. subtilis* chromosome map, and encodes a putative protein belonging to the Mrp family (Fig. 2). Unexpectedly, the putative ATP-binding motif in SalA was unrelated to its activity to regulate *aprE* expression through ScoC, although the motif is ubiquitous among the Mrp family proteins. The Mrp family proteins are conserved among the three kingdoms (3, 27, 37, 50), but their function is not known so far in any organisms. All we know at present is that NBP35, an Mrp family protein in *Saccharomyces cerevisiae*, is an essential protein (50). In the case of *salA*, disruption of the gene did not affect the viability of the host cell (data not shown).

SalA may not be a DNA-binding protein, since no such typical amino acid sequence was predicted in the molecule. This may suggest that the effect of SalA on *scoC*, and therefore on *aprE*, expression is indirect. The small difference in the expression levels of *aprE-lacZ* in *scoC* and *scoC salA* mutants (Fig. 4B) leaves open the possibility that some other factor under the regulation of SalA is also involved. Thus, the exact molecular mechanism of transcriptional regulation by SalA remains unknown. As for the transcriptional regulation of *scoC*, the only known factor that affects *scoC* expression is AbrB (39, 45). This factor, however, is not involved in the regulation of *aprE* by *salA* as shown by the epistatic analyses using *spo0A-abrB* mutants (Fig. 4A). Thus, the possibility that SalA influences *scoC* transcription through AbrB is excluded. SalA might affect expression of *scoC* by associating with some transcription factor or RNA polymerase itself.

Overproduction of ScoC in the wild-type cell results in inhibition of sporulation (33). It was shown that ScoC levels were higher in the *salA* mutant (Fig. 6), but the sporulation efficiency was not affected in the 2× SG medium. The different observations might be due to different levels of ScoC in the two cells. With respect to sporulation, we note that in the presence of 2% glucose in the 2× SG medium (31) the sporulation efficiency of the *salA* mutant was reduced to 10% of that observed for the wild-type strain (data not shown). The results imply that catabolite repression becomes more severe as the ScoC concentration is increased. This is in concert with the previous results that catabolite repression of sporulation is rescued by *scoC*-null mutations (4, 33).

Northern analysis showed that *scoC* transcripts were not detected after T1 in the wild-type cell (Fig. 5A). In contrast, the Western blot experiment revealed constant levels of the ScoC protein in the cells obtained at T0 and thereafter (Fig. 6). Since *aprE-lacZ* expression is increased after the end of growth phase (Fig. 3 and 4), there may be some mechanism by which ScoC is stabilized but becomes inactive after entry into stationary phase. There could be several explanations for these observations. (i) ScoC might be inactivated by some effector molecule, since ScoC belongs to the MarR family repressor, which is known to be inactivated by small molecular effectors (26). (ii) ScoC might be sequestered from the *aprE* promoter by some factor(s). (iii) ScoC binds to the *aprE* promoter, but its inhibitory function might be counteracted by a positive factor(s). Transcription of *aprE* is known to be positively regulated by phosphorylated DegU and auxiliary factors including *degR*, *degQ*, *senS*, *tenA*, and *proB* (21, 29, 30, 39). One or several of these factors might work as a factor antagonizing the ScoC function in stationary phase, although there is no data concerning this issue at present.

It has been noted that *salA* (*ybaL*) expression is induced by disulfide stress (22). It is not known, however, whether this phenomenon is correlated with either *scoC* or *aprE* expression.

ACKNOWLEDGMENTS

We thank Y. Kijima, S. Kinoshita, H. Kitoh, Y. Tuzuki, T. Inamori, and N. Takahashi for technical assistance and P. Zuber and K. Asai for plasmids. We also thank M. Takeuchi and T. Sato (Tokyo University of Agriculture and Technology), who independently discovered *ybaL* as an exoprotease-deficient mutant, for discussion.

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) Genome Biology and a Grant-in-Aid for Scientific Research (B) and (C) from the Ministry of Education, Science, and Sports and Culture of Japan.

REFERENCES

1. **Bai, U., I. Mandic-Mulec, and I. Smith.** 1993. SinI modulates the activity of SinR, a developmental switch protein of *Bacillus subtilis*, by protein-protein interaction. Genes Dev. **7:**139–148.
2. **Caldwell, R., R. Sapolsky, W. Weyler, R. R. Maile, S. C. Causey, and E. Ferrari.** 2001. Correlation between *Bacillus subtilis scoC* phenotype and gene expression determined using microarrays for transcriptome analysis. J. Bacteriol. **183:**7329–7340.
3. **Dardel, F., M. Panvert, S. Blanquet, and G. Fayat.** 1991. Locations of the *metG* and Mrp genes on the physical map of *Escherichia coli.* J. Bacteriol. **173:**3273.
4. **Dod, B., and G. Balassa.** 1978. Spore control (*sco*) mutations of *Bacillus subtilis.* Regulation of extracellular protease synthesis in the spore control mutations *scoC.* Mol. Gen. Genet. **163:**57–63.
5. **Ferrari, E., D. J. Henner, M. Perego, and J. A. Hoch.** 1988. Transcription of

Bacillus subtilis subtilisin and expression of subtilisin in sporulation mutants. J. Bacteriol. **170:**289–295.
6. **Ferrari, E., S. M. Howard, and J. A. Hoch.** 1986. Effect of stage 0 sporulation mutations on subtilisin expression. J. Bacteriol. **166:**173–179.
7. **Gaur, N. K., J. Oppenheim, and I. Smith.** 1991. The *Bacillus subtilis sin* gene, a regulator of alternate developmental processes, codes for a DNA-binding protein. J. Bacteriol. **173:**678–686.
8. **Gaur, N. K., E. Dubnau, and I. Smith.** 1986. Characterization of a cloned *Bacillus subtilis* gene that inhibits sporulation in multiple copies. J. Bacteriol. **168:**860–869.
9. **Gaur, N. K., K. Cabane, and I. Smith.** 1988. Structure and expression of the *Bacillus subtilis sin* operon. J. Bacteriol. **170:**1046–1053.
10. **Gavrilova, E. V., S. L. Mekhedov, A. A. Prozorov, and F. K. Khasanov.** 1992. Bacillus subtilis gene *rec223*: molecular cloning and proposed function of its protein product. Genetika **28:**29–39.
11. **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.
12. **Hata, M., M. Ogura, and T. Tanaka.** 2001. Involvement of stringent factor RelA in expression of the alkaline protease gene *aprE* in *Bacillus subtilis.* J. Bacteriol. **183:**4648–4651.
13. **Henner, D. J., E. Ferrari, M. Perego, and J. A. Hoch.** 1988. Location of the targets of the *hpr-97*, *sacU32(Hy*), and *sacQ36(Hy*) mutations in upstream regions of the subtilisin promoter. J. Bacteriol. **170:**296–300.
14. **Higerd, T. B., J. A. Hoch, and J. Spizizen.** 1972. Hyperprotease-producing mutants of *Bacillus subtilis.* J. Bacteriol. **112:**1026–1028.
15. **Itaya, M., K, Kondo, and T. Tanaka.** 1989. A neomycin resistance gene cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. Nucleic Acids Res. **17**:4410.
16. **Kallio, P. T., J. E. Fagelson, J. A. Hoch, and M. A. Strauch.** 1991. The transition state regulator Hpr of *Bacillus subtilis* is a DNA-binding protein. J. Biol. Chem. **266:**13411–13417.
17. **Kawamura, F., and R. H. Doi.** 1984. Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. J. Bacteriol. **160:**442–444.
18. **Kobayashi, K., S. D. Ehrlich, et al.** 2003. Essential *Bacillus subtilis* genes. Proc. Natl. Acad. Sci. USA **100:**4678–4683.
19. **Kobayashi, K., K. Shoji, T. Shimizu, K. Nakano, T. Sato, and Y. Kobayashi.** 1995. Analysis of a suppressor mutation *ssb* (*kinC*) of *sur0B20* (*spo0A*) mutation in *Bacillus subtilis* reveals that *kinC* encodes a histidine protein kinase. J. Bacteriol. **177:**176–182.
20. **Koide, A., M. Perego, and J. A. Hoch.** 1999. ScoC regulates peptide transport and sporulation initiation in *Bacillus subtilis.* J. Bacteriol. **181:**4114–4117.
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. **Leichert, L. I., C. Scharf, and M. Hecker.** 2003. Global characterization of disulfide stress in *Bacillus subtilis.* J. Bacteriol. **185:**1967–1975.
23. **Louie, P., A. Lee, K. Stansmore, R. Grant, C. Ginther, and T. Leighton.** 1992. Roles of *rpoD*, *spoIIF*, *spoIIJ*, *spoIIN*, and *sin* in regulation of *Bacillus subtilis* stage II sporulation-specific transcription. J. Bacteriol. **174:**3570–3576.
24. **Mandic-Mulec, I., N. Gaur, U. Bai, and I. Smith.** 1992. Sin, a stage-specific repressor of cellular differentiation. J. Bacteriol. **174:**3561–3569.
25. **Mandic-Mulec, I., L. Doukhan, and I. Smith.** 1995. The *Bacillus subtilis* SinR protein is a repressor of the key sporulation gene *spo0A.* J. Bacteriol. **177:** 4619–4627.
26. **Miller, P. F., and M. C. Sulavik.** 1996. Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in *Escherichia coli.* Mol. Microbiol. **21:**441–448.
27. **Nakashima, H., M. J. Grahovac, R. Mazzarella, H. Fujiwara, J. R. Kitchen, T. A. Threat, and M. S. Ko.** 1999. Two novel mouse genes—Nubp2, mapped to the t-complex on chromosome 17, and Nubp1, mapped to chromosome 16—establish a new gene family of nucleotide-binding proteins in eukaryotes. Genomics **60:**152–160.
28. **Ogura, M., and T. Tanaka.** 1996. Transcription of *Bacillus subtilis degR* is σ^D-dependent and suppressed by multicopy *proB* through σ^D. J. Bacteriol. **178:**216–222.
29. **Ogura, M., M. Kawata-Mukai, M. Itaya, K. Takio, and T. Tanaka.** 1994. Multiple copies of the *proB* gene enhance *degS*-dependent extracellular protease production in *Bacillus subtilis.* J. Bacteriol. **176:**5673–5680.
30. **Ogura, M., and T. Tanaka.** 1997. Expression of alkaline protease gene in *Bacillus subtilis* mutants that lack positive regulatory genes *degR*, *degQ*, *senS*, *tenA* and *proB.* Biosci. Biotechnol. Biochem. **61:**372–374.
31. **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.
32. **Ogura, M., L. Liu, M. Lacelle, M. M. Nakano, and P. Zuber.** 1999. Mutational analysis of ComS: evidence for the interaction of ComS and MecA in the regulation of competence development in *Bacillus subtilis.* Mol. Microbiol. **32:**799–812.
32a. **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.
33. **Perego, M., and J. A. Hoch.** 1988. Sequence analysis and regulation of the *hpr* locus, a regulatory gene for protease production and sporulation in *Bacillus subtilis.* J. Bacteriol. **170:**2560–2567.
34. **Robertson, J. B., M. Gocht, M. A. Marahiel, and P. Zuber.** 1989. AbrB, a regulator of gene expression in *Bacillus*, interacts with the transcription initiation regions of a sporulation gene and an antibiotic biosynthesis gene. Proc. Natl. Acad. Sci. USA **86:**8457–8461.
35. **Sekiguchi, J., K. Akeo, H. Yamamoto, F. K. Khasanov, J. C. Alonso, and A. Kuroda.** 1995. Nucleotide sequence and regulation of a new putative cell wall hydrolase gene, *cwlD*, which affects germination in *Bacillus subtilis.* J. Bacteriol. **177:**5582–5589.
36. **Shafikhani, S. H., I. Mandic-Mulec, M. A. Strauch, I. Smith, and T. Leighton.** 2002. Postexponential regulation of *sin* operon expression in *Bacillus subtilis.* J. Bacteriol. **184:**564–571.
37. **Shahrestanifar, M., D. P. Saha, L. A. Scala, A. Basu, and R. D. Howells.** 1994. Cloning of a human cDNA encoding a putative nucleotide-binding protein related to *Escherichia coli* MinD. Gene **147:**281–285.
38. **Shimotsu, H., and D. Henner.** 1986. Construction of a single copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis.* Gene **33:**103–119.
39. **Smith, I.** 1993. Regulatory proteins that control late-growth development, p. 785–800. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C
40. **Stragier, P., C. Bonamy, and C. Karmazyn-Campelli.** 1988. Processing of a sporulation sigma factor in *Bacillus subtilis*: how morphological structure could control gene expression. Cell **52:**697–704.
41. **Strauch, M. A.** 1995. In vitro binding affinity of the *Bacillus subtilis* AbrB protein to six different DNA target regions. J. Bacteriol. **177:**4532–4536.
42. **Strauch, M. A.** 1995. AbrB modulates expression and catabolite repression of a *Bacillus subtilis* ribose transport operon. J. Bacteriol. **177:**6727–6731.
43. **Strauch, M. A., and J. A. Hoch.** 1993. Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. Mol. Microbiol. **7:**337–342.
44. **Strauch, M., V. Webb, G. Spiegelman, and J. A. Hoch.** 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. Proc. Natl. Acad. Sci. USA **87:**1801–1805.
45. **Strauch, M. A., G. B. Spiegelman, M. Perego, W. C. Johnson, D. Burbulys, and J. A. Hoch.** 1989. The transition state transcription regulator *abrB* of *Bacillus subtilis* is a DNA binding protein. EMBO J. **8:**1615–1621.
46. **Tanaka, T., and M. Kawata.** 1988. Cloning and characterization of *Bacillus subtilis iep*, which has positive and negative effects on production of extracellular proteases. J. Bacteriol. **170:**3593–3600.
47. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
48. **Turgay, K., M. Persuh, J. Hahn, and D. Dubnau.** 2001. Roles of the two ClpC ATP binding sites in the regulation of competence and the stress response. Mol. Microbiol. **42:**717–727.
49. **Vagner, V., E. Dervyn, and S. D. Ehrlich.** 1998. A vector for systematic gene inactivation in *Bacillus subtilis.* Microbiology **144:**3097–3104.
50. **Vitale, G., E. Fabre, and E. C. Hurt.** 1996. NBP35 encodes an essential and evolutionary conserved protein in *Saccharomyces cerevisiae* with homology to a superfamily of bacterial ATPases. Gene **178:**97–106.
51. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1:**945–951.
52. **Wang, L.-F., and R. H. Doi.** 1990. Complex character of *senS*, a novel gene regulating expression of extracellular-protein genes of *Bacillus subtilis.* J. Bacteriol. **172:**1939–1947.
53. **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.