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Inhibition of *Bacillus subtilis scoC* Expression by Multicopy *senS*

Eiji Kawachi, Sadanobu Abe, and Teruo Tanaka*

Department of Marine Science, School of Marine Science and Technology, Tokai University, 3-20-1 Shimizuorido, Shizuoka 424-8610, Japan

Received 27 July 2005/Accepted 25 September 2005

The *Bacillus subtilis aprE* gene, which encodes the extracellular alkaline protease, is regulated by many positive and negative transcriptional regulators. SenS is one such positive regulator consisting of 65 amino acids. We found that the *senS* gene on a multicopy plasmid, pSEN24, caused an increase in *aprE* expression in strains carrying the upstream region of *aprE* up to −340 with respect to the transcription initiation site but not in a strain carrying the region up to −299, which is within the binding site of the negative regulator ScoC (Hpr). Epistatic analysis showed that the pSEN24 effect was lost in a *scoC*-deleted mutant. In accordance with these results, the *scoC* transcription level as assayed by a *scoC-lacZ* fusion and Northern analysis was greatly reduced in the cells carrying pSEN24. From these results we conclude that multicopy *senS* enhances *aprE* expression by suppressing the transcription of *scoC*.

Bacillus subtilis secretes degradative enzymes after the end of logarithmic growth, apparently for degrading high-molecular-weight materials around the cell to cope with adverse nutritional conditions (17, 23). Among the extracellular proteases produced by this organism, the alkaline and neutral proteases constitute the major part of the protease activities. Since these enzymes are produced on the order of grams per liter, expression of the genes coding for the enzymes (*aprE* and *nprE*, respectively) has to be strictly controlled to avoid extravagant use of energy and materials. Thus, the *aprE* gene is regulated by many positive and negative regulators. The positive regulators include Spo0A, the DegS-DegU two-component system, DegR, DegQ, ProB, TenA, RelA, SalA, and SenS, while the negative regulators include AbrB, ScoC (previously called Hpr), SinR, and Pai (2, 9, 10, 20). Among these factors, AbrB, DegU, ScoC, and SinR are known to regulate *aprE* expression directly by binding to upstream regions of the *aprE* coding sequence (1, 4, 11, 22). Therefore, it can be summarized that the major regulatory pathways controlling *aprE* expression are the routes via AbrB, ScoC, SinR, and DegU. The positive effect of Spo0A is through inhibition of *abrB* expression (20), while those of DegR, DegQ, ProB, and TenA are through functional DegU (6, 8, 9, 15). It was shown recently that disruption of *salA* caused a decrease in *aprE* expression, and this was attributed to the enhanced synthesis of ScoC (10). In addition, the stringent factor RelA was shown to be required for the efficient expression of *aprE* (2). The mode of action of the *pai* gene product remains to be studied.

The DNA-binding transcription regulators, such as ScoC, SinR, DegU, and AbrB, have their own specific target sites in the control region of *aprE*, i.e., for ScoC, these are nucleotides (nt) spanning −324 to −267 and −79 to −14; for SinR, −268 to −220; for DegU, −164 to −113 and/or −70 to −27; and for AbrB, −59 to +25 (3, 4, 19, 22).

Wong et al. discovered a *B. subtilis* Natto gene, *senN*, which on a multicopy plasmid enhances the expression of *B. subtilis aprE* (26). The authors later identified the *B. subtilis* counterpart of *senN* named *senS* that also functions as a positive regulator of *aprE* expression. SenS is a positively charged, 65-amino-acid protein with a helix-turn-helix motif in the molecule (25). It was demonstrated that SenS exerts its positive effect by acting on the region between nt −177 and −415 upstream of the transcription start site of *aprE* (5), but further details, including the target of SenS and the relationship with the other transcriptional regulators, have not been investigated. In this study, we show that multicopy *senS* enhances *aprE* expression by reducing the expression of *scoC*.

Stimulation of *aprE-lacZ* expression by multicopy *senS*. To study the effect of SenS on *aprE* expression, we used pSEN24 in which the Shine-Dalgarno sequence and the following *senS* coding region were placed under the control of the isopropyl-1-thio-β-D-galactopyranoside (IPTG)-inducible P*spac* promoter (Table 1). The plasmid was constructed in two steps. A PCR fragment prepared with the primer pair SENSF (5′-AGTTAAGCTTATCGTTTAGATAAGGGCC-3′) and SENSR (5′-AGTTGTCGACAAAAACCCGTTGTAGTCAGC-3′) and *B. subtilis* CU741 DNA as a template was digested with HindIII and SalI (sites are underlined) and inserted into pDG148 that had been treated with the same restriction enzymes. The ligated sample was transformed into strain CU741 as described previously (13), and the resultant Nm^r transformants were screened for the ability to produce larger halos on casein- and gelatin-containing Luria-Bertani plates (24) than those produced by the transformants carrying pDG148. The 5′ end of the *senS* region on pSEN24 is the 51st nucleotide upstream of the *senS* coding sequence (25).

When pSEN24 was introduced into *B. subtilis* OAM145 carrying *aprE-lacZ* at the *amyE* locus and the cells were grown in the presence of 0.2 mM IPTG, the β-galactosidase activity increased 3.7-fold around T3 (3 h after the end of logarithmic growth) compared with that in OAM145 carrying the vector pDG148 (Fig. 1A).

It has been well studied that many positive and negative transcription factors of *aprE* exert their effects at specific regions upstream of the *aprE* gene (see above). To locate the *cis*-acting site of multicopy *senS* in the control region of *aprE*,

* 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: 81-543-34-0411, ext. 2933. Fax: 81-543-34-9834. E-mail: teruot@scc.u-tokai.ac.jp.

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

Strain or plasmid	Genotype or description[a]	Reference or source
Strains		
CU741	*trpC2 leuC7*	S. A. Zhaler
OAM145	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r)	10
OAM146	*trpC2 leuC7 amyE*::*aprE-lacZ*(−340 [SG35.21], Cm^r)	10
OAM147	*trpC2 leuC7 amyE*::*aprE-lacZ*(−299, Cm^r)	10
OAM218	*trpC2 leuC7 amyE*::*aprE-lacZ*(−267, Cm^r)	10
TT715	*trpC2 leuC7 aprE-lacZ* (Cm^r)	7
MU38	*trpC2 leuC7 amyE*::*degR-lacZ* (Cm^r) *mecA*::Km^r *degU*::Sp^r	12
TU38	*trpC2 leuC7 aprE-lacZ* (Cmr) *degU*::Sp^r	MU38→TT715
OAM157	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *scoC*::Em^r (*lacZ*::Tc^r)	10
OAM221	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *sinR*::Pm^r	10
OAM169	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412 [SG35.18], Cm^r) *spoOA*::Sp^r *abrB*::Km^r	10
TSU2	*trpC2 leuC7 amyE*::*scoC-lacZ* (Cm^r)	pSCO1→CU741
KAW1	*trpC2 leuC7 amyE*::*scoC-lacZ* (Cm^r) *spoOA*::Sp^r	OAM169→TSU2
Plasmids		
pDG148	Multicopy *B. subtilis* and *Escherichia coli* plasmid; Ap^r, Km^r	21
pSEN24	pDG148 carrying *senS*	This study
pIS284	*E. coli* plasmid for insertion of *lacZ* fusions into the *B. subtilis amyE* locus	I. Smith
pSCO1[b]	pIS284 carrying *scoC-lacZ* at the *amyE* locus	This study

[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; Ap^r, ampicillin resistance. The concentrations of the antibiotics added to the medium were 1 μg/ml for erythromycin, 5 μg/ml for chloramphenicol and phleomycin, 15 μg/ml for neomycin, 10 μg/ml for tetracycline, and 100 μg/ml for spectinomycin.

[b] Plasmid pSCO1 was constructed as follows. A 561-bp PCR fragment spanning nt 76 in the *yhaH* gene located immediately upstream of *scoC* to nt 102 in *scoC* was prepared with primers YhaHF (5′-AGTTGAATTCGCACCTTCCTCAGGAAAGC-3′) and ScoCR (5′-AGTTGGATCCTTCTCGATCGATTTCC-3′) and CU741 DNA as a template, digested with EcoRI and BamHI (sites are underlined), and inserted into the EcoRI and BamHI sites of pIS284.

we used *aprE-lacZ* fusions with various deletions upstream of the transcription initiation site of *aprE* (3, 10) and examined the effect of multicopy *senS* on *aprE-lacZ* expression. The OAM145 strain used above carries a region up to −412 relative to the transcription initiation site of *aprE* (Table 1). In strain OAM146, in which the upstream region of *aprE* is deleted up to −340, the stimulating effect of multicopy *senS* was still exhibited (Fig. 1B), but in OAM147 and OAM218, carrying deletions up to −299 and −267, respectively, the positive effect was lost (Fig. 1C and D). The results show that the SenS target is located in a region upstream of or including −299. Since, among the known *aprE* regulators, ScoC is the only known regulator whose target DNA region includes −299 (4, 10), the results suggested that SenS might affect *scoC* expression. It should be noted that the deletions up to −299 and −267 resulted in derepression of *aprE* (Fig. 1), probably because part or all of the *scoC* target sequence is deleted in these strains. A similar deletion effect was observed previously (3).

Effect of multicopy *senS* on *aprE-lacZ* expression in mutants affecting *aprE* expression. If *scoC* is the target of SenS, it is expected that the positive effect of multicopy *senS* would be lost in a *scoC*-deleted strain. To investigate this possibility and also the relationship between SenS and the transcription regulators that directly affect *aprE* expression, we studied the multicopy effect of *senS* by quantifying *aprE-lacZ* expression in strains with deletions of the *degU*, *scoC*, *sinR*, and *spo0A-abrB* genes. The results presented in Fig. 2 show that, under the condition where there was 2.5- to 8.5-fold stimulation by

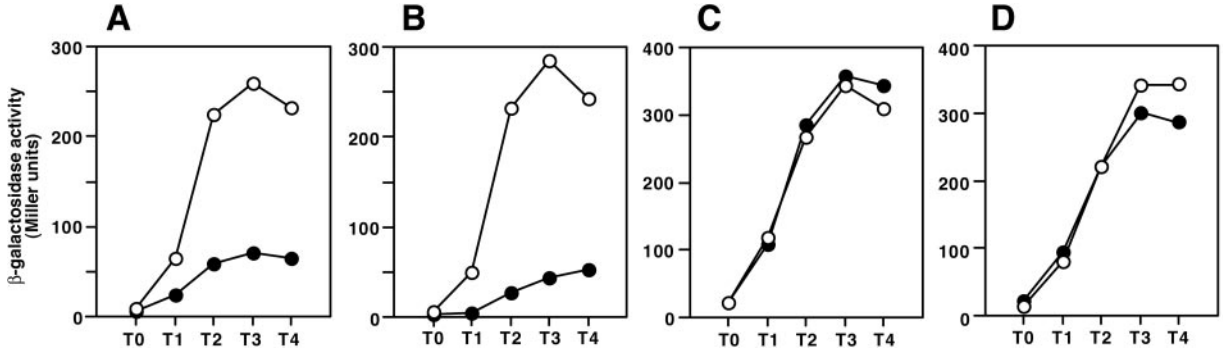

FIG. 1. Effects of deletions of the upstream region of *aprE* on stimulation of *aprE-lacZ* expression by multicopy *senS* on pSEN24. Deletions were up to −412 (A, strain OAM145), −340 (B, OAM146), −299 (C, OAM147), and −267 (D, OAM218). The solid and open circles indicate β-galactosidase activities in the cells carrying pDG148 and pSEN24, respectively. Cells were grown in Schaeffer's medium (18) containing 0.2 mM IPTG, and β-galactosidase activities were determined for the samples taken at the indicated times as described previously (10).

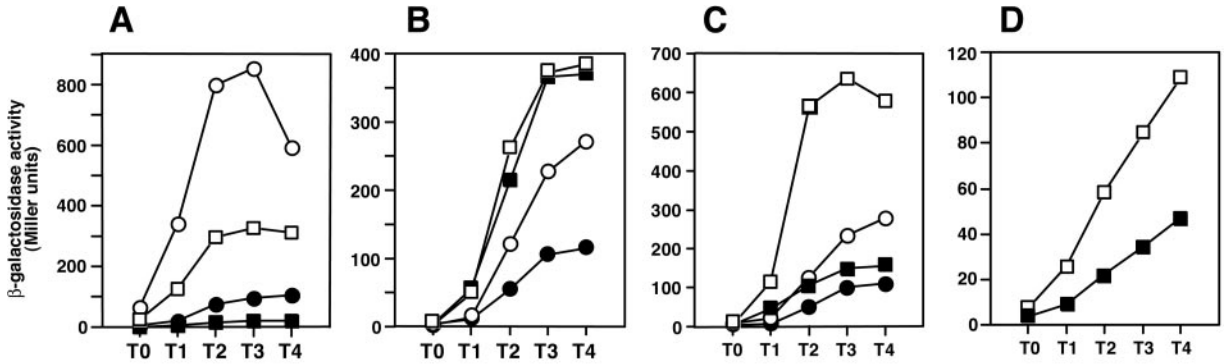

FIG. 2. Epistatic analysis of multicopy *senS* in mutants that affect *aprE-lacZ* expression. Cells were grown in Schaeffer's medium containing 0.2 mM IPTG. (A) ●, TT715 *degU*$^+$ (pDG148); ○, TT715 *degU*$^+$ (pSEN24); ■, TU38 *degU* (pDG148); □, TU38 *degU* (pSEN24). (B) ●, OAM145 *scoC*$^+$ (pDG148); ○, OAM145 *scoC*$^+$ (pSEN24); ■, OAM157 *scoC* (pDG148); □, OAM157 *scoC* (pSEN24). (C) ●, OAM145 *sinR*$^+$ (pDG148); ○, OAM145 *sinR*$^+$ (pSEN24); ■, OAM221 *sinR* (pDG148); □, OAM221 *sinR* (pSEN24). (D) ■, OAM169 *spo0A abrB* (pDG148); □, OAM169 *spo0A abrB* (pSEN24).

pSEN24 in the wild-type strains (compare circles), the enhancing effect was also observed in the *degU* (Fig. 2A), *sinR* (Fig. 2C), and *spo0A-abrB* (Fig. 2D) mutants but not in the *scoC* mutant (Fig. 2B) (compare squares). These results, together with those described in the previous section, support the notion that multicopy *senS* stimulates *aprE* expression through inhibition of *scoC* expression. The stimulatory effect of pSEN24 in strain TT715 (Fig. 2A) is somewhat stronger than that in strain OAM145 (Fig. 1A and 2B and C). This is probably due to the difference in the location of the *aprE-lacZ* fusions, i.e., the original *aprE* locus in TT715 and the *amyE* locus in OAM145 (Table 1), although the reason remains to be determined.

Inhibition of *scoC* expression by multicopy *senS*. We next investigated whether the enhancing effect of SenS on *aprE* expression is a result of inhibition of *scoC* transcription.

First, we constructed strain TSU2 carrying a *scoC-lacZ* fusion at the *amyE* locus and quantified the β-galactosidase levels in the cells harboring either pSEN24 or the pDG148 vector. As shown in Fig. 3A, the β-galactosidase activities in the pSEN24-carrying cells (open circles) were much lower than those in the cells carrying pDG148 (filled circles): the background β-galactosidase levels in strain CU741 lacking a *lacZ* fusion are also shown (diamonds).

It was shown previously that *scoC* expression reaches its highest level at the early stationary phase and that inactivation of *spo0A* results in overexpression of *scoC*, because *abrB*, a positive regulator of *scoC* expression, is negatively regulated by Spo0A (16). To test whether the AbrB-stimulated *scoC* expression is also reduced by multicopy *senS*, we introduced pSEN24 and pDG148 into strain KAW1 carrying a *scoC-lacZ* fusion in a *spo0A* background. It was shown that *scoC-lacZ* expression continued to increase after T1, which is in accordance with the previous result (16), and that this enhanced transcription was also inhibited by multicopy s*enS* on pSEN24 (Fig. 3B).

We further analyzed the effect of SenS on *scoC* expression by Northern analysis. RNA was prepared from strain CU741 carrying either pSEN24 or pDG148, and *scoC* mRNA was detected with a digoxigenin-labeled PCR fragment derived from a *scoC* coding region. As shown in Fig. 4, the intensity was lower in the RNA samples from the pSEN24-carrying cells than those from the cells harboring pDG148. The results sub-

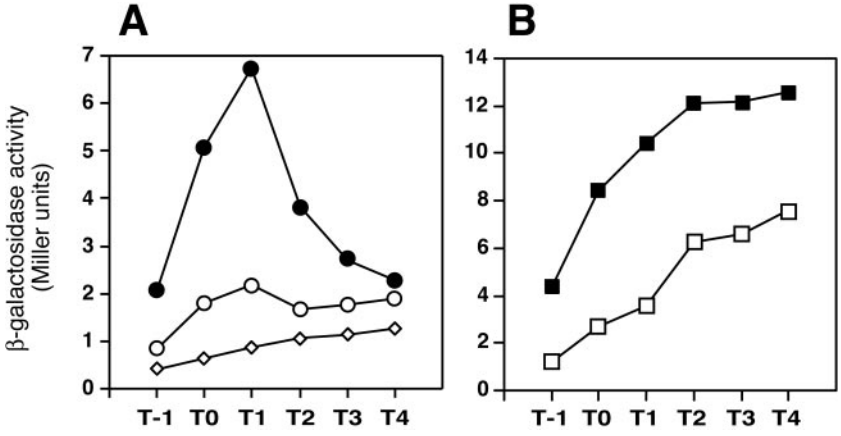

FIG. 3. Effect of multicopy *senS* on *scoC-lacZ* expression in wild-type and *spo0A* strains. Cells were grown in Schaeffer's medium containing 0.2 mM IPTG. The solid and open symbols indicate the values observed in strains carrying pDG148 and pSEN24, respectively. (A) TSU2 *spo0A*$^+$. (B) KAW1 *spo0A*. The diamonds in panel A indicate the values of the blank test with strain CU741.

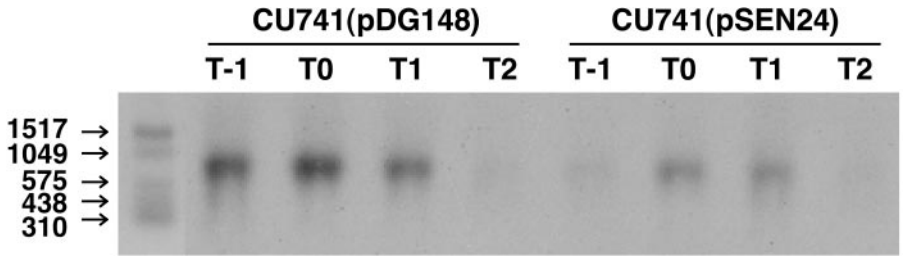

FIG. 4. Northern analysis of *scoC* mRNA in cells carrying the pDG148 vector and pSEN24. The leftmost lane shows the RNA markers, whose sizes are indicated by the numbers to the left of the panel. Strain CU741 carrying pDG148 or pSEN24 was grown in Schaeffer's medium containing 0.2 mM IPTG and harvested every hour from T − 1 to T2. RNA was prepared as described previously (27). The RNA samples (20 μg) were electrophoresed in a 1.2% agarose gel, and after the transfer of RNA to the membrane was verified by UV illumination, *scoC* mRNA was detected as described previously (10). A digoxigenin-labeled PCR fragment containing a coding region of *scoC* was prepared using a PCR DIG probe synthesis kit (Roche Diagnostics) with primer pair ScoC-1 (5′-ATCGAGTGGAACCGCCCTATGA-3′) and ScoC604R (5′-TTACAGGTTCGA GCTCTTCA-3′) and CU741 DNA as a template. The PCR was carried out by following the procedure provided by the supplier. The *scoC*-specific probe DNA was purified by agarose gel electrophoresis, and the specificity was confirmed by Southern analysis using DNAs from strains CU741 and OAM157. The size of *scoC* mRNA was determined by comparing with the size markers contained in RNA Molecular Weight Marker III obtained from Roche Diagnostics.

stantiate the *scoC-lacZ* fusion experiments described above. We note that the size of the *scoC* mRNA was around 750 nucleotides (Fig. 4), which is slightly larger than the *scoC* coding sequence (609 bp) (16). Since the size difference (around 140 bp) is less than the intergenic region (180 bp) between *scoC* and its upstream *yhaH*, it is likely that the transcriptional start site of *scoC* is within this region and *scoC* is transcribed in a single mRNA.

Binding of His-tagged SenS to the control region of *aprE*. It was predicted that SenS contains a DNA-binding, helix-turn-helix motif (24). We prepared a His-tagged SenS protein and subjected it to gel shift analysis with a DNA region spanning the C terminus of the upstream gene to an N-terminal region of *scoC*. Despite several attempts, however, no shifted band was detected.

The results described here show that multicopy *senS* stimulates *aprE* expression through inhibition of the expression of the negative regulator *scoC*. SenS is the first example that a positive regulator of *aprE* expression exerts its effect without the participation of DegU. However, since we failed to detect the binding of His-tagged SenS to the upstream region of *scoC*, it is not known at present whether the SenS effect on *scoC* expression is direct or via a second factor. An attempt was made to test the second possibility by microarray analysis, but no candidate was found (data not shown).

We have previously shown that *scoC* expression is regulated by SalA (9). It was shown, however, that the multicopy effect of *senS* on *scoC-lacZ* expression was still observed in a *salA*-deficient strain (data not shown), suggesting that SalA and SenS work in different pathways.

Although multicopy *senS* caused a decrease in *scoC* expression, resulting in overexpression of *aprE*, disruption of the chromosomal *senS* gene by insertion of the tetracycline resistance or neomycin resistance gene did not affect *aprE-lacZ* expression (data not shown), apparently indicating that the *senS* gene in a single-copy state is not expressed to a level that influences the expression of *aprE*. Although the SenS level may be low in the cells grown in the laboratory condition, i.e., growth in Shaeffer's sporulation medium, it is possible that the *senS* gene may play a role in some growth condition in the natural habitats of *B. subtilis*, the soil and the rhizosphere.

We have shown previously that a disruption of chromosomal *senS* by the spectinomycin resistance gene resulted in a decrease in *aprE-lacZ* expression (14). This negative effect on *aprE* expression, however, was observed only when *senS* was disrupted by the spectinomycin resistance gene in a specific orientation and was attributed to a secondary effect of spectinomycin resistance (data not shown).

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