***Bacillus subtilis* SenS exerts its activity through a site in the 5' flanking region of the *aprE* promoter**

PAULA M. MCCREADY† and ROY H. DOI*

Department of Biochemistry and Biophysics, University of California, Davis, CA 95616, USA

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The *Bacillus subtilis* gene *senS*, when present in high copy number, stimulates the expression of several extracellular protein genes during the onset of stationary phase, e.g. *aprE*. A novel integration vector, pINT, was constructed for transcription expression studies; it employed a unique method of promoter insert production for fusion with the *lacZ* reporter gene. Deletions were made of the 5' flanking region of the *aprE* promoter to localize the site responsible for SenS-mediated enhancement activity. pINT was used to translationally fuse *aprE* promoter deletion fragments with the *lacZ* reporter gene. A site between –177 and –415 with respect to the *aprE* start site of transcription was found to be required for the maximal SenS-mediated transcription increase from the *aprE* promoter. A multicopy vector containing the *senS* coding region without its native negative regulation was highly unstable in *B. subtilis*; this was due to the expressed *senS* insert.

Introduction

When *Bacillus subtilis* is subjected to an environmental stress, e.g. depletion of nutrients, it undergoes an ordered series of events which culminate in a heat-resistant spore. The expression of extracellular protein genes is closely associated with this phenomenon but not required for it. The regulation of the extracellular protein genes has been studied extensively and factors have been found which coordinately control their transcription (Gaur *et al.*, 1986; Higerd *et al.*, 1972; Lepesant *et al.*, 1972; Nagami & Tanaka, 1986; Pang *et al.*, 1991; Takagi *et al.*, 1990; Tanaka & Kawata, 1988; Wang & Doi, 1990; Yang *et al.*, 1986). The expression of the extracellular protease subtilisin has been used as a model in the study of the regulation of these genes. The gene encoding subtilisin, *aprE*, has been shown to be controlled by factors which also have an effect on sporulation (Gaur *et al.*, 1986; Perego & Hoch, 1988; Takagi *et al.*, 1990). Activity sites for the transcription factors AbrB and Sin have been mapped *in vivo* to the *aprE* 5' flanking region (Gaur *et al.*, 1991; Henner *et al.*, 1988). AbrB and Sin have been demonstrated to directly

bind *in vitro* to their *in vivo*-mapped sites on the *aprE* sequence (Gaur *et al.*, 1991; Strauch *et al.*, 1989) as well as to other promoter regions that are expressed in the later stages of cell growth (Strauch *et al.*, 1989).

SenS is a transcription factor which increases the expression of *aprE* during the onset of stationary phase when present in multiple copies (Wang & Doi, 1990). An increase in activity was also seen for other extracellular enzymes when multicopy *senS* was in the cell, e.g. neutral protease, amylase, and alkaline phosphatase (Wang & Doi, 1990). The purpose of this study was to show that SenS was acting on *aprE* at the level of transcription and to map a site(s) through which this activity was being exerted. Nested deletions were made of the *aprE* 5' flanking region and the corresponding fragments were ligated into the pINT integration vector. Integration strains demonstrated that SenS was in fact a transcriptional regulator and acted through a site upstream of the *aprE* promoter. SenS was also overproduced in *B. subtilis* and its effects on cell viability and *aprE* expression are discussed.

Methods

Strains. DB403 (*trpC2 npr aprE epr*) (Wang *et al.*, 1989) was used as host for *B. subtilis* DNA manipulations (Maniatis *et al.*, 1982), transformations (Anagnostopoulos & Spizizen, 1961) and integrations. XL-1 Blue (*recA1 endA1 gypA96 thi hsdR17 supE44 relA1 lac* {F' *proAB lac*^Q Δ M15, Tn10 (Tet^r)})) (Bullock *et al.*, 1987) was used for DNA

* Author for correspondence. Tel. (916) 752 3191; fax (916) 752 3085.

† Present address: Department of Microbiology, School of Medicine, Loma Linda University, Loma Linda, CA 92350, USA.

Abbreviation: SD, Shine–Dalgarno.

Fig. 1. Summary of vector constructions used in this study. SD, Shine-Dalgarno sequence; +1, start site of transcription.

manipulations and transformations in *Escherichia coli* (Cohen *et al.*, 1972). JM101 (*supE thi Δ(lac-proAB) {F' traD36 proAB lacI^q ΔM15}*) (Messing, 1979) was used as the *E. coli* host in which M13 cloning and single-stranded DNA preparation were carried out.

Media. Tryptose blood agar plates (TBAB; Difco) and 2 × SG (4% nutrient broth, 0.5 mM-MgSO₄, 6.7 mM-KCl, 1.0 mM-Ca(NO₃)₂, 0.1 mM-MnCl₂, 1.0 mM-FeSO₄, 0.1% glucose) sporulation agar plates were used for routine transformation and maintenance of *B. subtilis* strains. *B. subtilis* containing the integration constructs were selected

on TBAB containing 5 µg chloramphenicol ml⁻¹ and 100 µg X-Gal (US Biochemical) ml⁻¹. Transformants containing pWT-sen, pWL77 (Wang & Doi, 1990) and pWL95 were selected on TBAB containing 5 µg kanamycin ml⁻¹ and 100 µg X-Gal ml⁻¹. Cells for β-galactosidase assays were grown in 2 × SG liquid with and without selection.

DNA manipulations. Small-scale plasmid preparations were done by the rapid alkaline sodium dodecyl sulphate method (Rodriguez & Tait, 1983). Restriction enzymes and DNA modification enzymes were purchased from Bethesda Research Laboratories and Amersham and

used according to the recommendations of the manufacturers. DNA was sequenced by the chain-termination method (Sanger *et al.*, 1977) with Sequenase (US Biochemical) using single-stranded M13.

Plasmids. pWL95 contains a 620 bp fragment encompassing 240 bp of the *senS* 5' flanking region, promoter and coding region inserted into the *Hind*III site of pUB110. The promoter fragment for pEK-5 was derived from pEK112 (Park *et al.*, 1989) by digesting with *Hpa*I, ligating with *Sma*I linkers, and digesting with *Sma*I/*Eco*RI. The small fragment containing the *aprE* promoter and the subtilisin N-terminal sequence was ligated into the *Eco*RI/*Sma*I sites of pKS(−) (Stratagene), generating pEK-5. *aprE* deletions in pEK-5 were generated using *Bal*31 starting from the *Sma*I site, approximately 500 bp upstream from the start of transcription. Promoter deletions derived from pEK-5 were fused with the *lacZ* gene of pEK112 using the *Sca*I/*Sma*I fragments of both vectors. The large *Kpn*I/*Xho*I fragments were taken from the promoter deletion/*lacZ* fusion vectors and ligated with the large *Kpn*I/*Pvu*II fragment of pCOI, resulting in pCOI+7 (the 5' end of the *aprE* promoter fragment is at +7 with respect to the start of transcription), pCOI-60 (−60 with respect to the start of transcription) and pINT-500 (−500 with respect to the start of transcription). pCOI was constructed stepwise by cloning the ribosomal terminators, T1/T2 (Brosius *et al.*, 1981), on an *Eco*RI fragment into pKS(−) (Short *et al.*, 1988), giving pEK117. An *Eco*RI/*Sma*I fragment containing the *aprE* far 5' flanking region (−1600 to −800 with respect to the start of transcription) was cloned into the same site of pKS(−), resulting in pKW3. The *Sma*I/*Hind*III fragment of pKW3 was cloned into the *Hinc*II/*Hind*III site of pEK117, yielding pCOI. The large *Sma*I/*Not*I fragment of pINT-500 was ligated to the *Sma*I/*Not*I of pCOI and yielded pINT (Fig. 1).

***aprE* deletions.** pEK-5 was digested with *Eco*RI, treated with *Bal*-31 nuclease for 20 and 30 s, and the reaction stopped with EGTA. The samples were phenol extracted, and the aqueous phases were combined and GENECLAN purified. One A_{260} unit of *Eco*RI (dCCGGAATCCGG) or *Xba*I (dGCTCTAGAGC) linker was phosphorylated and the deleted pEK-5 DNA was ligated with either phosphorylated linker overnight at 14 °C. The ligation mix was digested with *Eco*RI or *Xba*I and ligated.

Polymerase-amplified deletions. Primers were synthesized (Pharmacia Gene Assembler) which were homologous to three parts of the *aprE* 5' flanking region. PM34 (5'-dGCCCCGGGAATTCGCATGACAT-TTCAGCATAATG-3') defined the −177 endpoint, PM33 (5'-dGCC-CCGGGAATTCATCACGATAATATCCATTGTT-3') defined the −280 endpoint, and PM41 (5'-dGCGCCCGGGAATTCGGCGG-CGCATCTGATG-3') defined the −415 endpoint. The primer for the opposite strand used in the amplification was the M13 universal primer (5'-dGTAAAACGACGGCCAGT-3'). The *aprE* promoter deletion fragments were polymerase-amplified using the above primers as described previously (Innis & Gelfand, 1990). The resulting fragments were digested with *Sma*I and ligated into *Sma*I-digested pINT.

The primers PM38 (5'-dCGCGGATCCTGAATACGGAAAA-CG-3') and PM39 (5'-dCGCGGATCCCGGGAGGTGATCATTATTGATGGGAGTCAAAAAGA-3') were used to polymerase-amplify the *senS* coding region, using pWL77 as the template. The recovered fragment was *Bam*HI digested and cloned into the *Bam*HI site of pWT18.

β -Galactosidase activity. β -Galactosidase activity was measured as described by Miller (1972). All assays were repeated a minimum of three times.

Southern blot analysis. Southern blot analysis was carried out according to the protocol for the membrane (Hybond, Amersham). Probes were labelled using a random DNA priming kit (Multiprime DNA Labeling System, Amersham).

Results

Construction of integration vector

The integration vector, pINT, was constructed for transcription expression studies in *Bacillus subtilis*. The vector was designed to be integrated into the chromosome through a single or double crossover event for the monitor gene (*lacZ*) to be stably expressed. The integration site used was the *aprE* upstream sequence between −1600 and −800 with respect to the *aprE* transcription start site. The vector includes the chloramphenicol resistance marker for screening of integrated strains and the *E. coli* bidirectional ribosomal terminators (T1/T2) were also cloned immediately downstream of the *aprE* far 5' to ensure that no readthrough was occurring from the 5' sequences. Promoters of interest were cloned just downstream of the terminators and fused in-frame to the eighth codon of the *lacZ* cartridge via a *Sma*I site (Fig. 1).

When a fragment containing a Shine–Dalgarno (SD) sequence and an AUG start codon was blunt-end ligated in-frame to the *lacZ* of pINT at the *Sma*I site, colonies of *E. coli* containing the construct turned dark blue in the presence of X-Gal. This facilitated the screening of large numbers of colonies.

Promoters of interest were initially cloned into a vector with universal and reverse primer sites adjacent to the polylinker, e.g. the pKS or pUC vectors. A primer was synthesized which contained a restriction site, the appropriate number of bases for in-frame fusion to the *lacZ* sequence, the complement of an AUG start codon, and the complement of an SD sequence. The 3' end of the primer was homologous to the universal or reverse primer site and used as one of the primers in a polymerase amplification reaction. This method of insert production for ligation into the pINT vehicles was successfully used for many promoter fragments and required the synthesis of only one extended primer.

Deletion analysis of the *aprE* 5' flanking region

pCOI+7 was constructed by the insertion of the *aprE* sequence between +7 and +60 (with respect to the start of transcription) into the pINT vector. This inserted fragment contained the SD sequence, and the AUG start codon, but was without a promoter. The ability of the translation signals within pCOI+7 to translate the β -galactosidase reporter gene was confirmed in *E. coli*. pCOI+7 has also been used in other studies with several promoter fragments without translation signals.

pINT-500 contained the fragment of the *aprE* promoter from −500 to +60 bp in front of the *lacZ* cassette of pINT and contained the most 5' flanking sequence of all the *aprE* inserts used in this study. pINT-500

contained all the signals relevant to regulation of the *aprE* promoter (Henner *et al.*, 1988).

Several deletions of the *aprE* promoter region were generated using the nuclease Bal-31 to localize a SenS-specific site of activation. The digestion started at -500 and a nested set of deletions was isolated down to $+7$. Deletion endpoints directly flanking the DegU/DegS target sites (-141 and -164) (Henner *et al.*, 1988) could not be isolated. Therefore primers were synthesized and the endpoints were amplified by PCR. The *aprE* promoter fragments were blunt-end ligated into the *Sma*I site of pINT. All promoter deletions were sequenced in order to define the exact endpoints generated and to confirm the primary sequence. The number of integrated constructs on the chromosome of each strain was determined by Southern blot analysis and confirmed to be one copy.

β -Galactosidase activity assay

The β -galactosidase activity of the recipient, *B. subtilis* DB403, containing the chromosomal *senS* gene or the *senS* gene in 30–50 copies per cell was less than 1 Miller unit during all phases of growth. This value was considered the background activity of the strain. The pCOI+7 'no promoter' integration strain with single or multiple copies of *senS* had activity profiles which were the same as that of the parental DB403 strain. This demonstrated that no readthrough transcription into *lacZ* was originating from upstream of the reporter, verifying that this site on the chromosome was transcriptionally silent.

The pINT-derivative constructions containing the promoter deletions were integrated into DB403 and the resulting strains were then transformed with the multicopy plasmid pWL77, which carries the *senS* gene and its promoter. These strains were assayed for β -galactosidase activity in order to localize a site at which the SenS activity was being exerted. The strain containing the pINT-500 integration with only the chromosomal copy of *senS* had activity of 1–2 Miller units during the vegetative phase of growth and showed a peak of 21 Miller units at the beginning of the stationary phase. The pINT-500 integration strain with pWL77 also had 1–2 Miller units of activity during the vegetative phase, increasing to 155 Miller units during the onset of the stationary phase. The pINT-500 strain with the *senS* gene in multiple copies had a seven- to eightfold greater activity than the strain without the *senS* gene in multiple copies. The timing of the *aprE* induction event in the strain with the *senS* gene in multiple copies was consistently 1 h earlier than in the strain without the multicopy *senS* gene. The integrated construct with 5'

Fig. 2. β -Galactosidase activity of the *aprE* promoter integrations with the *senS* gene in single copy and in multiple copies. $\diamond$, Growth curve; $\bullet$, pCOI+7 integration strain activity with *senS* in multiple copies; $\blacktriangle$, $\triangle$, pINT-500 integration strain activity with *senS* in single copy ($\blacktriangle$) and multiple copies ($\triangle$); $\blacksquare$, $\square$, pCOI-60 integration strain activity with *senS* in single copy ($\blacksquare$) and multiple copies ($\square$).

flanking sequence out to -415 , pINT-415, and the strain containing pINT-500 behaved identically.

The pCOI-60 (the -60 endpoint) integration strain containing only the chromosomal copy of *senS* had a vegetative activity of 1–2 Miller units, which increased during the onset of the stationary phase to 42 Miller units. There was no change in the timing of induction for the pCOI-60 strain with a single copy of *senS*. The pCOI-60 integration strain with plasmid harbouring the *senS* gene (pWL77) had the same vegetative-phase activity and showed an activity of 74 Miller units during the onset of the stationary phase (Fig. 2). Induction of the pCOI-60 integration strain with pWL77 was 1 h earlier than for the same strain with the *senS* gene in single copy. The pCOI-60 integration strain containing only the chromosomal copy of *senS* showed a twofold increase in stationary-phase activity over the integration strain containing the -500 endpoint. This was previously observed when regions between positions -340 and -244 were deleted (Gaur *et al.*, 1991; Henner *et al.*, 1988). The strains containing the *aprE* promoter with 5' flanking sequence out to -177 from the transcription start site gave the same results as pCOI-60.

The integration strain with the construct containing the 5' flanking sequence out to -415 showed a seven- to

Table 1. Summary of *aprE* deletion endpoint integration strains and the corresponding β -galactosidase activity

The *lacZ*-proximal endpoints of the deletions are fixed. The values in the last column were derived by dividing the values from *senS* in multicopy strains by the values from *senS* in single copies.

5' Distance from start of transcription	<i>aprE</i> expression* in presence of:		Increase (-fold) with multicopy <i>senS</i>
	Single-copy <i>senS</i>	Multicopy <i>senS</i>	
+7	0	0	0
-60	42	75	~2
-112	50	80	~2
-177	43	72	~2
-415	23	164	~8
-500	21	168	~8

* The β -galactosidase activity values are given in Miller units.

eightfold increase in activity, which contrasted with the 1.5–2-fold activity demonstrated by the strain with 5' sequence out to -177. This clearly demonstrated the existence of a SenS activity site between -177 and -415 (Table 1). The 1.5–2-fold greater activity of the -60 endpoint integration when *senS* was present in multiple copies versus when *senS* was present as a single copy indicated the presence of a second weak activity site between -60 and +79. These data confirm that *senS* is exerting its effect on the *aprE* gene at the level of transcription.

It has been shown that the *senS* upstream sequences are highly repressive, allowing only small amounts of transcript to be made when present in multiple copies (Wang & Doi, 1990). In order to produce more SenS protein molecules *in vivo*, the plasmid pWT-*sen*, which contains the strong vegetative promoter of the *rpoD* operon preceding an efficient SD sequence (Band & Henner, 1984) and the *senS* coding region without promoter region, was constructed. The *senS* insert of pWT-*sen* was found to be very unstable during the exponential phase when bacteria were grown without selection, in liquid medium, at 37 °C. Therefore, investigation of these strains was pursued using cleavage of X-Gal on solid medium with selection at 25 °C.

The parental integration strain, the integration strain with pWL77, and the integration strain with pWT-*sen* were gridded from freshly streaked plates onto TBAB and 2 × SG with X-Gal and 5 µg kanamycin ml⁻¹ and incubated at 25 °C overnight. The strain carrying pWT-*sen* turned very dark blue 2–3 h before the strain containing pWL77 started to appear blue, indicating stronger expression from the *aprE* promoter in the

presence of pWT-*sen*. The strain containing the *aprE* promoter with 177 bp of 5' flanking region and pWT-*sen* had similar expression patterns as the same deleted strain containing pWL77. These data again confirm that the 5' SenS activity site is required for the stimulatory effect of the *aprE* promoter.

Discussion

The activity sites of several transcription factors have been mapped to the *aprE* 5' flanking region (Gaur *et al.*, 1991; Henner *et al.*, 1988; Strauch *et al.*, 1989). Some of these factors have been shown to require the DegU/DegS activity site located between positions -141 and -164 in the 5' flanking region, e.g. DegQ (Henner *et al.*, 1988). SenS was shown to require a site between 20 and 40 bp upstream of the DegS/DegU site for full activity, suggesting that SenS may act differently from the other small regulatory polypeptides, such as DegQ.

The SenS-mediated enhancement of *aprE* transcription was shown to require a site located between -177 and -415 with respect to the start site of transcription. Both Hpr (Kallio *et al.*, 1991) and Sin (Gaur *et al.*, 1991) have been shown to bind within this region of DNA, at -267 to -324 and -220 to -268, respectively. In addition, there are indications that a site between -60 and +79 is active at a low level when SenS is overproduced. This also corresponds with the second region of DNA protected by Hpr, at -79 to -59 and -35 to -14 (Kallio *et al.*, 1991), and a region bound weakly by Sin *in vitro*, from -200 to +281 (Gaur *et al.*, 1991). It would therefore be reasonable to postulate that SenS may be interacting with Hpr or Sin at some level. Overproduction of SenS may be repressing the *hpr* or *sin* gene, leading to increased *aprE* expression. This possibility could be addressed by monitoring the expression of the *hpr* and *sin* genes when SenS is overproduced. The effect on *aprE* of overproducing SenS could also be examined in strains carrying deletions of the *sin*, *hpr*, *degS/degU* or *abrB* genes. This would distinguish whether or not the SenS effect is independent of these regulators. SenS may be negatively regulating the Hpr or Sin proteins, resulting in a stimulatory effect on *aprE* expression. The binding of the SenS factor to the DNA may exclude the binding of either or both of these proteins at their target sites in the *aprE* upstream. More detailed *in vivo* and *in vitro* analysis is required to clarify this matter further.

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