Cloning and Nucleotide Sequence of senN, a Novel ‘Bacillus natto’ (B. subtilis) Gene That Regulates Expression of Extracellular Protein Genes

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A new ‘Bacillus natto’ gene, senN, that regulates the expression of several extracellular proteins in B. subtilis has been cloned and sequenced. senN codes for a small, highly basic protein with an amino acid sequence different from the products coded by the regulatory genes sacQ, sacV, prtR and hpr. SenN stimulates gene expression at the transcriptional level. A putative homologous locus has been detected in the B. subtilis chromosome by Southern blotting.

INTRODUCTION

Several mutations in regulatory genes have been reported which cause a higher expression of extracellular proteins in ‘Bacillus natto’ (a variant of B. subtilis) and other Bacillus species. These include hpr (Higerd et al., 1972), sacU (Lepesant et al., 1972), sacQ (Amory et al., 1987; Lepesant et al., 1972; Yang et al., 1986), sacV (Martin et al., 1987) and prtR (Nagami & Tanaka, 1986; Tanaka et al., 1987; Yang et al., 1987). Most of these genes code for small proteins containing from 46 to 65 amino acid residues. The products of sacQ, sacV and hpr, when present in high quantities, stimulate the expression of genes at the transcriptional level. On the other hand hpr appears to encode a negative regulatory factor that represses gene expression (M. Perego and J. A. Hoch, personal communication). The pleiotropic effects of mutations in these genes suggest the existence of a rather general mechanism for regulating the synthesis of extracellular proteins.

We report in this paper the cloning and partial characterization of a novel ‘B. natto’ gene, senN, that regulates the expression of several extracellular protein genes.

METHODS

Bacterial strains, plasmids and media. Table 1 lists the B. subtilis strains used in this study. ‘B. natto’ strain BOSC 27A1 [amyE(+) amyR2 pro(H)] was used as a source for constructing a ‘B. natto’ chromosomal DNA library in plasmid pUBH. This is a pUB110 derivative containing the polylinker from pUC9 in place of the original EcoRI–BamHI region and was constructed by F. Kawamura in our laboratory. DB102 (see Table 1) was initially used as the recipient for the screening of genes which can enhance the production of subtilisin (aprA). pUB18 is a derivative of pUB110 containing the pUC18 polylinker in place of the EcoRI–PstI fragment (nucleotides 1–1035, see McKenzie et al., 1986) in the original pUB110 plasmid.

Protoplast transformants were selected on DM3 agar plates (l-1: 200 ml 4% w/v, agar; 500 ml 1 M-sodium succinate, pH 7.3; 100 ml 5% w/v, Casamino acids; 50 ml 10% w/v, yeast extract; 100 ml 3.5% w/v, K₂HPO₄ and 1.5%, w/v, KH₂PO₄; 25 ml 20%, w/v, glucose; 20 ml 1 m-MgCl₂; and 5 ml filter-sterilized 2% w/v, BSA) (Chang & Cohen, 1979). The sporation medium SG (l-1: 8 g nutrient broth; 1 g KCl; 0.25 MgSO₄; 1 ml 1 m-Ca(NO₃)₂; 1 ml 1 m-FeSO₄; 1 ml 0.1 m-MnCl₂; and 2 ml 50% w/v, glucose) was as described by Leighton & Doi (1971).

Preparation of plasmid and chromosomal DNA. Small scale plasmid preparation was done by the rapid alkaline sodium dodecyl sulphate (SDS) extraction method (Rodriguez & Tait, 1983). Large scale purification of plasmid

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>DB2(pUBHR)</td>
<td>DB2 containing pUBHR</td>
<td>This work</td>
</tr>
<tr>
<td>DB2(pUBHR-N1.8)</td>
<td>DB2 containing pUBHR-N1.8</td>
<td></td>
</tr>
<tr>
<td>DB102(pUBHR-N1.8)</td>
<td>DB102 containing pUBHR-N1.8</td>
<td></td>
</tr>
<tr>
<td>DB104(pUBHR-N1.8)</td>
<td>DB104 containing pUBHR-N1.8</td>
<td></td>
</tr>
<tr>
<td>MT221(pUBHR)</td>
<td>MT221 containing pUBHR</td>
<td></td>
</tr>
<tr>
<td>MT221(pUBHR-N1.8)</td>
<td>MT221 containing pUBHR-N1.8</td>
<td></td>
</tr>
<tr>
<td>MT441(pUB18)</td>
<td>MT441 containing pUB18</td>
<td></td>
</tr>
<tr>
<td>MT441(pUBHR-N1.8)</td>
<td>MT441 containing pUBHR-N1.8</td>
<td></td>
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</tbody>
</table>

was done by CsCl/ethidium bromide density gradient centrifugation (Davis et al., 1980). Chromosomal DNA from *'B. natto'* and *B. subtilis* was isolated according to Rodriguez & Tait (1983).

**Southern blot analysis.** Chromosomal DNA from *'B. natto'* and *B. subtilis* was digested by various restriction enzymes and then separated on a 0.6% agarose gel with Tris/borate-EDTA buffer by electrophoresis. The resolved DNA was depurinated and transferred to nitrocellulose by capillary blotting (Davis et al., 1980) overnight. The filter paper, after baking at 80°C for 2 h in a vacuum oven, was prehybridized with a solution containing 6 x SSC (20 x SSC contains 3 m-NaCl, 0.3 m-sodium citrate, pH 7.0, 0.5% SDS, 5 x Denhardt mix (50 x Denhardt mix contains 1% (w/v) each of BSA, Ficoll, and polyvinylpyrrolidone) and 100 µg ml⁻¹ sheared, heat denatured salmon sperm DNA at 65°C for 4 h and then hybridized in the hybridization mix (same composition as prehybridization mix with the addition of EDTA to a final concentration of 0.01 M and 3 x 10⁻⁶ c.p.m. of 32P-labelled probe) for 20 h at 65°C. The 1.8 kb EcoRI fragment from *'B. natto'* was used as the probe and labelled by nick translation. The filter was washed sequentially with 2 x SSC containing 0.5% SDS, and 2 x SSC containing 0.1% SDS at room temperature for 5 and 15 min, respectively. The filter was then washed twice in 0.1 x SSC containing 0.5% SDS at 65°C for a total of 2 h with the final wash in 2 x SSC at room temperature for 15 min. The air-dried filter was autoradiographed overnight.

**Enzyme assays.** Proteases present in the culture supernatant were assayed using the Hide powder azure method as described previously (Wang & Doi, 1987). The production of subtilisin from strain DB102 (*his nprR2 nprE18*) was assayed directly whereas the neutral protease activity produced by DB2 (*trpC2*) was assayed in the presence of 6 mM-phenylmethylsulphonyl fluoride (PMSF). The activities of alkaline phosphatase and α-amylase present in the DB104 culture were determined by the methods described by Torriani (1968) and Yamane et al. (1973), respectively.

**Other methods.** Nick translation kits, restriction enzymes, and calf intestinal alkaline phosphatase were purchased from Bethesda Research Laboratories and Boehringer Mannheim Corp. All were used according to the manufacturer’s specifications. Restriction mapping of the cloned *senN* gene was done by the double digestion method (Danna, 1980).

**RESULTS**

**Molecular cloning of the senN gene in *B. subtilis***

Purified *'B. natto'* chromosomal DNA was completely digested by EcoRI and ligated to the EcoRI-digested, alkaline-phosphatase-treated pUBHR plasmid in a 3:1 ratio (w/w). The ligation mixture was then transformed into *B. subtilis* DB102 by the protoplast method (Chang & Cohen, 1979). The transformants selected on DM3 agar plates containing 100 µg kanamycin ml⁻¹ were transferred to SG agar plates containing 1% (w/v) skim milk and 5 µg kanamycin ml⁻¹. Among about 2500 transformants, six showed enhanced production of subtilisin as judged by the presence of bigger haloes surrounding the colonies. Restriction mapping of the plasmid DNA isolated from these clones indicated that the plasmids all carried the same EcoRI 1.8 kilobase pairs (kb) insert. This fragment was named the 'N' fragment ('N' stands for 'natto')
Novel 'B. natto' regulatory gene, senN

Fig. 1. (a) Restriction map of the 'B. natto' senN region. (b) Deletion mapping to locate the senN gene within the 1.8 kb EcoRI fragment. Plasmids pND1-pND5 contain different deletions; these are indicated by parentheses. After the plasmids were transformed into DB102, each strain was tested for Sen activity. The results are shown in the right-hand column. The shaded region in (a) indicates the putative coding region deduced from the deletion mapping studies.

Table 2. Stimulatory effect of senN on the production of some extracellular enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Stimulation (-fold)</th>
</tr>
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<tbody>
<tr>
<td>Alkaline protease (subtilisin)</td>
<td>2.7</td>
</tr>
<tr>
<td>Neutral protease</td>
<td>3.7</td>
</tr>
<tr>
<td>a-Amylase</td>
<td>1.9</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>1.8</td>
</tr>
</tbody>
</table>

The degree of stimulation for each enzyme was calculated from the peak specific activity during the time course of expression obtained from strains DB2, DB102 or DB104 carrying pUBHR-N1.8 divided by the specific activity from the same strains carrying the control plasmid pUBHR at the same growth stages, which were 3 to 4 h after the onset of sporulation in sporulation medium 2 × SG (Leighton & Doi, 1971).

while the gene on this N fragment that is responsible for the enhanced production of subtilisin was named 'sen' for 'subtilisin (secretion) enhancer'. The plasmid containing this fragment was named pUBHR-N1.8.

The restriction map of the N fragment was determined by the double digestion method and the results are shown in Fig. 1 (a). In order to further localize the senN gene within this fragment, deletion mapping was done, either by removing a certain region from pUBHR-N1.8 or by subcloning shortened fragments into pUB18. The Sen phenotype was then tested by examining their stimulatory effect on aprA expression in DB102. The results are presented in Fig. 1 (b). Removal of the left arm of the fragment by EcoRI/EcoRV digestion (pND1) or the right arm by BamHI/EcoRI digestion (pND5) was found not to affect the enhancing activity. These deletions (see Fig. 1b) suggested that the senN gene was localized within an EcoRV–BamHI fragment of about 600 bp in length. SstI digestion of the N fragment split the fragment into two. Neither the left arm (pND2) nor the right arm (pND4), when subcloned into the pUBHR plasmid, was able to enhance subtilisin production.

Effect of multicity senN on the production of several extracellular enzymes

When it was initially screened for, the senN gene was present in high copy number and the production of subtilisin in B. subtilis was stimulated 2.7-fold. In order to know whether this Sen effect was specific to aprA or, like other stimulatory genes such as sacQ and prtR, whether it also affected other extracellular protein production, the expression of neutral protease, alkaline phosphatase and a-amylase in the presence and absence of high copy 'B. natto' senN (in plasmid pUBHR-N1.8) was examined. The results are summarized in Table 2. The stimulatory effect of
Fig. 2. Nucleotide and deduced amino acid sequence of the 'B. natto' senN gene. The DNA sequence of the coding strand is given from 5' to 3', numbered from nucleotide 1 at the EcoRV site. The deduced amino acid sequence is given below the DNA sequence with the N-terminal methionine as residue number 1. Locations of important restriction sites are shown above the DNA sequence. The putative promoter site, ribosomal binding site (SD) and -independent transcription terminator are underlined.

**senN** was not specific only to aprA gene expression: it stimulated nprE expression even more (3-7-fold). However, compared to the stimulatory effects of high copy numbers of sacQ, sacV or prtR, which are in the order of 10-40-fold, the stimulation by senN was much less. We do not know whether this is due to the inherent properties of the senN product or whether the expression of senN itself is regulated in such a way that its full expression could not be achieved under the conditions of our tests.

**Nucleotide sequence analysis of the senN region**

The DNA sequence of the EcoRV-BamHI fragment (the shaded region in Fig. 1) was determined for both strands by the dideoxy method of Sanger et al. (1977) using M13mp18 and 19 vectors. The results are shown in Fig. 2. The base sequence revealed an open reading frame that encoded a 60 amino acid protein with an $M_\text{r}$ of 7219. The direction of translation of this putative senN gene is consistent with the finding that readthrough promoter activity from the pUBHR plasmid could increase the Sen effect in one orientation (from EcoRV to BamHI, the same orientation as for the transcription of the kanamycin resistance gene in the plasmid) but not in the other (data not shown). The deduced protein product is highly charged (44% charged amino acids), very basic (29% basic amino acids) and lysine-rich (18% lysine residues). There is no amino acid homology of SenN with PrtR (Nagami & Tanaka, 1986; Yang et al., 1987), SacQ (Amory et al., 1987; Yang et al., 1986), SacV (Martin et al., 1987) or Hpr (M. Perego & J. A. Hoch, personal communication). The putative translation initiation codon (ATG) was preceded by a strong ribosomal binding site ($AG' = -25.0$ kcal mol$^{-1}$; $-104.6$ kJ mol$^{-1}$). senN was flanked by typical prokaryotic transcription-initiation and -termination signals (underlined in Fig. 2). The putative promoter site was determined in vivo by inserting fragments upstream of
Novel ‘B. natto’ regulatory gene, senN

Fig. 3. Determination of the senN promoter region in vivo. The upper part of the figure is a physical map of the senN region indicating the location of the putative promoter (arrow), open reading frame (shaded region) and terminator (stem-loop) of the senN gene. The fragments used to probe for promoter activity in pWP19 are indicated by the solid lines below with the arrow head indicating the direction toward the reporter gene (aprA) in the promoter probe vector pWP19 (Wang & Doi, 1987). The promoter activity of these constructs was monitored on SG skim milk plates as described previously (Wang & Doi, 1987). pWP-H1 was constructed by a partial HindIII digestion. The results on the right side indicate that only the insert in clone pWP-S4 shows promoter activity, suggesting that the promoter was located between HindIII and Sau3A or overlapped the HindIII site.

the open reading frame into a promoter probe plasmid pWP19 (Wang & Doi, 1987). The results clearly indicated that the promoter activity lay within the HindIII–Sau3A region or spanned the HindIII site (Fig. 3). There is a putative terminator site at the 3' end of the open reading frame as revealed by sequence analysis (Fig. 3); this region (SsrI–BamHI) functions as a terminator in both orientations when tested in vivo using the terminator probe plasmid pWT19 (Wang & Doi, 1987) (data not shown).

senN regulates gene expression at the level of transcription

High copy numbers of senN caused the stimulation of expression of neutral protease, subtilisin, alkaline phosphatase and α-amylase (see Table 2). Time-course analyses of these genes in the presence and absence of high copy numbers of the senN gene indicated that the timing of expression was not changed, rather the levels of expression were enhanced (data not shown). The mechanism of senN stimulation of the subtilisin gene was studied further by using the constructs of Tanaka et al. (1987), in which the effect was examined of a high copy number of senN on the expression of the extracellular β-lactamase gene controlled by either a promoter for subtilisin (aprA, strain MT221) or a promoter for an intracellular protein gene (dfrA, strain MT441) (Tanaka et al., 1987). The results (Fig. 4) showed that senN only stimulated expression of the β-lactamase gene when it was controlled by the subtilisin promoter. The results also confirmed our previous observation that the effect of SenN does not change the timing of the temporal expression of the subtilisin promoter. Two other promoters for intracellular protein genes, the promoters for the sigA (rpoD) operon (Wang & Doi, 1987) and the citG gene (Moir et al., 1984), were not stimulated by SenN when tested in vivo in similar constructs (data not shown). Thus the SenN effect is exerted at the transcriptional level and so far has only stimulated the expression of extracellular protein genes. Furthermore these results indicated that SenN was not regulating a secretory function of the cell.
Fig. 4. Effect of SenN on promoters for an extracellular and an intracellular protein gene. B. subtilis strains MT221 (his nprR2 nprE18 aprA3 apr::pLK221) (a) and MT441 (his nprR2 nprE28 aprA3 dfmA::pKDFl) (b), carrying pUB18 (a derivative of pUB110 containing pUC18 polylinker, constructed by L.-F. Wang) or pUBHR-N1.8 were grown in 2× SG (Leighton & Doi, 1971) liquid culture containing chloramphenicol and kanamycin (each 5 μg ml⁻¹) for analysis of the time course of expression. The β-lactamase activity [μmol substrate consumed min⁻¹ ml⁻¹ (Klett unit)⁻¹] present in the culture supernatant at different growth stages was assayed as described (Tanaka et al., 1987). ○, ○, β-lactamase activity of strains carrying pUBHR-N1.8 and pUB18, respectively. The subscripts of t represent time in hours after the initiation of sporulation.

Detection of a senN homologue in B. subtilis

In order to see whether a sen homologue also exists in B. subtilis, Southern blot analysis was done for B. subtilis DB2 (trpC2) strain using the ‘B. natto’ 1-8 kb EcoRI fragment as probe. When the DB2 chromosomal DNA was completely digested with EcoRI and hybridized to the probe, a 2-8 kb band was detected under stringent conditions (Fig. 5), indicating that the B. subtilis chromosome has a senS (‘S’ for ‘subtilis’) region very homologous to the ‘B. natto’ senN region. Cloning and characterization of the senS locus and the investigation of the Sen stimulation mechanism are in progress and will be reported separately.

DISCUSSION

A new regulatory gene, senN, has been cloned from ‘B. natto’, which is similar to but not identical with the previously reported regulatory genes such as sacQ, sacU, sacV, prtR and hpr. Its mode of action appears to be related to that reported for prtR (Tanaka et al., 1987; Yang et al., 1987), sacQ (Amory et al., 1987; Yang et al., 1986) and sacV (Martin et al., 1987), since a high copy number of the gene increases the production of several extracellular enzymes. However, multiple copies of senN do not have as dramatic a stimulatory effect as high copy numbers of the other reported genes. This may be related to its inherent function, to a relatively weak promoter, or to other regulatory elements that prevent high expression of the gene even in high copy number. The results with prtR showed that the amount of stimulation by this gene at the same copy number varied from 8- to 100-fold depending on the promoter utilized to express the indicator gene (Yang et al., 1987). As with the other regulatory genes, the senN gene seems to function at the level of transcription. It is significant that a family of similar genes exists that code for these small regulatory proteins, perhaps similar in function to eukaryotic transcription factors.
Regulatory genes of this type appear to be common among the bacilli since they have been identified in \textit{B. subtilis}, \textit{‘B. natto’} and \textit{B. amyloliquefaciens}. The detection of the homologous \textit{senS} locus in \textit{B. subtilis} indicated that the \textit{sen} genes, like the other small regulatory protein genes, may be highly conserved.

The exact role of these regulatory gene products should be tested \textit{in vitro} by DNA ‘footprinting’, transcription studies, and other related techniques, since all of these genes seem to affect gene expression at the transcriptional level. If the effect is a direct, positive, transcriptional one, it is possible that all the genes that are activated by these regulatory proteins may have some common or homologous sequence features in their regulatory sites such as the promoter region or regions close to or overlapping the promoter. But, so far no such common features have been found, suggesting that the direct positive regulation of different genes may be highly specific. On the other hand, the stimulatory effect may affect transcription in an indirect way, e.g. by negating the effects of repressors. If that is the case, the \textit{in vitro} identification of their functions would be much more difficult. The availability of cloned genes (Wong \textit{et al.}, 1984; Yang \textit{et al.}, 1983, 1984), whose expression is stimulated by these regulatory factors should make \textit{in vitro} regulatory studies feasible.

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REFERENCES


