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Cloning and Characterization of a Pair of Novel Genes That Regulate Production of Extracellular Enzymes in *Bacillus subtilis*

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Two novel *Bacillus subtilis* genes that regulate the production of several extracellular enzymes were cloned and characterized. These two genes are organized as part of an operon. When cloned in a multicopy plasmid, the first gene (*tenA*, transcription enhancement) stimulates alkaline protease production at the transcriptional level. The second gene (*tenI*) exerts an opposite effect to reduce alkaline protease production. The production of neutral protease, levansucrase, and alkaline protease can be stimulated up to 11- to 55-fold. Thus, *tenA* is a new member of the *deg* (regulatory genes for degradative enzymes) family in *B. subtilis*. A functional *degS* product is required to observe the stimulatory effect from *tenA*. Between the promoter and the ribosome-binding site of *tenA*, there exists a terminatorlike structure. Deletion of this structure doubles the expression of *tenA*. Neither *tenA* nor *tenI* is essential for cell growth and the production of extracellular enzymes. However, inactivation of these genes causes a delay in sporulation. This operon is located close to *tre* on the genetic linkage map. The overall organization of this operon and its relationship with other known regulatory factors in the *deg* family are discussed.

Bacillus subtilis is capable of secreting a wide variety of extracellular enzymes (proteases, α-amylase, levansucrase, and β-glucanases) directly into the medium (22). Several mutants that can stimulate the production of extracellular enzymes have been isolated. These mutants carry mutations in *degQ* and *sacU* (12, 14). Structural genes encoding *degQ* and *sacU* have been cloned (1, 8, 11, 34, 36, 45). Furthermore, the cloning of several regulatory genes from *B. subtilis*, *B. natto*, and *B. stearothermophilus* has been shown to enhance the production of extracellular enzymes when these genes are in a multicopy plasmid. These genes are *sacV* (17), *degR* (19, 47), *senS* (38), *senN* (39, 42), and *degT* (33). The size of the protein products derived from these genes varies from small polypeptides (46 to 65 amino acids for the *degR*, *sacV*, *senS*, and *senN* products) to larger polypeptides (372 amino acids for the *degT* product). Although these gene products show no sequence homology with each other, they all stimulate the production of extracellular enzymes. The target genes encoding extracellular enzymes such as *aprE* (alkaline protease) (30, 41), *nprE* (neutral protease) (46), *amyE* (α-amylase) (44), and *sacB* (levansucrase) (32) have also been cloned. With these mutants and the cloned genes available, the mechanism for the enhanced production of extracellular enzymes can be studied in detail. *degR*, *senN*, *degQ*, and *sacU* have been shown to exert their stimulatory effects at the transcriptional level (28, 35, 42). However, in the *sacU* minus mutant strain, no enhanced production of extracellular enzymes can be observed with cells carrying either *degR* or *degQ* on a multicopy plasmid (1, 34).

In this report, we describe in detail the cloning, nucleotide sequence, genetic mapping, and organization of a set of two novel genes from *B. subtilis* that can regulate the production of several extracellular enzymes. They were isolated by using a shotgun cloning approach and were selected by their abilities to stimulate the production of alkaline protease. The characterization of these genes and their roles in regulating gene expression are discussed.

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MATERIALS AND METHODS

Plasmids. Plasmid pUB18 (42), a derivative of pUB110, was used for routine subcloning in *B. subtilis*. pPQ is a pUB18 derivative carrying *degQ* (40). Bluescribe plasmid from *Escherichia coli* was used for cloning and double-stranded DNA sequencing. Standard DNA transformation was performed by the competent cell method in *B. subtilis* (29) and *E. coli* (16).

Media. Tryptose blood agar plates (TBAB; Difco) and Schaeffer (SG) sporulation agar plates (13) were used for routine transformation and maintenance of *B. subtilis* strains. For detecting enhanced protease production from *B. subtilis* DB102 (*his nprR2 nprE18*) (10), SG agar plates containing 1% (wt/vol) skim milk were used. For *B. subtilis* carrying pUB18 and its derivatives, kanamycin was added to the media at a final concentration of 10 μg/ml. C-medium (21) was used to culture *B. subtilis* for levansucrase assay. Schaeffer (13) and synthetic (20) media were used for sporulation studies. L broth and YT medium were used for routine culture of *E. coli* DH5α(ϕ80d*lacZ*ΔM15) [*endA1 recA1 hsdR17* (r^- m^-) *supE44 thi-1* λ^- *gyrA relA1* F^- Δ(*lacZYA-argF*)*U169*].

DNA manipulations. Small-scale plasmid preparations were made by the rapid alkaline-sodium dodecyl sulfate method (24), and large-scale plasmid preparations were made by CsCl-ethidium bromide centrifugation (6). Chromosomal DNA was prepared as described previously (24). Restriction enzymes and DNA modification enzymes were purchased from Bethesda Research Laboratories and Boehringer Mannheim and were used according to the recommendations of the manufacturers. DNA was sequenced by the chain termination method with Sequenase (4, 25). Double-stranded plasmid DNA was used as the templates. dGTP was replaced by dITP in some reaction mixtures to over-

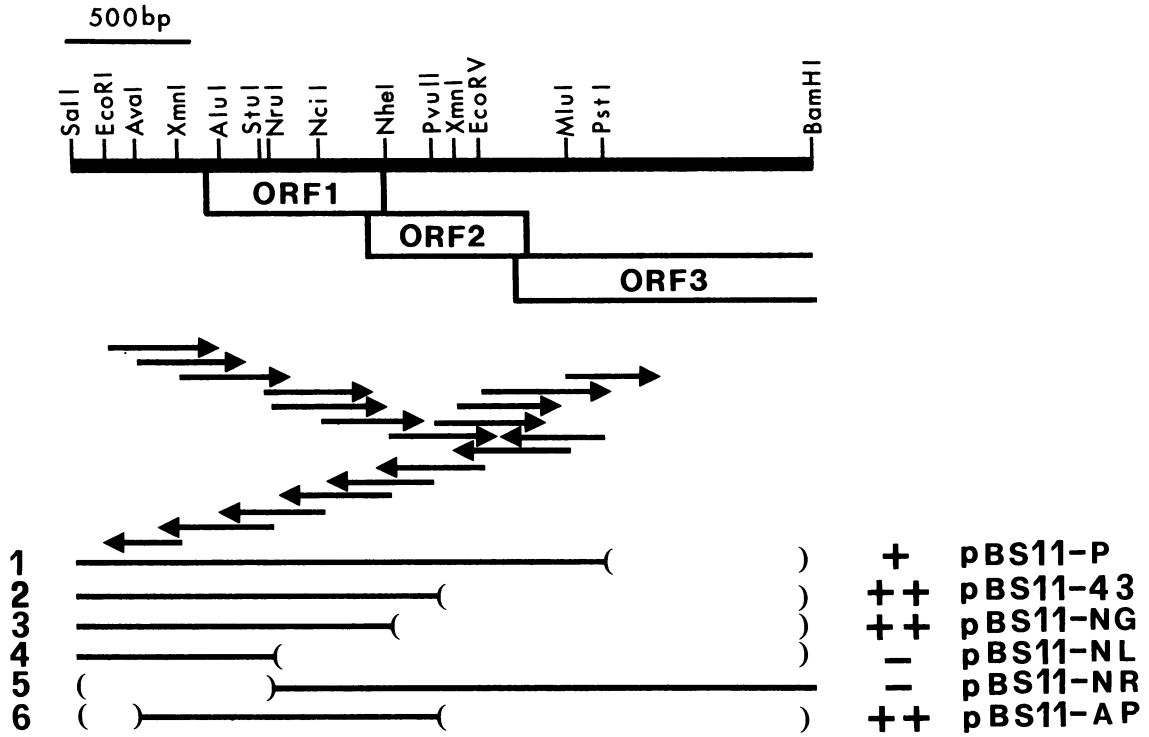

FIG. 1. Restriction map, sequencing strategy, and organization of the *ten* operon. The arrows indicate the direction of sequencing and the lengths of the subcloned fragments that were sequenced. The bracketed zones indicate that the sequence in that area was deleted. The positive and negative signs indicate whether the stimulatory effect for the production of alkaline protease was observed.

come compression. Restriction mapping of the cloned DNA fragment was done by the double-digestion method (5).

Construction of β-galactosidase fusion. A 3-kb *Sma*I-*Pst*I fragment carrying the *lacZ* cassette was obtained from pMC1871 (27). This fragment was inserted into the *Sma*I- and *Pst*I-digested pUB18 to generate pUB-lacZ or inserted into the *Eco*RV- and *Pst*I-double-digested pBS11 (see the Results for the construction of pBS11) to generate pUBP12. pUBP12 carried *lacZ* with its expression under the control of the regulatory region of *tenA* (see Fig. 3). To construct pUBΔP12, we digested pUBP12 with *Xba*I and *Nru*I. After the fill-in reaction with the large fragment of DNA polymerase I, the plasmid was reclosed by ligation and transformed into *B. subtilis* DB104.

Enzyme assays. Levansucrase and β-lactamase assays were performed as described previously (40). One unit of levansucrase activity was defined as the change in 1 absorbance unit per min. Protease assays were determined by using azocasein (3) and resorufin-labeled casein (43) as substrates. Alkaline protease and neutral protease activities were assayed in the presence of either 20 mM EDTA or 2 mM phenylmethylsulfonyl fluoride. β-Galactosidase activity in *B. subtilis* was measured as previously described (18). Cells were collected every hour to determine enzyme activity.

Determination of plasmid copy number. *B. subtilis* strains carrying different plasmids were cultured in Schaeffer medium up to the early stationary phase (1 h after the end of exponential growth). Whole-cell lysates were prepared by lysing cells (5×10^8) with 100 μl of lysing buffer (20 mM Tris [pH 8.0], 10 mM EDTA, 100 mM NaCl, 20% [wt/vol] sucrose, 20 mg of lysozyme per ml, 10 mg of RNase per ml), and the plasmid copy number was determined by the fluorescence densitometry method (23).

Determination of sporulation efficiency. The sporulation efficiency was measured by the method of Ochi et al. (20) in both synthetic and Schaeffer media. Cells were cultured in these media in the absence of any antibiotics. Spores were counted by plating the cells on a TBAB plate after heat treatment at 75°C for 20 min.

Mapping of the *ten* operon. Genetic mapping of the *ten* locus was performed by pBS1 transduction (9). Cm^r transductants were scored for the linkage to several loci from the set of reference strains described Dedonder et al. (7).

Nucleotide sequence accession number. The nucleotide sequence reported in this article has been assigned GenBank accession number M37776.

RESULTS

Shotgun cloning of regulatory genes that can enhance protease production. Chromosomal DNA (5 μg) from *B. subtilis* 168 *trpC2* (DB2) was partially digested with *Sau*3AI. DNA fragments in the range of 3 to 5 kb were size fractionated by agarose electrophoresis and electroeluted. Chromosomal DNA was ligated to *Bam*HI-digested pUB18 in a 2:1 (wt/wt) ratio. The ligated DNA was transformed into *B. subtilis* DB102 *his nprR2 nprE18* (10), which is an alkaline protease-positive and neutral protease-deficient strain. Transformants were transferred to SG-skim milk agar plates containing 10 μg of kanamycin per ml. Of 4,000 transformants, 2 showed enhanced production of alkaline protease (subtilisin) as judged by the presence of a bigger halo surrounding the colonies. *Bam*HI and *Sal*I double digestion indicated that

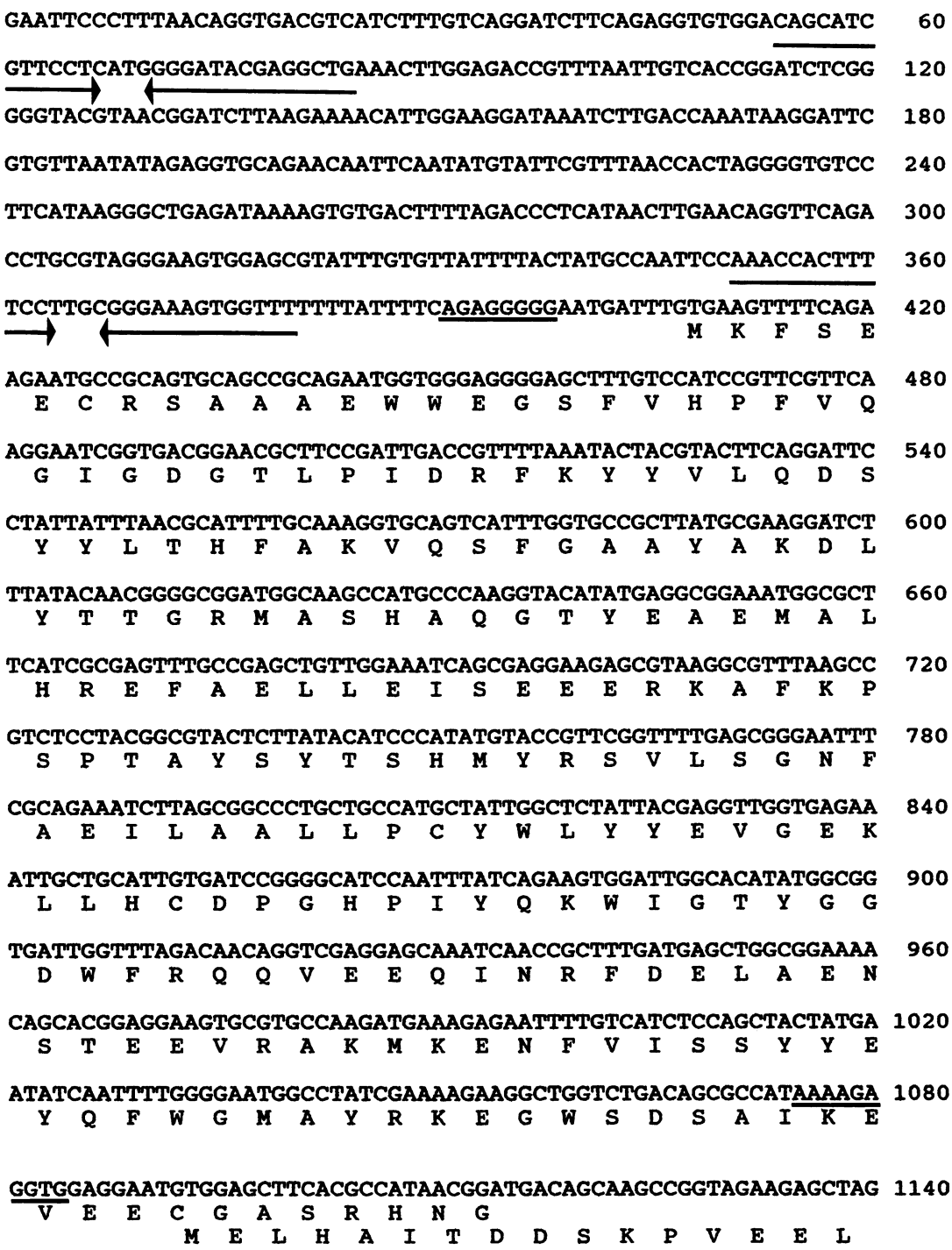

FIG. 2. Nucleotide sequence of the *ten* operon. ORF1 (*tenA*) and ORF2 (*tenI*) were translated. Underlined sequences indicate the potential ribosome-binding sites for *tenA* and *tenI*. The two terminatorlike sequences are marked by arrows (→ ←).

these two clones carried the same 2.8-kb insert. Subcloning this 2.8-kb fragment into pUB18 generated pBS11. DB102(pBS11) produced subtilisin at a higher level. Transformation of pBS11 into DB104 (Npr^-, Apr^-) did not result in higher levels of protease production. This indicated that the cloned gene is not coding for a protease. Instead, it encodes a regulatory factor that enhances alkaline protease production.

Localization of regulatory genes. A restriction map of the cloned 2.8-kb fragment was determined (Fig. 1). Deleting the *Bam*HI-*Pst*I fragment from the right arm (pBS11-P) still showed the stimulatory effect. However, deletion of either a *Pvu*II-*Bam*HI fragment or an *Nhe*I-*Bam*HI fragment from the right arm (pBS11-43, pBS11-NG) resulted in a further enhancement for subtilisin production. This effect could still be observed when the *Ava*I-*Sal*I fragment from pBS11-43

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CGAGAATCATCATTACGATTCAGAATGAAGTTGATTTTATTCACATTCGGGAACGCTCAA 1200
A  R  I  I  I  T  I  Q  N  E  V  D  F  I  H  I  R  E  R  S

AATCGGCGGCGGATATTTTGAAACTGCTCGATCTTATTTTTGAAGGCGGTATAGATAAAC 1260
K  S  A  A  D  I  L  K  L  L  D  L  I  F  E  G  G  I  D  K

GAAAATTGGTGATGAACGGGCGCGTGGATATTGCACTTTTCTCTACTATTCACCGCGTGC 1320
R  K  L  V  M  N  G  R  V  D  I  A  L  F  S  T  I  H  R  V

AGCTGCCAAGCGGCAGCTTTTCACCGAAGCAGATCAGAGCCAGATTTCCACACCTTCATA 1380
Q  L  P  S  G  S  F  S  P  K  Q  I  R  A  R  F  P  H  L  H

TCGGGAGGTCGGTGCATTCACTGGAGGAAGCGGTTCAAGCAGAAAAGGAAGACGCGGACT 1440
I  G  R  S  V  H  S  L  E  E  A  V  Q  A  E  K  E  D  A  D

ACGTGCTGTTCGGCCATGTGTTTGAAACGGATTGCAAAAAGGGTCTTGAAGGCAGAGGAG 1500
Y  V  L  F  G  H  V  F  E  T  D  C  K  K  G  L  E  G  R  G

TATCTTTGCTGTCAGATATCAAACAGCGGATTTCCATCCCGGTTATAGCCATCGGGGGGA 1560
V  S  L  L  S  D  I  K  Q  R  I  S  I  P  V  I  A  I  G  G

TGACGCCGGACAGGTTAAGAGACGTAAAACAAGCAGGGGCAGACGGCATTGCTGTTATGT 1620
M  T  P  D  R  L  R  D  V  K  Q  A  G  A  D  G  I  A  V  M

CAGGAATTTTCTCTTCCGCTGAACCTTTGGAAGCAGCCAGACGATATTCCCGCAAGCTAA 1680
S  G  I  F  S  S  A  E  P  L  E  A  A  R  R  Y  S  R  K  L

AGGAGATGCGCTATGAAAAGGCATTATGAAGCAGTGGTGATTGGAGGCGGAATTATCGGT 1740
K  E  M  R  Y  E  K  A  L

TCCGCAATTGCTTATTATTTGGCAAAGGAAAACAAAAACACCGCATTGTTTGAAAGCGGA 1800

ACAATGGGCGGCAGAACGACAAGTGCCGCTGCCGGAATGCTGGGCGCCCATGCCGAATGC 1860

GAGGAACGTGACGCGTTTTTTGATTTCGCTATGCACAGTCAGCGTCTGTACAAAGGTCTT 1920

GGAGAAGAGCTTTATGCATTATCCGGTGTGGATATCAGGCAGCATAACGGCGGTATGTTT 1980

AAGCTTGCATTTTCTGAAGAAGATGTGCTGCAG                              2010
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FIG. 2—*Continued*

(pBS11-AP) was deleted. Thus, the positive regulatory gene should be located between *Nhe*I and *Ava*I. By using *Nru*I as a reference point, subcloning of either the *Bam*HI-*Nru*I fragment from the right arm (pBS11-NR) or the *Nru*I-*Sal*I fragment from the left arm (pBS11-NL) showed no enhancement effect. This result indicated that the *Nru*I site is located within the structural gene encoding the positive regulatory factor.

Nucleotide sequence determination. The DNA sequence of the 2-kb *Eco*RI-*Pst*I fragment was determined from both strands by the dideoxy method. Sequence analysis revealed the presence of three open reading frames (ORFs), and each was preceded by a potential ribosome-binding site (Fig. 2). ORF1 and ORF2 have the capacity to code for proteins of 236 and 205 amino acid residues, respectively. ORF3 remains open up to the end of the sequence. Since ORF1 resided within the 848-bp *Xmn*I-*Nhe*I fragment and the *Nru*I site is located inside ORF1, ORF1 should code for a regulatory factor that can enhance the production of subtilisin. Deletions affecting ORF2 resulted in higher levels of subtilisin production (Fig. 1). The same result was achieved by the insertion of an 8-bp *Sal*I linker at the *Nhe*I site (after the fill-in reaction) of ORF2. The insertion caused a shift in the reading frame of ORF2. The structural genes encoding the ORF1 and ORF2 products were designated *tenA* and *tenI*, respectively. Deletions affecting ORF3 showed no effect on subtilisin production. A homology search was made through the SWISS-PROT data bank. None of the *tenA*, *tenI*, or ORF3 products showed any significant homology with other known proteins. Two transcription terminator-like structures were found at nucleotides 54 to 84 and 351 to 380. The second one is located upstream of *tenA* and is immediately followed by the ribosome-binding site of *tenA*.

***tenA* and *tenI* are arranged in the same operon.** Since the coding sequences for *tenA* and *tenI* partially overlap and there is no obvious transcription terminator sequence present between these two genes, it is likely that these two genes are arranged in the same operon. To confirm this arrangement, we constructed a set of *lacZ* translation fusions (Fig. 3). Plasmid pUBP12 carried the *tenA* sequence and the sequence corresponding to the first 142 amino acids of *tenI*. This sequence was fused in frame to codon 8 of *lacZ*. For DB104(pUBP12), 204 U of β-galactosidase activity were detected. However, for DB104(pUBΔP12), only the back-

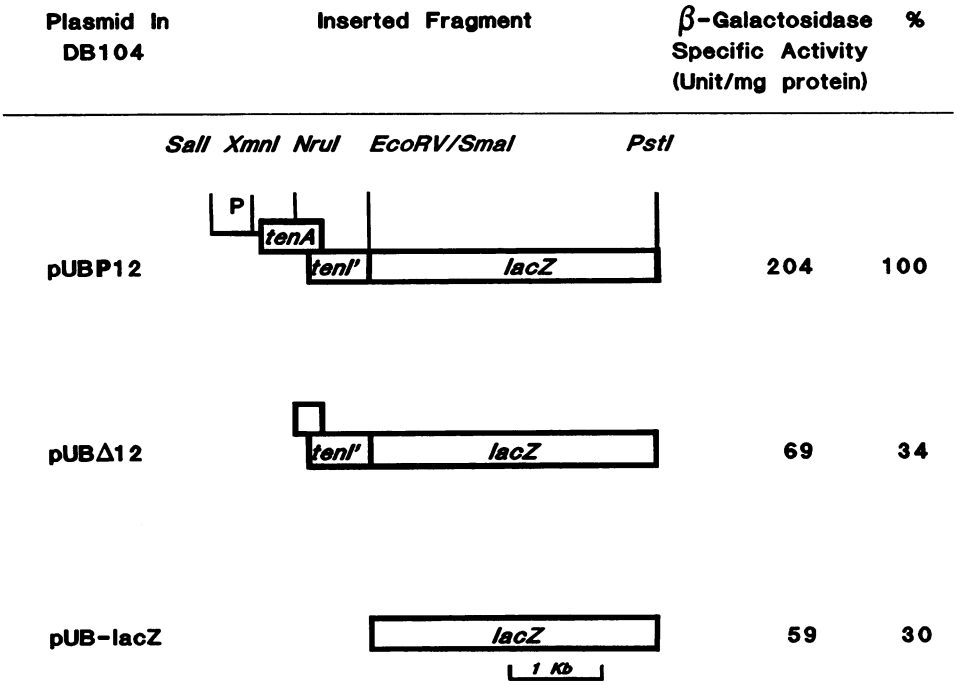

FIG. 3. *tenA* and *tenI* are organized in the same operon. The *Sal*I-*Eco*RV fragment carrying the upstream regulatory sequence of the *ten* operon, the coding sequence for *tenA*, and the first 142 codons of *tenI* (symbolized as *tenI'*) was fused in frame with codon 8 of *lacZ* in pUB-lacZ to generate pUBP12. A subsequent deletion was made to remove the *Sal*I-*Nru*I fragment which carries the promoter region and the first 133 codons of *tenA* to generate pUBΔ12. Specific activity (Miller units per milligram of protein) of β-galactosidase was determined from DB104(pUB-lacZ), DB104(pUBP12), and DB104(pUBΔ12) and was expressed as a percentage in reference to the activity obtained from DB104(pUBP12).

ground level (69 U) of β-galactosidase activity was observed. Plasmid pUBΔP12 was constructed by the deletion of an *Xba*I-*Nru*I fragment from pUBP12. This deletion removed the promoter region and part of the *tenA* sequence. No internal promoter can be found within the *tenA* sequence. This was confirmed by the insertion of a 376-bp *Xmn*I-*Nru*I *tenA* fragment into a promoter-probe plasmid, pWP19 (37). This plasmid carries a promoterless subtilisin gene (*aprE*) as a reporter gene. No extra subtilisin activity could be detected with the insertion of the *Xmn*I-*Nru*I fragment. On the basis of these observations, we conclude that both *tenA* and *tenI* are arranged in the same operon.

Localization of promoter region and regulation of gene expression through the transcription terminator-like structure. To determine whether the transcription terminator-like structures play any regulatory roles in controlling the expression of *tenA*, we determined the location of the promoter for this transcription unit in vivo by using the promoter-probe plasmid pWP19. Insertion of a 457-bp *Eco*RI-*Alu*I fragment carrying the two terminatorlike structures activated *apr* expression (pWP19-EA) (Fig. 4). Deletion of the first transcription terminator showed no effect on subtilisin expression (pWP19-AA). However, deletion of the second transcription terminator resulted in a twofold increase in subtilisin production (pWP19-EX). Subcloning of a 172-bp *Ava*I-*Xmn*I fragment into pWP19 demonstrated that the promoter resided in this region and was located between the two transcription terminator-like structures (pWP19-AX). It is likely that the first terminatorlike structure functions as a transcription terminator for the upstream genes. The second one plays a role in reducing the expression of *tenA* and *tenI*.

Effect of *tenA* and *tenI* products on production of extracellular enzymes. By using DB2(pUB18) as a control, DB2(pBS11) (carrying both *tenA* and *tenI* on the same plasmid) showed an eight- to ninefold increase in neutral protease, alkaline protease, and levansucrase activities (Table 1). Further increases in neutral protease, levansucrase, and alkaline protease activities up to 11-, 33-, and 55-fold, respectively, could be observed in the supernatant from DB2(pBS11-43) (carrying only *tenA*). Only the expression level of these enzymes was enhanced; the timing for expression was not altered. No significant enhancement for the production of α-amylase, xylanase, and cellulase could be detected from DB2(pBS11-43). Thus, *tenA* and *degR* share similar specificity in enhancing the production of extracellular proteins.

Copy number of pBS11. When *tenI* was present in the plasmid pBS11, the stimulatory effect exerted by *tenA* was reduced. To determine whether *tenI* exerts its effect by reducing the copy number of the plasmid, the copy numbers of pUB18, pBS11, and pBS11-43 in DB2 were determined and found to be 88 ± 12, 79 ± 2, and 83 ± 1, respectively. Thus, the presence of *tenI* in pBS11 showed no significant effect in reducing the copy number of the plasmid.

Stimulation of subtilisin production at the transcriptional level. To determine whether *tenA* product exerts its stimulatory effect at the transcriptional level or at the secretional level, we used a pair of strains constructed by Tanaka et al. (35) for further studies. Both MT221 (*his nprR2 nprE18 aprAΔ3 aprA*::pLK221) and MT441 (*his nprR2 nprE18 aprAΔ3 dfrA*::pKDF1) carried a modified TEM β-lactamase gene (*bla*) which was integrated into the *apr* locus in the chromosome. This modified *bla* has the ribosome-binding

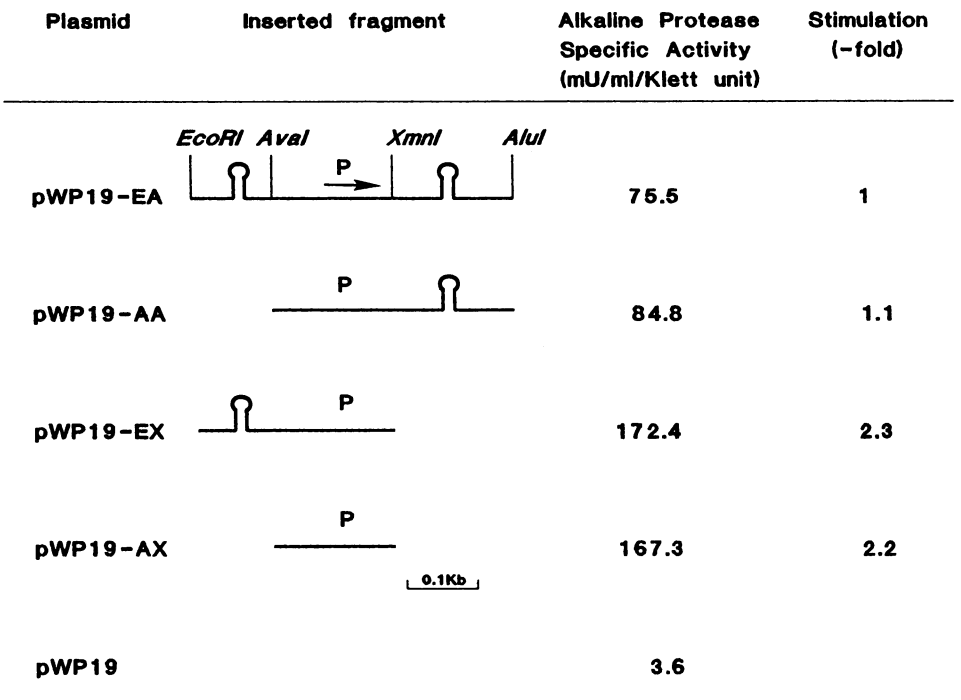

FIG. 4. Regulation of *ten* operon through a terminatorlike structure. pWP19 is a promoter probe plasmid with promoterless *aprE* (alkaline protease) as a reporter gene. P represents the promoter region of the *ten* operon, and the two terminatorlike structures are symbolized as Ω . The arrow indicates the direction of transcription.

site derived from *B. subtilis apr* and the signal peptide sequence derived from *B. amyloliquefaciens amy* (α-amylase). The expression of *bla* in MT221 and MT441 was controlled by the promoters derived from *apr* (sequence derived from an extracellular enzyme) and *dfr* (sequence derived from an intracellular enzyme, dihydrofolate reductase), respectively. If stimulation is exerted at the secretional level, enhanced production of β-lactamase should be observed in both strains MT221(pBS11) and MT441(pBS11), regardless of the promoters applied to express *bla*. However, a higher level of β-lactamase production was observed with MT221(pBS11) only (Fig. 5). This indicated that the stimulation exerted by *tenA* is promoter specific and that it acts at the transcriptional level. In comparison with MT221(pBS11), MT221(pBS11-43) showed a twofold enhancement for β-lactamase production. MT221(pUB18) and MT441(pUB18) served as controls.

TABLE 1. Effects of *tenA* and *tenI* on the production of extracellular enzymes in *B. subtilis*[a]

Plasmid	Relative enzymatic activity		
	Alkaline protease	Neutral protease	Levansucrase
pUB18	1	1	1
pBS11	9.6	8.3	9.7
pBS11-43	55.0	11.0	33.0

[a] DB2 transformants carrying different plasmids were cultivated in SG medium to determine the alkaline and neutral protease activities and in C-medium for the assay of levansucrase activity. Cells were collected every hour for enzyme activity determination. The highest specific activity was used for comparison. Alkaline protease activity (0.05 mU/ml per Klett unit), neutral protease activity (0.16 mU/ml per Klett unit), and levansucrase activity (14 U/ml per Klett unit) from DB2(pUB18) were set as 1.

Stimulatory effect of *tenA* requires functional *degSU* products. Functional gene products from the *sacU* locus have been shown to play a central role in stimulating the production of extracellular proteins. Stimulatory effects exerted by *degQ* and *degR* can be observed only in SacU$^+$ strains. To determine whether a similar requirement is needed for *tenA* to exert its stimulatory effect, we transformed pBS11, pPQ (*degQ* in pUB18 [40]), and pUB18 (control) into QB254 (*hisA1 sacA321 sacU42 trpC2*), a SacU$^-$ strain, and DB2. Transformants were spotted on an SG-skim milk plate. Similar to *degQ*, no stimulatory effect on protease production could be observed from QB245(pBS11) (Fig. 6).

Chromosomal inactivation of *tenA* and *tenI*. To determine whether *tenA* and *tenI* are essential for growth, sporulation, and the production of extracellular enzymes, we constructed two integration plasmids, pΔTENA and pΔTENI. A 270-bp *Alu*I fragment and a 379-bp *Nhe*I-*Eco*RV fragment (after the fill-in reaction), which are internal to the coding regions of *tenA* and *tenI*, respectively, were inserted into *Sma*I-digested pAZ111 to generate pΔTENA and pΔTENI. pAZ111 is a pUC19 derivative carrying the *cat* gene (a 1.3-kb *Hpa*II-*Cla*I fragment from pC194) at the *Acc*I site. Intact pΔTENA and pΔTENI were transformed into *B. subtilis* 168. Integration of these plasmids into *tenA* and *tenI* loci through a single reciprocal recombination was confirmed by Southern hybridization (data not shown). The resulting strains, WBΔTENA and WBΔTENI, grew normally in both the synthetic and Schaeffer media. The production of extracellular enzymes including subtilisin and neutral protease was not affected (data not shown). However, a delay in sporulation was observed for these mutant strains. The sporulation frequency of the mutant strains collected at T_8 (8 h after the beginning of the stationary phase) dropped 3 to 4

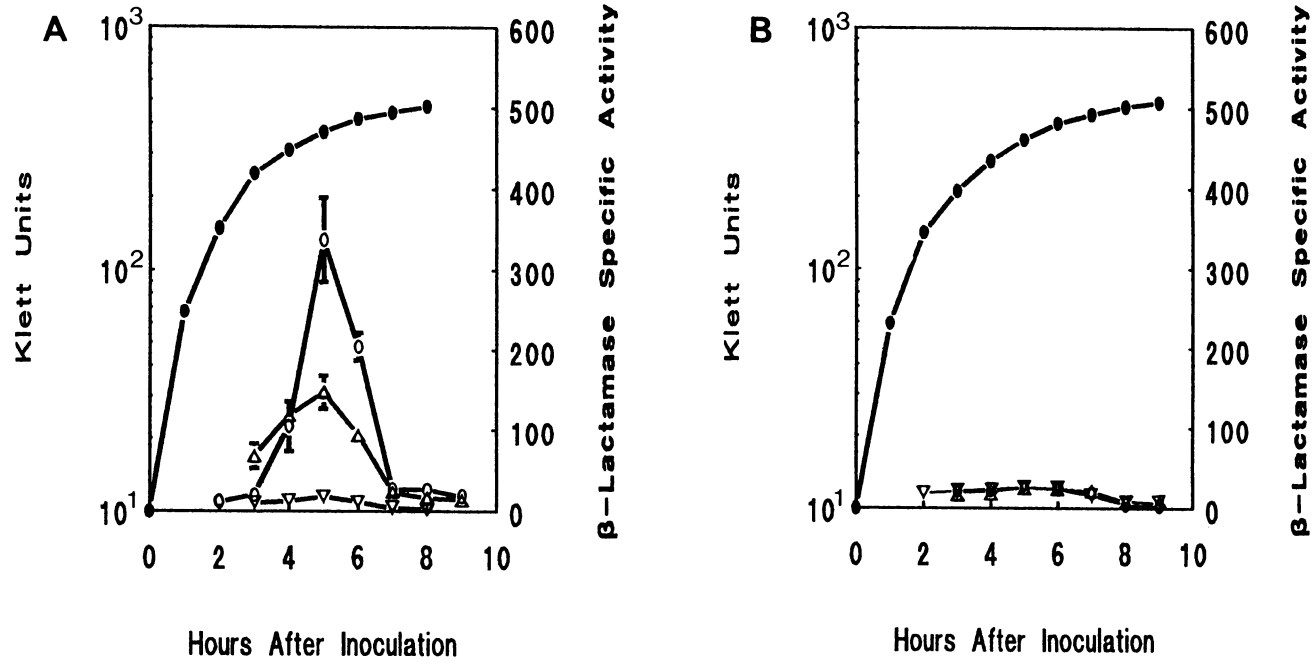

FIG. 5. *tenA* exerts its stimulatory effect at the transcriptional level. MT221 (A) and MT441 (B) carrying various plasmids were cultured in SG medium. Symbols: ●, growth curve of the cells; ▽, △, and ○, β-lactamase activity during growth by cells carrying pUB18, pBS11, and pBS11-43, respectively. Each assay was determined five times. Standard deviations are shown in the graph.

orders of magnitude in comparison with that of the wild-type strain (Table 2). No significant difference in sporulation frequency could be observed when cells were collected at T_{20}.

Location of *ten* operon in *B. subtilis* chromosome. To map the *ten* operon in the *B. subtilis* chromosome, we used WBΔTENA, which contained the *cat* gene inserted at the *tenA* locus, as a donor for pBS1 transduction mapping. The transduction results indicated that the inserted *cat* gene is tightly linked to *tre* (97% cotransduction), *glyB* (88% cotransduction), and *purB* (86% cotransduction) and is linked to *dal* (64% cotransduction), *aroI* (26% cotransduction), and *metC* (16% cotransduction). These data indicated that *tenA* is closely linked to *tre* and is located between *purB* and *glyB*. Besides *senS*, no other known regulatory gene is mapped within this region. Sequence analysis clearly indicated that the *ten* operon is structurally distinct from *senS*.

DISCUSSION

We reported the cloning of a pair of novel genes (*tenA* and *tenI*) from *B. subtilis* that regulate the production of extracellular enzymes. Functionally speaking, *tenA* is similar to *degQ*, *degR*, *senS*, and *senN*. It can stimulate the production of several extracellular enzymes at the transcriptional level. Structurally speaking, neither the *tenA* nor the *tenI* product shares no significant homology with any known regulatory proteins. This observation is not too unexpected, since none of the *degQ*, *degR*, and *senN* products share any significant homology with each other. Similar to *degQ* and *degR*, the stimulatory effect exerted by *tenA* requires a functional *sacU* product. Recent characterization of the *sacU* locus indicates that two ORFs encoding *degS* and *degU* products are responsible for the SacU phenotype (8, 11). Based on the homology with other two-component sensor-regulator sys-

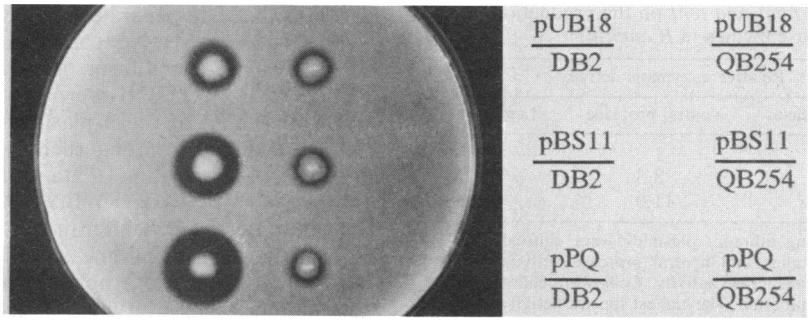

FIG. 6. Stimulatory effect of *tenA* required a functional *degS* product. DB2 and QB254 carrying pUB18, pBS11, and pPQ were spotted on the SG-skim milk agar plate. The plate was incubated overnight at 37°C.

TABLE 2. Effect of chromosomal inactivation of *tenA* and *tenI* on sporulation

Stage	*B. subtilis* 168		WBΔTENA		WBΔTENI	
	Cells/ml	Spores/ml	Cells/ml	Spores/ml	Cells/ml	Spores/ml
T_8	1.3×10^9	1.0×10^9	3.4×10^8	2.2×10^5	2.2×10^9	6.6×10^4
T_{20}	8.3×10^8	7.1×10^8	8.8×10^8	6.3×10^8	7.6×10^9	7.5×10^8

tems, the products of *degS* and *degU* may function as a sensor and a transcription activator, respectively (8, 11). The *degS* product also shows homology with the catalytic domain of several procaryotic and eucaryotic protein kinases (18) and is likely to be a protein kinase. *B. subtilis* QB254 used in the present studies carries the *sacU42* mutation. This mutation is a result of a G-to-A transition (Glu-300 to Lys) in *degS* (18). Dependence on a functional *degS* product suggested that *tenA* acts indirectly to enhance the production of extracellular enzymes. It may stimulate the protein kinase activity of the *degS* product and result in an increase in the level of the activated *degU* transcription activator. Alternatively, *tenA* may regulate the expression of the *degS-degU* operon. Further characterization of these aspects would be informative.

When *tenI* was present in a multicopy plasmid, the stimulatory effect exerted by *tenA* was reduced. It is possible that the expression of *tenI* resulted in a reduction of the copy number of the plasmid and, consequently, that fewer *tenA* products were synthesized to exert the stimulatory effect. Plasmid copy number determination does not support this hypothesis since pBS11 (carrying both *tenA* and *tenI*) has a copy number similar to that of pBS11-43 (carrying *tenA* only). Alternatively, the *tenI* product may function as a negative regulatory molecule, with its effect being mediated at either the RNA or the protein level. Computer analyses suggested that *tenI* is unlikely to function as an antisense RNA to interfere with *tenA* expression. However, insertion of a *Sal*I linker which shifted the reading frame of *tenI* abolished the inhibitory effect of *tenI*. This suggested that the *tenI* effect is mediated at the protein level. As possible modes of action, the *tenI* product could act directly to bind to the regulatory region of *aprE* and other target genes, or it could modify the *tenA* product through chemical modifications such as phosphorylation (2) or through the formation of a *tenI-tenA* protein complex. Further characterizations in these directions are needed.

Chromosomal inactivation of either *tenA* or *tenI* does not affect the production of neutral protease and alkaline protease. It is possible that *tenA* and *tenI* under physiological conditions play no functional roles in regulating protease production. The observed enhancement of protease production may have simply been a cross-talk effect when these genes were cloned in a multicopy plasmid. However, it is important to note that *B. subtilis* has several genes such as *degQ*, *degR*, and *senS* which have similar effects on the production of extracellular enzymes. It is possible that when *tenA* is inactivated, the others can compensate for *tenA* in regulating the expression of target genes. It would be important to construct a strain that has the chromosomal copies of *degQ*, *degR*, and *senS* inactivated. The regulatory roles of *tenA* and *tenI* could then be studied without interference. Deletions in either *tenA* or *tenI* resulted in a delay in sporulation (Table 2). This delay may be directly related to the loss of *tenA* and *tenI* products. However, ORF3 is likely to be organized in the same operon with *tenA* and *tenI*. The delay in sporulation because of the loss of ORF3 expression by a polar effect cannot be excluded.

It is interesting to observe that a transcription terminator-like structure is located between the promoter sequence and the ribosome-binding site of *tenA*. Similar organizations have been reported in at least three other cases, i.e., *sacB* (32) and *senS* (38) in *B. subtilis* and *bgl* (26) in *E. coli*. The transcription terminator-like structure has been demonstrated to function as a regulatory device in controlling the induction of *sacB*, the structural gene for levansucrase (28, 31, 48). A similar induction mechanism operates to control the *bgl* operon in *E. coli* (2, 15). In the third case, *senS*, a homolog of *senN*, also carries an upstream terminatorlike structure. If the terminatorlike structure is deleted, it is lethal for *B. subtilis* (38). For the *ten* operon, the presence of the terminatorlike structure reduces the expression of the operon. It is likely to play a functional role similar to that in *senS*.

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