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Cloning and Characterization of *Bacillus subtilis iep*, Which Has Positive and Negative Effects on Production of Extracellular Proteases

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We have isolated a DNA fragment from *Bacillus subtilis* 168 which, when present in a high-copy plasmid, inhibited production of extracellular alkaline and neutral proteases. The gene responsible for this activity was referred to as *iep*. The open reading frame of *iep* was found to be incomplete in the cloned DNA fragment. When the intact *iep* gene was reconstructed after the missing part of the *iep* gene had been cloned, it showed an enhancing effect on the production of the extracellular proteases. The open reading frame encodes a polypeptide of 229 amino acids with a molecular weight of ca. 25,866. Deletion of two amino acids from the N-terminal half of the putative *iep* protein resulted in dual effects, i.e., a decrease in the inhibitory activity shown by the incomplete *iep* gene and a slight increase in the enhancing activity shown by the complete *iep* gene. These results show that the *iep* gene product is a bifunctional protein, containing inhibitory and enhancing activities for the exoprotease production in the N-terminal and C-terminal regions, respectively. It was found by genetic and functional analyses that *iep* lies very close to *sacU*.

Production of the *Bacillus subtilis* extracellular proteases are under positive and negative controls. Hyperproduction of the proteases is brought about by mutations such as *hpr* (7), *sacQ*(Hy) (12), and *sacU*(Hy) (12) or by multiple copies of *prtR* or *sacQ* cloned on a multicopy plasmid (14, 28, 30). Disruption of the *sacQ* and/or *prtR* genes in the chromosome does not affect the level of the extracellular proteases, suggesting that there exists another element(s) which compensates for the defects of *prtR* and *sacQ* (30). Mutation of the *sacU* gene results in two phenotypes, SacU(Hy) and SacU$^-$, which show overproduction and reduced synthesis of the extracellular proteases and levansucrase, respectively. Presence in a SacU$^-$ strain of the *sacQ* and *prtR* genes in multicopy does not enhance the production of extracellular proteases and levansucrase (1; T. Tanaka, unpublished result), suggesting that among the many regulatory factors, the *sacU* gene product plays a central role in the regulation.

To help in understanding the mechanism of this complex regulation of the protease synthesis, we thought that a negative regulator would be useful, since in contrast to the number of the positive regulators discovered so far, *sin* is the only known negative regulator (3). As the first step toward this end, we looked for a gene capable of inhibiting production of exocellular protease. During this screening program, we found a gene, *iep*, which showed such activity and which also suppressed the enhancing activity of *prtR*. The open reading frame (ORF) of *iep* was not complete in the cloned DNA fragment, and, therefore, the downstream region was cloned to reconstruct the intact *iep* gene. In contrast to the inhibitory effect observed for the incomplete *iep*, the intact *iep* gene showed an enhancing effect on the protease production.

By genetic analysis, *iep* was found to fall in the region close to the *sacU* locus.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study are listed in Table 1. The cloning vector used was a kanamycin resistance (Km^r) plasmid, pUBH1, a derivative of pUB110 (10). pTL12 is a *B. subtilis* plasmid carrying the trimethoprim resistance (Tmp^r) gene *dfrA* derived from *B. subtilis* 168 (27), and pNT5 is a derivative of pT12 carrying the *prtR* gene (14). The *E. coli* plasmids pGEM1 and pGEM2 (2.9 kilobases [kb]) are the plasmids constructed by Promega Biotec Co. and were supplied by M. Itaya.

For construction of pAA2104 and pAA2109, the deleted DNA fragments carrying a *Hin*dIII linker ($\frac{GAAGCTTC}{CTTCGAAG}$) at the 5′ end and the *Eco*RI site at the 3′ end were first ligated with the 29-base-pair *Eco*RI-*Hin*dIII fragment of pBR322 (25) and then cleaved with *Eco*RI and introduced into the *Eco*RI site of pUBH1 in the same orientation as pAA1. pEH1 and pHH1 were obtained by cleavage of pAA1 with *Eco*RI plus *Hpa*I and *Hin*dIII plus *Hpa*I, respectively, followed by filling in and ligation. To construct pAA2204, the deleted DNA fragment carrying an *Aat*I site at the 5′ end and the *Hin*dIII linker at the 3′ end was inserted into the *Acc*I site filled in with the Klenow DNA polymerase I and *Hin*dIII sites of pUBH1. For the construction of pSH1006 and pSH1010, deletion was introduced from the *Hin*dIII site of pSHC1, and the *Hin*dIII linker was attached. From the constructed plasmids, the *Hin*dIII-*Eco*RI fragments carrying part of the *iep* gene were inserted into the *Hin*dIII and *Eco*RI sites of pAA1. (For the deletion endpoints in pAA2104, pAA2109, pAA2204, pSH1006, and pSH1010, see Fig. 5.)

Construction of pHIPΔ2 was performed as follows. The *Hin*dIII-*Bam*HI fragment of pSHC1 (Fig. 1) was replaced by the *Hin*dIII-*Bam*HI fragment derived from the multicloning site of pGEM1, resulting in construction of pHIP11. Then, the amino acids Met and Asp (see Fig. 3) were removed from pHIP11 by linking the *Eco*RV site and the *Bcl*I site filled in with the Klenow DNA polymerase I. pAAΔ2 was constructed by replacing the *Eco*RI-*Bam*HI fragment of pHIPΔ2 with that of pAA1. pSCHΔ3 was constructed by deletion of the DNA region between the *Eco*RV and *Hpa*I sites from

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference[a]
B. subtilis		
MI112	*arg-15 leuB8 thr-5 recE4 hsmM hsrM*	This laboratory (26)
CU741	*leuC7 trpC2*	S. Zahler (29)
T24	*trpE*	P. Lovett (8)
TT711	*leuC7 trpC2 iep::cat*[b]	pSCM2 → CU741
OA101	Prototroph	T24 DNA → CU741
OA102	Prototroph; Cm^r	pOU1 → OA101
OA103	*iep::cat*	TT711 DNA → OA101
QB255	*sacA321 trpC2 hisA1 sacU52*	Y. Sadaie (12)
TT2513	*sacA321 hisA1 sacU52*	OA103 → QB255
NIG1155	*his div-341 uvrA10*	Y. Sadaie (18)
E. coli		
JA221	*hsdR hsdM*$^+$ *ΔtrpE5 leuB6 lacY recA1*	Laboratory stock
Plasmid[c]		
pC194	Cm^r	9
pUBH1	Km^r	R. Doi (10)
pTL12	Tmp^r	This laboratory (27)
pNT5	pTL12 carrying *prtR*	This laboratory (14)
pBR322	Ap^r Tc^r	25
pGEM1	Ap^r	M. Itaya
pGEM2	Ap^r	M. Itaya

[a] Arrows designate transformation of the plasmids or chromosomal DNA into the chromosome of the respective strains.
[b] *cat* indicates the pC194 chloramphenicol acetyltransferase gene.
[c] Plasmids constructed in this study are described in Materials and Methods, the legend to Fig. 1, and Table 7. r, Resistance to antibiotic.

pHIP11. (For other plasmids constructed in this study, see Fig. 1 and Table 5.)

Media and reagents. The media used in this study were LB broth, LB agar (13), Antibiotic medium 2 (Difco Laboratories), Penassay broth (Difco Laboratories), and Spizizen's minimal medium (23). LBCG is the LB broth containing 10 g each of casein and gelatin per liter. The synthetic C medium (12) containing 50 μg of auxotrophic requirements per ml was used for the assay of levansucrase production.

Concentrations of the antibiotics added to the media were 10 μg/ml for trimethoprim and kanamycin, 50 μg/ml for ampicillin, 0.05 μg/ml for mitomycin C, and 5 μg/ml for chloramphenicol. Restriction endonucleases, Klenow DNA polymerase I, T4 DNA ligase, and alkaline phosphatase were purchased from Toyobo Co., Takara Syuzo Co., and Boehringer Mannheim Biochemicals. [α-^{35}S]dCTP and a dideoxy sequencing kit were obtained from Amersham Corp. and Toyobo Co., respectively. The 20-mer synthetic DNAs of the T7 and SP6 promoter regions were from Pharmacia and Boehringer Mannheim Biochemicals, respectively.

Transformation. The polyethylene glycol-induced protoplast transformation procedure of Chang and Cohen (2) was used for the shotgun cloning experiment. The transformed protoplasts were regenerated on DM3 plates (2) containing trimethoprim (1 μg/ml) and kanamycin (150 μg/ml). Transformation of *B. subtilis* competent cells was performed as described previously (27). Preparation of competent cells and transformation of *Escherichia coli* were described previously (11).

Chromosomal DNA was prepared by the method of Saito and Miura (19).

Assay of inhibiting and enhancing effects of plasmids on the production of extracellular protease. For testing inhibitory activity, MI112 cells carrying either pUBH1 or a plasmid to be tested were made competent, transformed by pNT5, and plated on the LBCG plates containing trimethoprim and kanamycin. When the halo size around the colony carrying the test plasmid and pNT5 was smaller than that around the colony carrying pUBH1 and pNT5, the plasmid was regarded as having an inhibitory activity. For testing enhancing activity, halo sizes around the colonies were compared between the cells carrying pUBH1 and those carrying the plasmid tested.

Nucleotide sequencing. After the *Aat*I-*Hin*dIII fragment of pSA1 and the *Hpa*I-*Hin*dIII fragment of pSHC1 (Fig. 1), each containing part of *iep*, were excised from an agarose gel and their cohesive ends were filled in with Klenow DNA polymerase I, they were cloned in the *Sma*I site of pGEM2 and the *Hin*cII site of pGEM1, respectively, in both orientations. The pGEM2 and pGEM1 derivatives were cleaved with *Bam*HI plus *Pst*I and *Bam*HI plus *Sac*I, respectively, and deletions were introduced by exonuclease III and nuclease S1 (6). Supercoiled plasmids carrying deletions were denatured and reannealed with the 20-mer primers of the T7 or SP6 promoter region, and the nucleotide sequence was determined by the dideoxy sequencing method (20) as described by Hattori and Sakaki (5). All the sequence was determined for both strands.

RESULTS

Cloning of *iep*, which regulates production of extracellular protease. To search for a gene capable of inhibition of protease production, we used the following strategy. A Tmp^r plasmid, pNT5, carrying *prtR* was introduced into *B. subtilis* MI112. Presence of *prtR* on a multicopy plasmid enhances the production of exoprotease (14) and thus permitted visual examination of the protease production easily on casein-containing plates. By shotgun cloning of the chromosomal DNA into these cells with a compatible plasmid, we tried to obtain a gene which might antagonize the *prtR* action. The chromosomal DNA of CU741 was cleaved with *Eco*RI, ligated with pUBH1 which had been cut with *Eco*RI and dephosphorylated with calf intestine alkaline phosphatase, and transformed into the protoplasts of MI112 cells carrying pNT5 (Fig. 1). The protoplasts were regenerated on DM3 plates containing trimethoprim and kanamycin. Transformants thus obtained were transferred by toothpick plating onto LBCG plates containing trimethoprim and kanamycin. After 2,500 colonies were examined, one showed a significantly reduced level of protease (Fig. 2b) compared with the control cell (Fig. 2a). The plasmids contained in the clone were prepared and transformed into MI112 cells. From a transformant showing Km^r alone, a plasmid which carried a 2.4-kb *Eco*RI fragment, pSA1, was obtained (Fig. 1). From a transformant showing only Tmp^r, a plasmid indistinguishable from pNT5 with respect to structure and function was obtained, indicating that the *prtR* gene on pNT5 did not suffer alteration during its coexistence with pSA1. It was found by Southern blot analysis that when the *Aat*I-*Eco*RI fragment of pSA1 carrying the *Hpa*I site (Fig. 1) was used as a probe, it hybridized to the 2.4-kb region of the *Eco*RI digest of the CU741 chromosomal DNA (data not shown), indicating that the cloned *Eco*RI fragment had not undergone gross structural alteration. The gene specifying the inhibitory activity was designated *iep*. To reduce the size of pSA1, the 1.5-kb DNA region from the *Aat*I to *Acc*I sites of pSA1 (Fig. 1) was deleted, and pAA1 was constructed. The plasmid retained the inhibitory activity, as determined by the method described in Materials and Methods. The extent of inhibition by pAA1 was estimated by using the culture

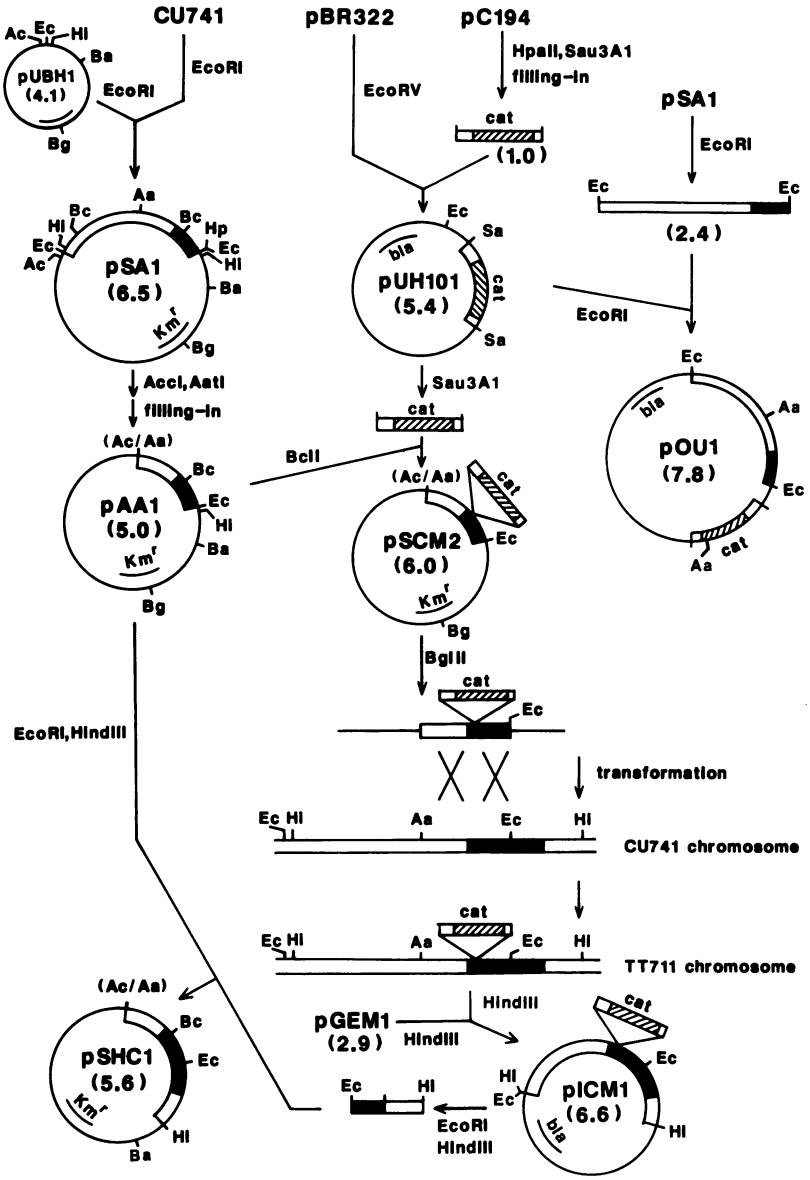

FIG. 1. Construction of plasmids and a *B. subtilis* strain. Symbols: =, DNA region derived from the *B. subtilis* CU741 chromosome; ■ and ▨, ORFs of *iep* and *cat*, respectively. Cleavage of pC194 with *Hpa*II and *Sau*3A1 gives a 1,031-base-pair DNA fragment (9). In pUH101, only relevant *Sau*3A1 sites are shown. Abbreviations: Aa, *Aat*I; Ba, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; Ec, *Eco*RI; Hi, *Hin*dIII; Hp, *Hpa*I; Sa, *Sau*3A1. Numbers in parentheses indicate the molecular sizes in kilobases.

supernatants obtained after 8, 24, and 32 h of growth. Without *prtR*, pAA1 reduced the protease activity to 20 to 40% of the control level (Table 2, lines 1 and 2), whereas in the presence of *prtR*, the protease level was reduced to 2% of the control level by pAA1 (Table 2, lines 3 and 4). The protease activities of the supernatants obtained from the pNT5- and pAA1-carrying cells (Table 2, line 4) were only twice the level found for the cells carrying pTL12 and pAA1 (Table 2, line 2). It was also found that the production of both the alkaline and neutral proteases was inhibited by *iep* to the same extent (data not shown). The results show that *iep* on pAA1 inhibits the production of exoprotease and antagonizes the enhancing effect of *prtR*.

Nucleotide sequence determination for the 0.9-kb region between the *Aat*I and *Eco*RI sites revealed that the region contained an ORF preceded by a Shine-Dalgarno sequence (21). The ORF, however, was found to be truncated by *Eco*RI (see below). Therefore, to obtain the intact *iep* gene, the DNA region downstream from the *Eco*RI site was cloned as outlined in Fig. 1. In brief, the 1-kb DNA fragment containing the *cat* gene of pC194 (9) was obtained from pUH101 and inserted into the *Bgl*II site of pAA1, generating pSCM2. After pSCM2 was linearized, the *iep* gene region carrying *cat* was integrated into the CU741 chromosome by a double-reciprocal crossover event, generating strain TT711. We found by Southern hybridization analysis that the *iep* region was in a 2.7-kb *Hin*dIII fragment in the TT711 chromosome (data not shown). Therefore, the 2.7-kb *Hin*dIII fragment was cloned in pGEM1 by selecting for chloramphenicol resistance (Cm^r), and pICM1 was thus constructed (Fig. 1). The entire *iep* gene was assembled by insertion of the 600-bp *Eco*RI-*Hin*dIII fragment of pICM1

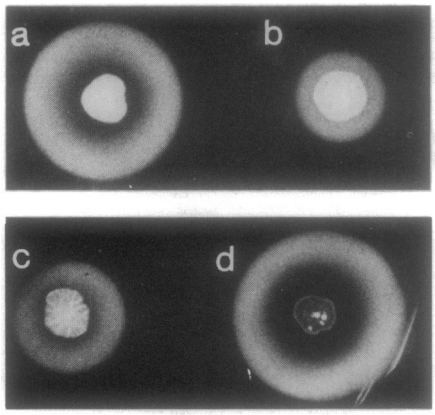

FIG. 2. Inhibition by pSA1 and enhancement by pSHC1 of the protease production from MI112 cells. Transformants were selected on LBCG plates as described in Materials and Methods and spread on the same plates. The colonies thus obtained were transferred by toothpick plating onto fresh plates containing either trimethoprim and kanamycin (a and b) or kanamycin alone (c and d). (a) MI112 (pNT5, pUBH1); (b) MI112(pNT5, pSA1); (c) MI112(pUBH1); (d) MI112(pSHC1).

into the *Hin*dIII and *Eco*RI sites of pAA1 (Fig. 1). Unexpectedly, when pSHC1 thus constructed was introduced into MI112, the transformants showed enhanced production of exoprotease, as shown by the larger halo around the colony (Fig. 2d). By quantitative analyses, the extent of enhancement was shown to be two- to fivefold, depending on the culture time (Table 3). Both the alkaline and neutral protease levels were found to be increased to the same extent (data not shown).

Nucleotide sequence and deletion analysis of *iep*. The nucleotide sequence of the *iep* gene together with its flanking DNA region was determined (Fig. 3). There is an ORF between nucleotides 486 and 1172 which encodes a polypeptide of 229 amino acids with a molecular weight of 25,866. It is preceded by a Shine-Dalgarno sequence (21) and is followed by a sequence which could form a hairpin structure and a cluster of Ts, i.e., the structure typical for the rho-independent transcription terminator (17). A typical amino acid sequence found for various DNA-binding proteins (15) is identified almost in the middle of the ORF (nucleotides 768 to 800).

To correlate the function of the putative *iep* gene product and the nucleotide sequence, deletion and insertion analyses were performed with either pAA1 or pSHC1 as the starting material (Fig. 4). Frameshift mutation introduced by filling in at the *Bcl*I site of pAA1 (pAA11) abolished the inhibitory activity, indicating that the ORF from the initiation codon to the *Eco*RI site is responsible for the inhibitory activity. The DNA region from nucleotide 421 to the *Eco*RI site (pAA2104) had the inhibitory activity, but further deletion extending to nucleotide 559 and thus removing the 25 N-terminal amino acids (pAA2109) inactivated the inhibitory activity, suggesting again that the ORF is necessary for inhibition. It was possible that the inhibitory activity was generated by fusion of the ORF with the nucleotide sequence downstream from the *Eco*RI site which was derived from pUBH1. Therefore, the C-terminal amino acid sequence was changed by cleavage of pAA1 (Fig. 1) with either *Eco*RI plus *Hpa*I or *Hin*dIII plus *Hpa*I, followed by filling in with Klenow DNA polymerase I and ligation. The plasmids thus obtained, pEH1 and pHH1, still retained inhibitory activity comparable to that of pAA1, indicating that the amino acid sequence as far as the *Hpa*I site is capable of inhibition of the exoprotease production. Deletion from the *Eco*RI site up to nucleotide 792 (pAA2204) (i.e., beyond one of the amino acids for the putative DNA binding sequence) abolished the activity. These results show that the inhibitory activity resides in the N-terminal part of the *iep* gene product. Neither pAA1 nor its derivatives described here showed the enhancing activity (Fig. 4).

Next, we examined the DNA region in pSHC1 necessary for the enhanced production of protease. Filling in at the *Bcl*I (pSBC1) or the *Eco*RI site (pSEC1) destroyed the enhancing activity (Fig. 4), suggesting that the ORF is necessary for the activity. Deletion from the *Hin*dIII site of pSHC1 to nucleotide 1233 (pSH1006) did not affect the enhancing activity. Further deletion up to nucleotide 1155 (pSH1010), removing the five C-terminal amino acids, abolished the enhancing activity, suggesting that the C-terminal end region is necessary for this activity.

To determine how internal deletion affects the regulatory activity of *iep*, two in-phase deletions were introduced. One plasmid, pHIPΔ2, contains a deletion from nucleotides 648 to 653, the region corresponding to Met and Asp (Fig. 3), and the other, pSHCΔ3, carries a deletion from the *Eco*RV to the *Hpa*I site which removes 80 amino acids including the putative DNA binding sequence. A *B. subtilis* MI112 colony harboring pHIPΔ2 exhibited a slightly higher level of exoprotease than that carrying the parental plasmid pHIP11, as shown by the halos around the colonies (Fig. 5a and b). To test whether the deletion of Met and Asp affects the inhibitory activity of *iep*, we constructed a plasmid, pAAΔ2, which is identical to pAA1 except that the two amino acids were deleted. It was found that MI112 carrying pAAΔ2 and pNT5 (Fig. 5e) produced a higher level of protease than did the cells carrying pAA1 and pNT5 (Fig. 5d) but had a

TABLE 2. Inhibition of exoprotease production by pAA1 in the presence or absence of *prtR* on a multicopy plasmid[a]

Plasmids	Enzyme activity (U/ml) at culture time (h)[b]		
	8	24	32
pTL12, pUBH1	0.004	0.24 (1.0)	0.70 (1.0)
pTL12, pAA1	0.002	0.095 (0.4)	0.13 (0.19)
pNT5, pUBH1	0.20 (1.0)	12.0 (1.0)	14.0 (1.0)
pNT5, pAA1	0.006 (0.03)	0.19 (0.016)	0.3 (0.02)

[a] MI112 cells carrying the sets of plasmids were spread on LBCG plates containing trimethoprim and kanamycin and grown overnight at 37°C. LB medium containing the antibiotics was inoculated by the colonies, incubated overnight, and transferred to fresh LB media (0.5% inoculation) containing the antibiotics. At the indicated times, samples were taken for the protease assay (14).

[b] Data in parentheses indicate the extent of inhibition when the control value is taken as 1.0.

TABLE 3. Enhancement of exoprotease production by pSHC1[a]

Plasmid	Enzyme activity (U/ml) at culture time (h)[b]		
	8	24	32
pUBH1	0.013 (1.0)	0.168 (1.0)	0.420 (1.0)
pSHC1	0.053 (4.1)	0.870 (5.2)	0.871 (2.1)

[a] MI112 cells carrying either pUBH1 or pSHC1 were cultured for the indicated times. The conditions used were the same as those described in footnote a of Table 2, except that the culture media contained only kanamycin.

[b] Data in parentheses indicate the extent of production when the control value is taken as 1.0.

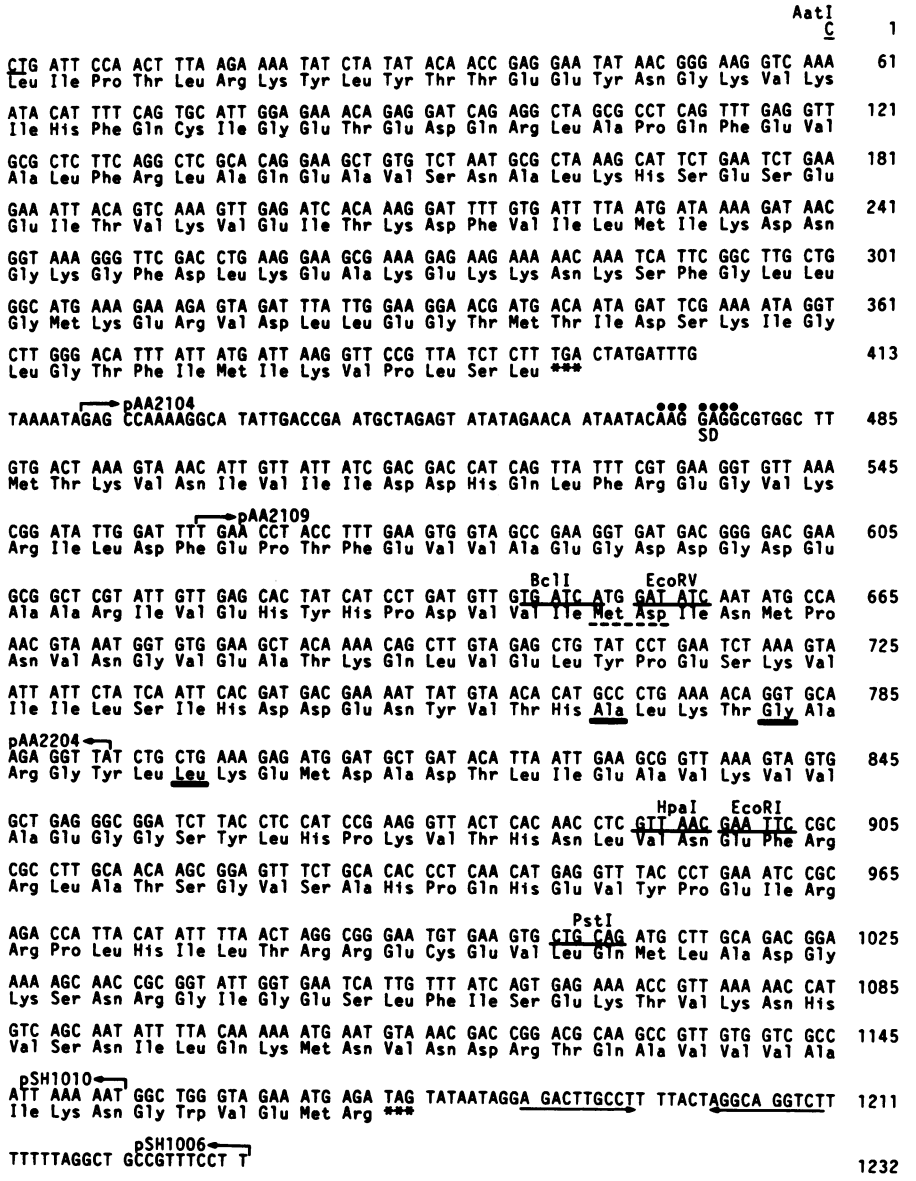

FIG. 3. Nucleotide sequence of the *iep* gene and its flanking regions. Symbols: · · · · · ·, putative ribosomal binding site (Shine-Dalgarno sequence [21]); _ _ _ _ _, amino acids removed in pHIPΔ2 and pAAΔ2 (Fig. 6); ▬, amino acids which show a putative DNA binding sequence (15). The arrows downstream from the ORF (nucleotides 1185 to 1194 and nucleotides 1201 to 1210) indicate the inverted repeat sequences. pAA2104 and pAA2109 carry the DNA regions from the nucleotides indicated by the appropriate arrows to the *Eco*RI site (nucleotide 897), whereas pAA2204, pSH1010, and pSH1006 carry the regions from the nucleotides indicated by arrows to the *Aat*I site (nucleotide 1) (see also Fig. 4).

significantly reduced level of protease compared with the cells harboring pUBH1 and pNT5 (Fig. 5c). These results show that removal of the two amino acids results in partial inactivation of the inhibitory activity of *iep* and causes slight activation of the enhancing activity of the *iep* protein. The enhancing activity of pSHCΔ3 was very low (Fig. 4) compared with the activity shown by pSHC1.

Functional analysis of *iep*. Production of levansucrase from MI112 cells was inhibited by pAA1 and enhanced by pSHC1 (data not shown), as shown by the chromogenic assay described by Lepesant et al. (12).

To determine whether the *iep* gene on a multicopy plasmid affects the SacU$^-$ phenotype of strain QB255, pSH1006 (Fig. 3 and 4) was introduced into this strain and the protease productivity was examined. pSH1006 was used in this study to neglect possible involvement of the unknown DNA sequence downstream from the intact *iep* gene. Strain QB255 *sacU52* transformed with pSH1006 showed enhanced production of exoprotease (Fig. 6). It is possible that the pSH1006 transformants producing a higher amount of exoprotease arouse by two independent events: (i) the integration into the *sacU* locus of the contaminating *sacU*$^+$ chromosomal DNA of MI112 from which pSHC1 was isolated, and (ii) the transformation of pSHC1 itself into the host. Among the 30 colonies tested, all the Km^r transformants showed the hyperproduction phenotype and carried the plasmid, excluding the possibility discussed above. These results suggest that the protease-overproducing phenotype

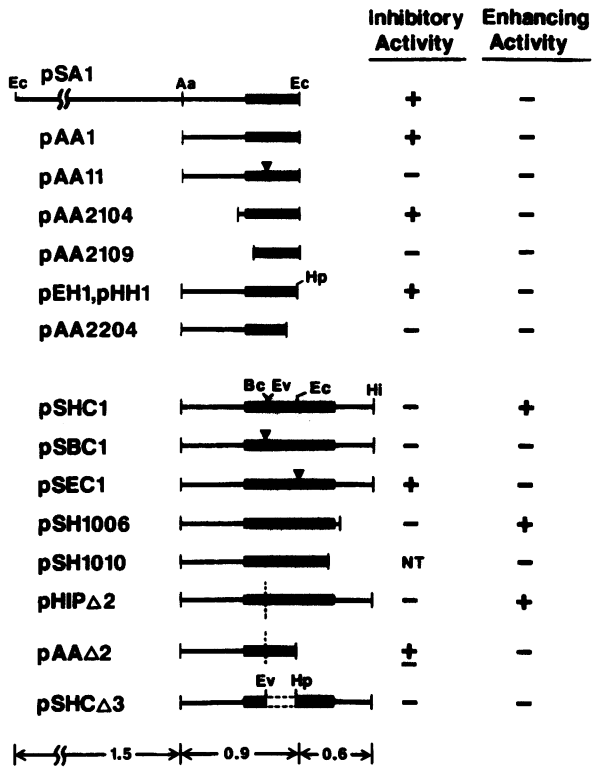

FIG. 4. Effect of deletion of the *iep* gene on protease production. DNA regions other than the vector part are shown. Inhibitory activity and enhancing activity indicate the inhibition and enhancement of exoprotease production, respectively, by the plasmids shown. The criterion for inhibitory or enhancing effect is based on halo-forming ability as described in Materials and Methods. pSA1 and pAA1 are shown in Fig. 1. Symbols: ▼, *Bcl*I and *Eco*RI sites of pAA1 and pSHC1 cleaved by these enzymes and filled in with the Klenow DNA polymerase I; +, inhibitory or enhancing activity observed; ±, reduced level of inhibitory activity observed; −, no activity observed; NT, not tested. Numbers at the bottom show the distances in kilobases between the restriction sites.

of strain QB255 was due, at least in part, to complementation of the *sacU* mutation by the cloned *iep* gene.

Strain TT711 carrying *iep*::*cat* did not produce a detectable level of levansucrase as assayed by levan synthesis around the colonies on the C-medium plate (data not shown). The exoprotease level of TT711 was 1/10 that of CU741 (Table 4), indicating that the *iep* gene product is involved in the production of exoprotease.

The effect of *iep* on the production of α-amylase was examined on the minimal plate containing starch (1). MI112 cells harboring pAA1 or pUBH1 gave halos indistinguishable in size (data not shown). MI112 cells carrying pSHC1 did not grow very well on the same plate, precluding the comparison

TABLE 4. Inhibition of the exoprotease production by disruption of the *iep* gene[a]

Strain	Enzyme activity (U/ml) at culture time (h)		
	8	24	32
CU741	0.015	0.475	0.588
TT711	0.006	0.050	0.060

[a] The strains were cultured in the same way as described in footnote a of Table 2, except that the culture media contained no antibiotic.

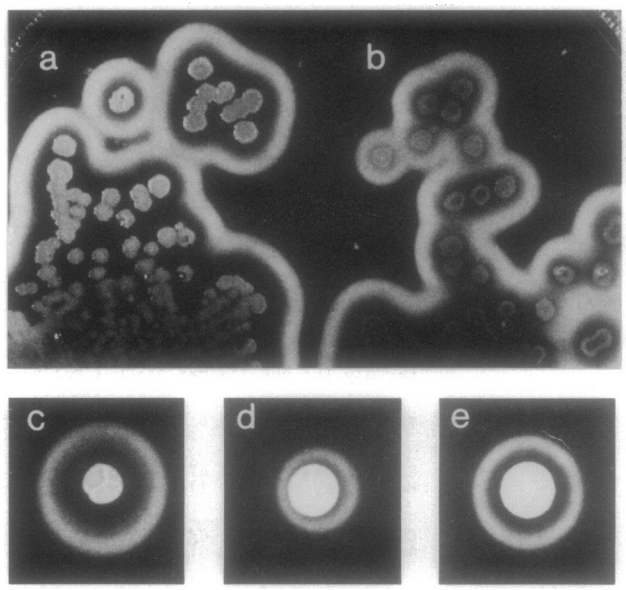

FIG. 5. Enhancement by pHIPΔ2 and pHIP11 and inhibition by pAA1 and pAAΔ2 of exoprotease production. MI112 cells carrying the plasmids were spread on LBCG plates containing kanamycin (a and b) or trimethoprim and kanamycin (c through e). (a) MI112 (pHIPΔ2); (b) MI112(pHIP11); (c) MI112(pNT5, pUBH1); (d) MI112(pNT5, pAA1); (e) MI112(pNT5, pAAΔ2).

of the α-amylase production by these cells with that by cells containing pUBH1.

Strain TT711 was not transformable (Table 5). On the other hand, MI112(pSHC1), which carries the *iep* gene on a multicopy plasmid and therefore is supposed to overproduce the *iep* gene product, was transformable, although the efficiency was reduced to approximately 4% of the control level (Table 5).

Genetic mapping of *iep*. The 2.4-kb *Eco*RI fragment of pSA1 was introduced into an integration vector, pUH101, and the resultant plasmid, pOU101 (Fig. 1), was integrated by Campbell-type recombination into the chromosome of OA101, producing Cm^r strain OA102. Transduction with the PBS1 phage grown in OA102 showed that *iep* linked to *hisA1* (299 degrees on the *B. subtilis* genetic map [16]) but not to *thr-5* (290 degrees) (data not shown), suggesting that the *iep* locus lies at a higher position than *hisA1*. Fine mapping was performed by transformation with the markers *div-341* and *uvrA10* and the chromosomal DNA from OA103 carrying *iep*::*cat*. A three-factor transformation cross indicated that the gene order was *iep–div-341–uvrA10* (Table 6), and the

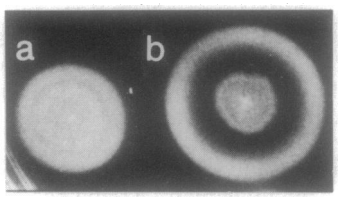

FIG. 6. Enhanced production of exoprotease by pSH1006 in a SacU$^-$ strain. QB255 cells carrying either pUBH1 or pSH1006 were spread on LBCG plates containing kanamycin, and the single colonies thus obtained were transferred to the same fresh plate with toothpicks. The halo formation was observed after incubation at 37°C for 40 h. (a) QB255(pUBH1); (b) QB255(pSH1006).

TABLE 5. Transformability of *B. subtilis* strains[a]

Strain[b]	Relevant genotype or description	No. of transformants to:	
		Km^r	Tmp^r
CU741	*iep*$^+$	25,000	
TT711	*iep*::*cat*	<10	
MI112(pUBH1)	*iep*$^+$		8,650
MI112(pSHC1)	*iep*$^+$ on pUBH1		350

[a] Competent cells were prepared as described in Materials and Methods. CU741 and TT711 were transformed to Km^r with pUBH1, whereas MI112 (pUBH1) and MI112(pSHC1) were transformed to Tmp^r with pTL12. Plasmid DNA used was 0.1 μg/ml.

[b] Plasmids carried in MI112 are shown in parentheses.

distance between *iep* and *div-341* was 47 (100% − percent cotransformation). Since the distance between *sacU32*(Hy) or *pap-9* and *div-341* is ca. 60 to 50 and the gene order around *sacU* is *sacU–div-341–uvrA10* (18), the results described above suggest that *iep* lies very close to *sacU*.

Next, we examined the relative positions of *iep* and *sacU* by transformation of SB255 SacU$^-$ Trp$^-$ with plasmids containing various parts of the ORF and the upstream flanking region of *iep*. The DNA regions examined were first cloned in *E. coli*, and the plasmids thus constructed were used for transformation, since plasmids obtained from *B. subtilis* may be contaminated by the *sacU*$^+$ chromosomal DNA. Since SacU$^+$ transformants cannot be selected directly, we first selected Trp$^+$ transformants on the C-medium plates containing histidine and then examined the levan synthesis around the Trp$^+$ colonies. It was found that neither pSAG21 containing the *Aat*I-*Eco*RI region (nucleotides 1 to 897) nor pBCH10 carrying the *Bcl*I-*Hin*dIII region of pSHC1 (nucleotide 644 through the *Hin*dIII site containing the *Eco*RI site) transformed SacU$^-$ to SacU$^+$ (Table 7). On the other hand, pOU2 carrying the region upstream from the *iep* gene had the SacU$^+$-transforming ability (Table 7). These observations show that *sacU52* and *iep* are present in close proximity but are distinct from each other.

DISCUSSION

This paper describes cloning and characterization of a gene which we named *iep*. The *iep* gene was found to encode a protein of 229 amino acids carrying two functional regions. The N-terminal region contains inhibitory activity for the production of exoprotease and levansucrase, whereas the C-terminal region carries the enhancing activity for the enzymes. The gene product itself, however, was found to be a positively regulating factor. The presence of a DNA binding sequence in the *iep* gene suggests that the gene product regulates the production of exoprotease and levansucrase by binding to DNA. By genetic analyses, the *iep* gene was found to be present downstream from the *sacU52* locus.

TABLE 6. Mapping of *iep* by transformation[a]

Phenotype	No. of colonies
Cm^r Div$^-$ UVR$^-$	56
Cm^r Div$^+$ UVR$^-$	38
Cm^r Div$^+$ UVR$^+$	41
Cm^r Div$^-$ UVR$^+$	14

[a] Transformants were selected for Cm^r on Antibiotic medium 2 containing chloramphenicol. Div$^+$ and UVR$^+$ phenotypes were examined on Antibiotic medium 2 incubated at 45°C and in the presence of mitomycin C, respectively. The recipient was NIG1155 (*div-341 uvrA10*), and donor DNA was from OA103 (*iep*::*cat*). The deduced order is *iep–div-341–uvrA10*.

TABLE 7. Transformation of QB255 SacU$^-$ to SacU$^+$ with DNA containing various parts of the *iep* gene and the 5′ flanking region[a]

DNA source[b]	DNA region[c]	No. of transformants	
		Trp$^+$ SacU$^-$	Trp$^+$ SacU$^+$
pOU2	Ec-Aa (pSA1)	1,353	36
pSAG21	Aa-Ec (pSA1)	1,714	0
pBCH10	Bc-Hi (pSHC1)	1,150	0
OA103	Chromosome	1,257	30

[a] Transformation was performed with 0.1 μg of the chromosomal DNA of strain TT2513 and 1 μg of the plasmid DNA per ml. For the experiment with OA103, 0.1 μg of the chromosomal DNA per ml was used.

[b] pOU2 was constructed from pOU1 (Fig. 1) by removing the *Aat*I fragment containing parts of *iep* and *cat*. pSAG21 was constructed by insertion into pGEM2 of the *Aat*I-*Hin*dIII fragment of pSA1 containing the N-terminal region of *iep*. pBCH10 was constructed by insertion of the *Bcl*I-*Hin*dIII fragment of pSHC1 (Fig. 1) into the *Bam*HI and *Hin*dIII sites of pGEM1.

[c] DNA regions examined for *sacU*$^+$-transforming ability (Fig. 1). Plasmids in parentheses are the origins from which the DNA regions derived.

It was found that strain TT711 carrying *sacU*::*cat* (insertion of *cat* at the *Bcl*I site [nucleotide 644]) did not produce a detectable level of levansucrase and had a reduced level of exoprotease (Table 4). Since filling in at the *Bcl*I site of pAA1 and pSHC1 destroyed the inhibitory and enhancing activities of *iep*, respectively, it is likely that the *iep* gene product of strain 711 is completely inactivated, indicating that the *iep* gene product is required for the production of extracellular protease and levansucrase. These phenotypes of strain TT711 resemble those of the *sacU* mutants (12). One interpretation of these results is that there are at least two genes involved in the production of exoprotease and levansucrase in this chromosomal region, one *sacU* and the other *iep*, and that both gene products are necessary for the production of these enzymes. Alternatively, the *iep* gene is in an operon and its expression is dependent on a promoter upstream from the *iep* gene. If *sacU52* was a promoter mutation, expression of the downstream *iep* gene would be reduced, resulting in low levels of the exoenzymes.

Strain QB255 is transformable, as was previously demonstrated (24) and as was confirmed in this study, whereas strain TT711 was nontransformable (Table 5). The result suggests two possibilities. One is that there is a gene downstream from *iep*, which is involved in competence development and is cotranscribed with the *iep* gene, although a transcription termination sequence is present after the *iep* gene. If the interruption of the *iep* gene by the *cat* gene resulted in reduction of mRNA for the putative competence gene, competence development would be impaired. It should be noted that one *com* gene has been mapped around *hisA1* (299 degrees [16]) (4). Another possibility is that the *iep* gene product is necessary for competence development. It has been reported that some of the *pap* mutations which map in the *sacU*(Hy) locus render the host cell nontransformable (31). Although *sacU*(Hy) and *sacU* mutation loci are reported to be very close to each other (24), it has not been demonstrated whether they are within the same gene. Since the N-terminal region of the *iep* gene product was shown to have inhibitory activity for the production of exoprotease, mutations in this region would inactivate the inhibitory function of *iep* and generate an *iep* protein with increased enhancing activity. The demonstration that pAAΔ2 and pHIPΔ2 showed a decrease in the inhibitory activity and a slight increase in the enhancing activity for the exoprotease production, respectively, is in agreement with this idea. Therefore, it might be possible that

some of the *sacU*(Hy) mutations reported so far (12) map in *iep* and that nontransformability is due to inactivation of the inhibitory activity present in the N-terminal region of the *iep* protein. Sequence analysis of various *sacU* mutations will clarify this question.

The *sacQ* gene on a multicopy plasmid does not stimulate the production of levansucrase when the host carries the *sacU52* mutation (1). A similar result was obtained when pNT5 was transformed into strain QB255 *sacU52* (data not shown). These results show that the *sacU* gene product is necessary for the *sacQ* and *prtR* gene functions. In contrast, pSHC1 stimulated the protease production by QB255, suggesting that *iep* can complement, at least in part, *sacU* deficiency. It was also found (T. Tanaka, unpublished data) that pAA1 inhibited the enhanced production of exoproteases caused by *sacU*(Hy) and that the inhibition was at the transcription level. Detailed studies are in progress.

Questions are raised of whether the positive and negative effects of *iep* are functioning in vivo, and if so, how they participate in the regulation of the exoprotease production. Positive regulation by *iep* may be exerted in vivo, since disruption of *iep* resulted in a 90% decrease in the exoprotease production. On the other hand, for the negative effect of *iep* to be effective, the C-terminal region of the *iep* gene product must be inactivated in some way or cleaved off. In this respect, it has been postulated that the *spo0A* gene product might inactivate the *abrB* gene product, which is supposed to be involved in negative regulation of the protease production (32). Further study including the mode of action of the *iep* gene product is under way.

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ADDENDUM IN PROOF

Recently it was found that the *sacU* region consists of two ORFs (D. Henner, personal communication) and that the *iep* gene corresponds to the downstream ORF.

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