JOURNAL OF BACTERIOLOGY, Mar. 1986, p. 837–842
0021-9193/86/030837-06$02.00/0
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Vol. 165, No. 3

Secretion of a Heterologous Protein from *Bacillus subtilis* with the Aid of Protease Signal Sequences

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Received 2 October 1985/Accepted 9 December 1985

Secretion vectors based on the genes from *Bacillus amyloliquefaciens* P for alkaline protease (apr_{BamP}) and neutral protease (npr_{BamP}) were constructed. With both apr_{BamP} and npr_{BamP}, a unique restriction site was introduced 3′ of the predicted signal coding region by using the technique of oligonucleotide-directed mutagenesis. The new sites enabled us to fuse a heterologous gene to the expression and secretion elements. We used the protein A gene (*spa*) from *Staphylococcus aureus* as a heterologous gene. *Bacillus subtilis* cells carrying the resulting *apr-spa* or *npr-spa* gene fusions synthesized the fusion protein. *B. subtilis* cells were also capable of removing the signal peptide from the fusion protein, as indicated by the appearance of processed protein A into the growth medium. In addition, these gene fusions allowed us to identify the signal processing site of both the APR-SPA and NPR-SPA proteins.

Bacilli have been used in fermentation technology as a source of a variety of extracellular proteins. In the last few years, the potential of *Bacillus subtilis* as a host organism for the commercial production and secretion of heterologous proteins has been examined (12, 13). Secretion vector based on the α-amylase genes from *Bacillus amyloliquefaciens* and *B. subtilis* have been successfully used to construct *B. subtilis* strains which are able to secrete β-lactamase, protein A, or human interferon into the growth medium (3, 12, 13, 20). Here, we report the development of secretion vectors based on the genes for two extracellular proteases of *B. amyloliquefaciens* and the ability of *B. subtilis* strains carrying such vectors to secrete large amounts of heterologous protein.

We have previously reported the isolation of the *B. amyloliquefaciens* P genes for an alkaline protease (apr_{BamP}) and a neutral protease (npr_{BamP}) (18, 19). *B. subtilis* strains carrying either of these genes on a multicopy plasmid produce and secrete large amounts of the corresponding protein. To take advantage of the efficient expression and secretion properties of these two genes, we were interested in constructing secretion vectors based on each. However, biochemical and genetic studies showed that, in each case, the primary translation product of the protease gene is a preproprotease (19). Since the proprotease form of the enzyme has not been isolated (the mature protein was found to be the predominant form in the growth medium), it was not possible to determine the location of the signal peptide cleavage site by comparison of the N-terminal amino acid sequence of the primary translation product and the processed product. Thus, we used heterologous gene fusions to analyze the signal sequence functions of the protease genes.

MATERIALS AND METHODS

Strains. *Escherichia coli* GX1210 [F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q}/Δ*lacZM15* Δ(*lac-pro*) *supE thi zig*::Tn*10 hsdR2*] was used throughout (19). *B. subtilis* GX4935, a derivative of strain BR151 (*trpC2 metB10 lys-3*) was constructed by S. Fahnestock; it has a deletion in the chromosomal subtilisin gene (*aprE*) and lacks the neutral protease due to an uncharacterized UV-induced mutation (10).

Plasmids. pGX251, pGX2109, and pSPA1 have been described earlier (8, 19).

Growth media. *B. subtilis* was grown in synthetic medium (17). *E. coli* was grown in L broth (19) or YT medium (22). Methods of plasmid isolation and transformation have been described previously (5, 19). DNA sequence analysis was performed by J. Strasser and D. Filpula by the dideoxy method (1).

Oligonucleotide-directed mutagenesis. A phage vector, MGX100, which contains the entire apr_{BamP} fragment, has been described previously (19). MGX120 was constructed by cloning a 2.3-kb *Eco*RI-*Eco*RV fragment from pGX2109 between the *Eco*RI and *Sma*I sites of M13mp19 (19, 21). Site-directed mutagenesis to introduce a *Bam*HI site was performed on a single-stranded MGX100 or MGX120 template by the method of Norris et al. (11) with the following modifications. The mutagenic reactions were set up in duplicate. The mutagenic oligomer (33-mer) and the M13 universal primer (17-mer) were annealed to the single-stranded template at a molar ratio of 20:12:1. The annealing reaction was incubated at 55°C for 5 min and then at 23°C for 20 min. For the elongation reaction, the Klenow fragment of DNA polymerase I and T4 DNA ligase were added together, and incubation was continued for 2 h at 23°C. The elongation reaction was terminated by heat inactivation for 20 min at 65°C. The entire mutagenic reaction mix was then diluted 50-fold and digested with restriction enzymes (*Eco*RI and *Sal*I) for 1 h at 37°C. One microgram of predigested (*Eco*RI and *Sal*I) shuttle vector (pGX251) was added as the carrier DNA. The DNA mixture was deproteinized before ethanol precipitation (19). The precipitated DNA was suspended in 10 μl of distilled water, and the duplicate samples were pooled. One-half of the DNA was subjected to gel electrophoresis to verify that the elongation and digestion had occurred; the other half of the DNA was treated with T4 DNA ligase at 12°C for 8 h. One-third of the ligated DNA was used to transform competent *E. coli* cells (GX1210). Two of the 20 ampicillin-resistant transformants obtained contained the new restriction site. This was further confirmed by DNA sequence analysis.

Construction of the *apr-spa* and *npr-spa* gene fusions. Plas-

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mid pGX2912 contains the entire *spa* gene and was constructed by S. Fahnestock by deleting a 6.8-kilobase *Pvu*II fragment from pSPA1 (8). A 1,850-base-pair fragment encoding mature protein A lacking the first 22 amino acids was isolated as a *Bcl*I-*Pvu*II fragment from pGX2912. Plasmid pGX2134 (which carries apr_{BamP}) was digested with *Bam*HI and *Pvu*II; pGX2138 (which carries npr_{BamP}) was similarly digested with *Bam*HI and *Eco*RV. The purified *Bcl*I-*Pvu*II *spa* fragment or the *Bcl*I-*Pvu*II-digested pGX2912 was ligated to each vector in a volume of 15 μl (70 ng of DNA per μl). The ligated DNA was used to transform competent *E. coli* GX1210 cells. Plasmid DNA was isolated from the transformants and analyzed by restriction analysis. The purified plasmid DNA from *E. coli* was used to transform competent *B. subtilis* GX4935 cells.

In vivo labeling of proteins. A *B. subtilis* culture was grown in synthetic medium at 37°C to an A_{600} of 0.5. The cells were washed with the prewarmed synthetic medium lacking methionine and then suspended in synthetic medium lacking methionine and starved for 15 min by shaking at 37°C. To 2 ml of cell suspension, [^{35}S]methionine (200 μCi) was added, followed after 3 min by 30 μl of a chase solution (1 mg of puromycin per ml, 5 mg of methionine per ml in water). Samples (500 μl) were withdrawn and centrifuged for 15 s in a microcentrifuge. The supernatant was transferred to a tube containing bovine serum albumin (5 μg) as the carrier protein. The cell and supernatant fractions were precipitated by trichloroacetic acid (5% final concentration). The trichloroacetic acid-precipitable material was isolated, washed with 80% acetone, and suspended in 50 μl of buffer (19). The samples were processed in a manner similar to the immunoprecipitation procedure described previously (15) except that, instead of antibody, 50 μl of Immunobeads (rabbit anti-mouse immunoglobin beads; Bio-Rad Laboratories, Richmond, Calif.) was incubated with the sample for 2 h at 23°C with intermittent shaking. The immunoglobulin G (IgG)-protein A complex was collected by centrifugation and washed three times with the immunoprecipitate washing buffer to remove nonspecifically bound proteins (15). The protein A was solubilized by boiling the protein A-IgG complex in 2% sodium dodecyl sulfate (SDS) for 5 min followed by boiling in Laemmli sample buffer for 5 min (7). The solubilized proteins were separated on a 10% polyacrylamide–SDS gel. The gel was soaked in Autofluor (National Diagnostics) for 1 h and dried down onto 3 MM paper (Whatman, Inc., Clifton, N.J.). The dried gel was exposed to X-ray film X-Omat APS; Eastman Kodak Co., Rochester, N.Y.) at −80°C.

Microsequence analysis of secreted protein. *B. subtilis* cells carrying *apr-spa* or *npr-spa* plasmids were grown in synthetic medium at 37°C to an A_{600} of 0.5. The cells from 1 ml of culture were collected by centrifugation, washed once with prewarmed synthetic medium without lysine, and suspended in the synthetic medium lacking lysine but containing 5 mM phenylmethylsulfonyl fluoride. After 15 min of incubation at 37°C to starve for lysine, [^{3}H]lysine (500 μCI) was added. The cells were labeled for 5 min, and then 15 μl of a chase solution (1 mg of puromycin per ml, 5 mg of lysine per ml) was added. Incubation was continued for 10 min longer. Cells and supernatant were separated and processed as described earlier. The protein A was released from the protein A-IgG complex by boiling in 2% SDS. A sample of the solubilized protein A was subjected to polyacrylamide gel electrophoresis to verify that no proteolysis had occurred. The rest of the solubilized protein A was precipitated by adding 8 volumes of acetone; the precipitate was washed with acetone. The amino-terminal sequence analysis of the protein was performed by Mark Hermodson, Purdue University, by the method of Mahoney et al. (9).

Materials. [^{35}S]methionine (1,098 Ci/mmol) and L-[4,5-^{3}H]lysine hydrochloride (90.8 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Restriction enzymes were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. or New England Biolabs, Inc., Beverly, Mass., and were used as recommended by the manufacturers. Puromycin was purchased from Sigma Chemical Co., St. Louis, Mo. Mutagenic oligomers were synthesized by Steven Pulford on a DNA synthesizer (model 380A; Applied Biosystems, Foster City, Calif.).

RESULTS

Creation of a restriction site in apr_{BamP} and npr_{BamP}. The location of the putative signal processing site in the apr_{BamP} and npr_{BamP} gene products was predicted by the hypothesis of Perlman and Halvorson (14). By using site-directed mutagenesis, we created a restriction site (*Bam*HI) by insertion of a six-base sequence 3′ to this putative signal coding region in both apr_{BamP} and npr_{BamP} (Fig. 1). The mutagenesis method of Norris et al. was modified as described in Materials and Methods (11). The modifications alleviated the need to use a labeled oligonucleotide as a probe to detect the desired mutations. The frequency of mutants isolated in this way varied between 10 and 50%. The creation of the *Bam*HI site in both cases was confirmed by DNA sequence analysis.

Construction of *apr-spa*. The DNA sequence of the entire

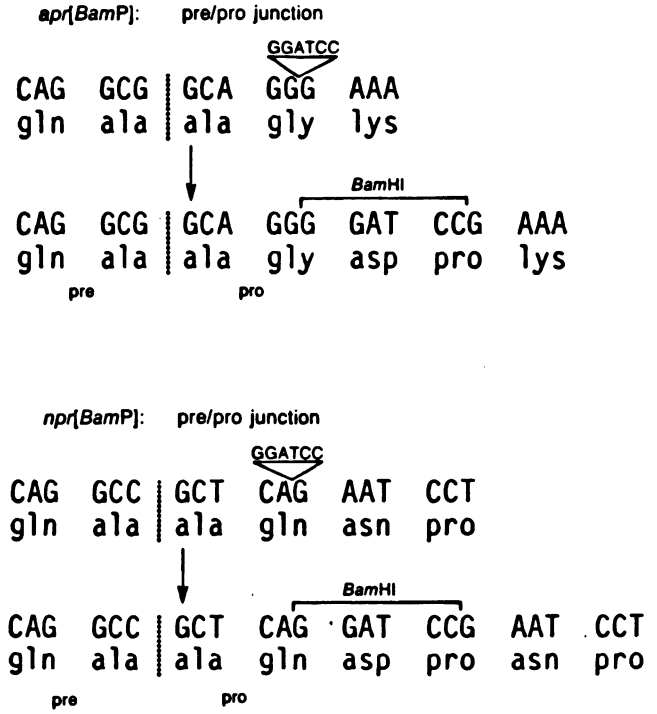
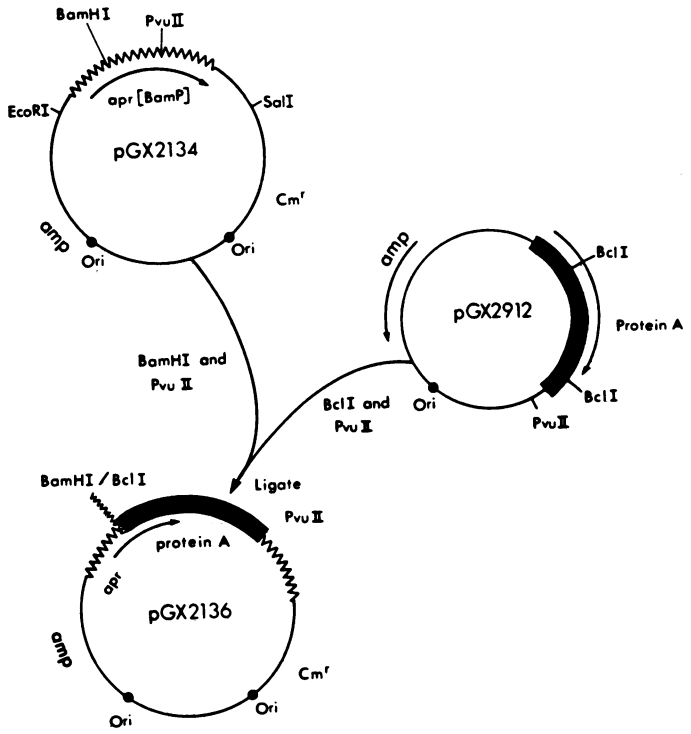


FIG. 1. DNA sequence across the pre-pro junction of apr_{BamP} (*apr*[*BamP*]) and of npr_{BamP} (*npr*[*BamP*]). The complete DNA sequences of apr_{BamP} and npr_{BamP} have been published (19). A *Bam*HI site was created two codons 3′ to the putative signal-coding region by site-directed mutagenesis as described in Materials and Methods. Each mutation was confirmed by DNA sequence analysis. The mutated DNA sequence and the deduced amino acid sequence are shown.



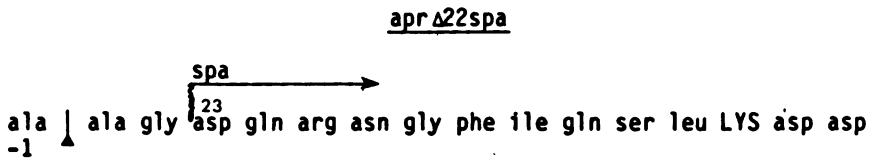


FIG. 2. Construction of the *apr-spa* fusion. pGX2134 is an *E. coli-B. subtilis* shuttle vector containing apr_{BamP} (apr[BamP]) on an *Eco*RI-*Sal*I fragment; there is a unique *Bam*HI site at the pre-pro junction. Symbols: ~~~, apr_{BamP} sequence; ▬, the mature protein A (*spa*) gene. pGX2912 has been described (2, 3). The DNA sequence across the fusion junction was analyzed, and the deduced amino acid sequence is shown at the bottom of the figure.

spa gene has been published (8, 16). There are two *Bcl*I sites in the coding region of mature protein A, corresponding to residues 22 through 23 and 428 through 429, respectively. pGX2912 was first partially digested with *Bcl*I and then completely digested with *Pvu*II. From this digest, a fragment of *spa* lacking the region coding for signal peptide and the N-terminal 22 amino acids of the mature protein was isolated. pGX2136 (*apr-spa*) contains this *Bcl*I-*Pvu*I *spa* fragment in place of the smaller *Bam*HI-*Pvu*II fragment of pGX2134 (Fig. 2). *B. subtilis* GX4935 containing pGX2136 was designated GX2811. An *Eco*RI-*Sal*I fragment containing *apr-spa* from pGX2136 was also cloned between the *Eco*RI and *Sal*I sites of pGX2104 (19), resulting in pGX2143.

npr-spa. For the construction of *npr-spa*, the partial *Bcl*I-plus-*Pvu*II digest of pGX2912 was ligated (without fragment purification) to pGX2138 which had been digested with *Bam*HI and *Eco*RV (Fig. 3). From this mixture, pGX2140 and pGX2141 were isolated. In pGX2140, the internal *Bcl*I fragment of the protein A gene replaced the smaller *Bam*HI fragment of pGX2138. pGX2141 contains the *Bcl*I-*Pvu*II fragment at the *Bam*HI-*Eco*RV site of pGX2138. The *npr-spa*-containing *Eco*RI-*Sal*I fragment from pGX2140 was cloned between the *Eco*RI and *Sal*I sites of pGX2104 (19), resulting in pGX2144.

The proteins encoded by pGX251 (vector), pGX2136 (*apr-spa*), pGX2141 (*npr-spa*), and pGX2912 (*spa*) were analyzed in a coupled transcription-translation system with an *E. coli* S30 extract (Amersham Corp., Arlington Heights, Ill.). A protein band of approximately 62 kilodaltons was produced from pGX2912. In the case of pGX2136 and pGX2141, a protein band of around 59 kilodaltons was present (Fig. 4). The greater mobility of pGX2136 (*apr-spa*) and pGX2141 (*npr-spa*) gene products reflects the absence of the 22 N-terminal amino acids of mature protein A.

Expression of *apr-spa* and *npr-spa* in *B. subtilis*. Strains GX2855(pGX251), GX2811(pGX2136), and GX2836(pGX 2141) were labeled for 5 min with [^{35}S]methionine, followed by a 5-min chase in the presence of nonradioactive methionine and puromycin, an inhibitor of protein synthesis. Strains GX2811(pGX2136) and GX2836(pGX2141) synthesized a protein that reacted with Immunobeads (Fig. 4). This protein was found in both the cellular and supernatant fractions with an apparent mobility of 56 kilodaltons which was faster than that of the protein that was made in vitro (59 kilodaltons) possibly due to the removal of signal peptide. The protein A in the supernatant seemed to have a slightly slower mobility than cellular fraction, possibly due to the salts in the medium. When the protein A from both cellular and supernatant fraction were pooled before being loaded onto the gel, a single band was observed.

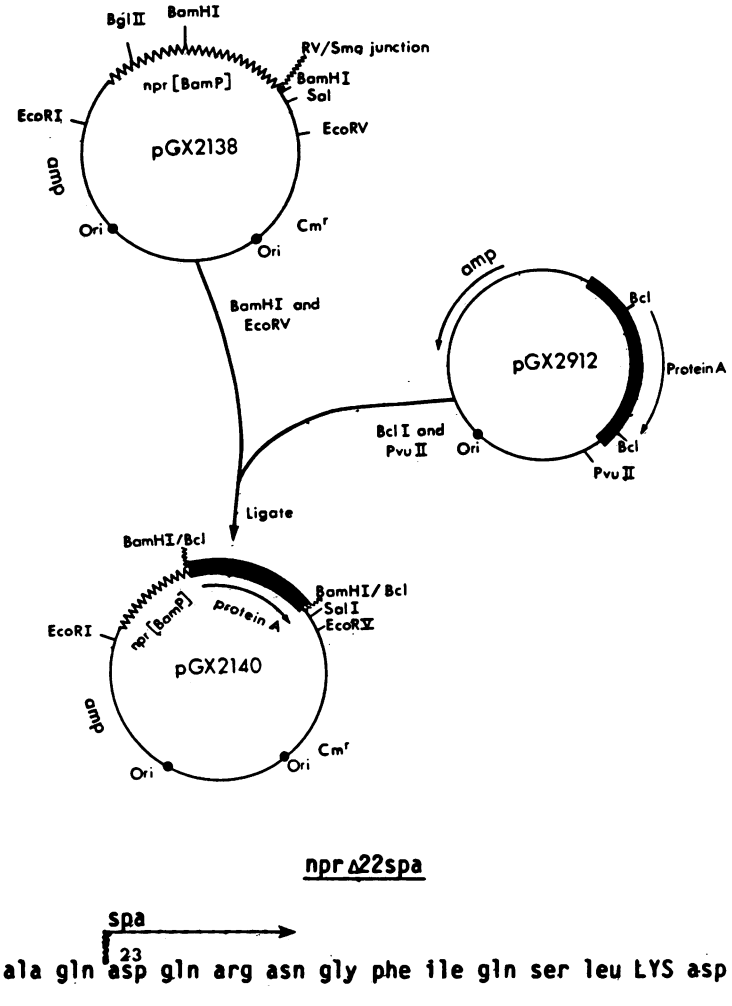


FIG. 3. Construction of the *npr-spa* fusion. pGX2138 is an *E. coli-B. subtilis* shuttle vector containing part of npr_{BamP} (npr[BamP]) (19). There is a *Bam*HI site at the pre-pro junction. npr_{BamP} (~~~) and mature protein A gene, *spa*, (▬) are shown. pGX2140 contains only the internal *Bcl*I fragment of *spa*. pGX2141 (not shown) contains the *Bcl*I-*Pvu*II fragment of *spa* from pGX2912 at the *Bam*HI-*Eco*RV site of pGX2138. Analysis of the DNA sequence across the fusion junction led to the deduced amino acid sequence which is shown at the bottom of the figure.

Identification of the signal-processing site of *apr-spa* and *npr-spa*. Both apr_{BamP} and npr_{BamP} encode proteins which are considerably larger than the mature, extracellular proteases. These have been termed preproproteases, but the proprotease forms of the enzymes have not been isolated. Therefore, neither the amino-terminal sequence of proAPR nor that of proNPR has been determined directly, and the mechanism of secretion of the proteases is not understood. In particular, although the amino-terminal peptide encoded by both open reading frames resembles a signal peptide, it is not known for either protease whether this putative signal sequence is removed during secretion, and if it is, where the signal processing occurs. The results described in the preceding section suggest that the protease signal sequences could be processed from the APR-SPA and NPR-SPA fusion proteins. Further analysis of the secreted product allowed us to determine the site of processing in each case.

B. subtilis cells carrying *apr-spa* (pGX2143) or *npr-spa* (pGX2144) were labeled with [^{3}H]lysine, and the secreted protein A was isolated from the culture supernatant. A sample of the isolated protein A was analyzed by polyacrylamide gel electrophoresis and shown to be a single electrophoretic species in each case. The rest of the protein A was subjected to Edman degradation, and the radioactivity released in the first 15 cycles was determined (Fig. 5a and b). In both cases, the label was found to be predominantly associated with sequenator cycle 13. These results are consistent with a location of the signal-processing site at the Ala-Ala junction which had been predicted for both apr_{BamP} and npr_{BamP} (Fig. 1). However, in the case of the protein A secreted from GX2815(pGX2143), radioactivity was also released in cycles 14 and 15 (Fig. 5a). This could be due to multiple processing sites or proteolytic degradation (see Discussion) or both.

DISCUSSION

Secretion vectors based on the α-amylase genes of either *B. amyloliquefaciens* or *B. subtilis* have been used in the development of *B. subtilis* strains capable of secreting heterologous proteins (12, 13, 20). For example, Fahnestock et al. have shown that *B. subtilis* cells can secrete protein A under the influence of either the native protein A signal sequence or the *B. amyloliquefaciens* α-amylase signal sequence (2, 3). We wanted to develop secretion vectors based

on protease genes to study the effect of different signal sequences on the secretion of heterologous proteins in *B. subtilis*.

The apr_{BamP} and npr_{BamP} genes both contain a large open reading frame in the DNA sequence preceding the sequence encoding the mature protein. At the 5′ end of each gene, there is a sequence which encodes a polypeptide which appears to be a signal sequence. In each case, this polypeptide has a number of positively charged amino acid residues at the amino terminus (three in the case of apr_{BamP} and two in the case of npr_{BamP}). The polypeptides also each have a stretch of hydrophobic amino acids. By using the hypothesis of Perlman and Halvorson, a small number of plausible locations for the signal-processing site of each gene could be predicted (14). To determine whether these polypeptides were signal sequences and whether each was sufficient to translocate a heterologous protein across the cell membrane, we constructed gene fusions which linked the signal sequence of each protease to mature protein A. *B. subtilis* strains carrying such gene fusions were able to synthesize protein A and secrete it into the growth medium. The signal sequences of apr_{BamP} or npr_{BamP} were also each capable of translocating β-lactamase into the growth medium (S. R. Fahnestock, unpublished data). Thus, the amino-terminal sequences of apr_{BamP} and npr_{BamP} can act as signal sequences.

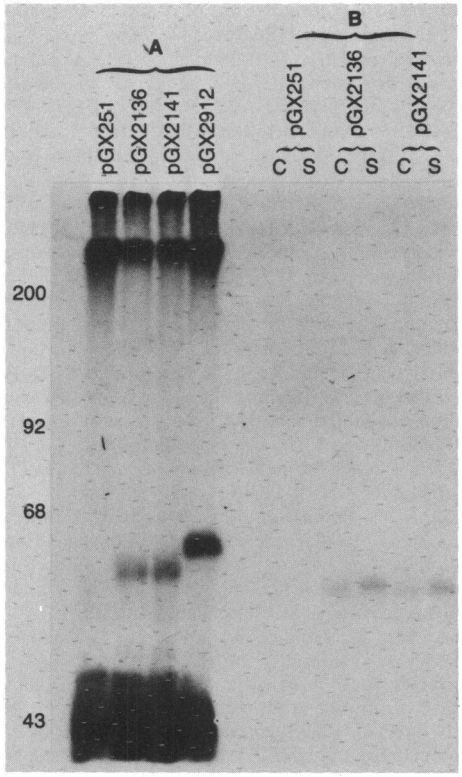


FIG. 4. Identification of *apr-spa* (pGX2136) and *npr-spa* (pGX2141) gene products. (A) Proteins made in vitro with *E. coli* S30 extract in a coupled transcription-translation system by pGX251 (vector), pGX2136 (*apr-spa*), pGX2141 (*npr-spa*), and pGX2912 (*spa*) were analyzed in a 10% polyacrylamide–SDS gel electrophoresis. (B) Synthesis of protein A in *B. subtilis*. Strains carrying pGX251, pGX2136, or pGX2141 were grown in synthetic medium and pulse-labeled with [^{35}S]methionine for 5 min and chased for 5 min. The cell-associated (lanes C) and extracellular (lanes S) protein A was isolated based on its ability to bind to Immunobeads and electrophoresed. Numbers (200, 93, 68, and 43) to the left of the gel refer to the mobilities of those prestained molecular weight standards.

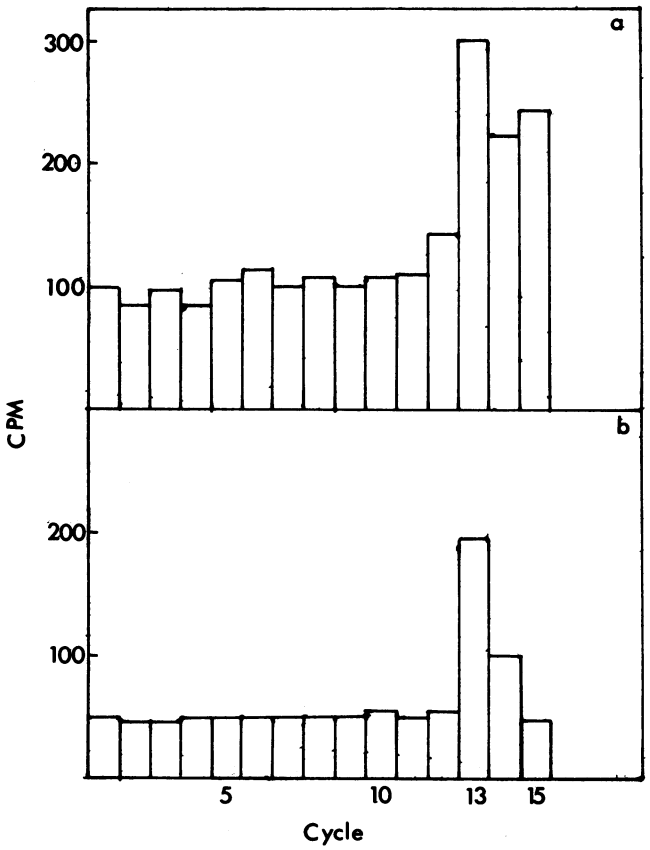


FIG. 5. Location of [^{3}H]lysine-containing residues in the amino-terminal residues of sectreted protein A from (a) GX2815(pGX2143) (a) and GX2834 (pGX2144) (b). GX2815 and GX2834 were grown in synthetic medium and labeled for 5 min with [^{3}H]lysine in the presence of 5 mM phenylmethylsulfonyl fluoride as described in Materials and Methods. The secreted protein A was purified, solubilized, and subjected to Edman degradation (9). The amount of radioactivity released in each cycle was counted in a scintillation counter.

We then attempted to determine the site at which each signal sequence was removed from the product of the gene fusion. The results allowed us to identify a site which was consistent with a site in the primary translation product of the wild-type gene (npr_{BamP}) which had been predicted to be a processing site. However, *B. subtilis* cells containing *apr-spa* secreted protein A with heterogeneous termini. The amino acid sequence around the putative signal-processing site of apr_{BamP} is Ser-Ala-Gln-Ala-Ala, wherein we had identified the Ala-Ala bond as the most plausible cleavage site. It is conceivable that there are multiple signal-processing sites, as we cannot rule out the possibility that the signal processing primarily occurred at the Ala-Gln bond and that there was proteolytic degradation. However, it should be noted that, although both pGX2143 (*apr-spa*) and pGX2144 (*npr-spa*) fusions are in the same host background (GX4935) and the radiolabeling experiments were performed in the presence of 5 mM phenylmethylsulfonyl fluoride under identical conditions, the protein A secreted from GX2834 (pGX2144) had a predominantly homogenous N terminus.

B. subtilis cells carrying the *spa* gene fusions accumulate the full-length precursor (signal peptide fused to the mature protein A) during a short labeling (N. Vasantha, unpublished data). At least 30% of the signal processing seems to occur after the completion of the synthesis of the protein A. Josefsson and Randall had observed full-length precursors for a variety of secreted proteins in *E. coli* (6).

We also found a lag between the time of appearance of the mature protein A in the cell and the time of its appearance in the culture supernatant. Gould et al. observed a 15-min lag between the synthesis and secretion of amylase and protease in *B. amyloliquefaciens* cells (4). The lag was not observed with protoplasts, from which the cell wall had been removed (4). The lag we observe also might reflect the time taken for the protein to pass through the cell wall of *B. subtilis*.

Strains GX2815(pGX2143) and GX2834(pGX2144) accumulate protein A in amounts ranging from 10 mg to 2.9 g per liter in the growth medium, depending upon the components of the medium and the culture conditions (L. D. Thompson and N. Vasantha, *in* A. T. Ganesan and J. Hoch, ed., *Genetics and Biotechnology of Bacilli*, in press). Those results, along with the signal processing data, demonstrate that the protease signal sequences can be used to obtain efficient heterologous protein secretion from *B. subtilis*.

B. subtilis cells can secrete two procaryotic heterologous proteins (protein A and β-lactamase) when the appropriate gene is fused to a variety of signal sequences (2, 3, 13, 20). Thus, despite minor variations such as the number of positive charges in the signal sequence and the exact amino acid sequence at the signal-processing site, *B. subtilis* cells have the intrinsic ability to express a fused gene to translocate a heterologous protein across the cell membrane and to secrete the heterologous protein to the growth medium. Thus, with the identification of the signal-processing site for the two protease genes, it is now feasible to study the effects of different signal sequences on the secretion of eucaryotic proteins from *B. subtilis*.

ACKNOWLEDGMENTS

We thank Mark Guyer for useful discussions and critical review of the manuscript; Stephen Fahnestock, Ethel Jackson, and Charlie Saunders for helpful discussions; Martin Lindberg and Pharmacia, AB, for providing the cloned Protein A gene; Brian Schmidt for providing competent *B. subtilis* cells; Jeanne Strasser and David Filpula for DNA sequencing; Steve Pulford for synthesizing the oligonucleotides; and Sally Young for typing the manuscript.

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