JOURNAL OF BACTERIOLOGY, July 1993, p. 4203–4212
0021-9193/93/134203-10$02.00/0

Vol. 175, No. 13

Structural Requirements of *Bacillus subtilis* α-Amylase Signal Peptide for Efficient Processing: In Vivo Pulse-Chase Experiments with Mutant Signal Peptides

YOSHIKIYO SAKAKIBARA, KIYOFUMI TSUTSUMI, KOUJI NAKAMURA, AND KUNIO YAMANE*

Institute of Biological Sciences, University of Tsukuba, Tsukuba-shi, Ibaraki 305, Japan

Received 28 December 1992/Accepted 3 May 1993

The *Bacillus subtilis* α-amylase signal peptide consists of 33 amino acids from its translation initiation site. To analyze the structural requirements for efficient processing of the signal peptide, single and repeated Ala-X-Ala sequences and their modifications were introduced into *B. subtilis* α-amylase signal peptides of different lengths and the mature thermostable α-amylase. Then the cleavage positions and processing rates of the signal peptides were analyzed by the NH_2-terminal amino acid sequences of the exported thermostable α-amylases and by in vivo pulse-chase experiments. In *B. subtilis*, the most efficient cleavage site was located at the peptide bond between Ala-33 and amino acid X at position 34, even though Val-X-Ala and six repeating Ala-X-Ala sequences were present around the cleavage site. However, the cleavage site was shifted to the peptide bond between Ala-31 and amino acid X when Ala-33 was deleted, and it was also shifted to Ala-35 and X when Ala-33 was replaced with Val-33. The shorter signal peptide consisting of 31 amino acids reduced the processing rate and α-amylase production. In contrast, those signal peptides were cleaved preferentially at the peptide bond between Ala-31 and amino acid X in *Escherichia coli*. In addition to the presence of an Ala residue at the −1 amino acid position, the length of the signal peptide was another important requirement for efficient processing.

Most exported proteins are synthesized as precursors with an NH_2-terminal extension, the signal peptide, which is removed by a specific enzyme. The signal peptide and signal peptidase mechanisms for the secretion of exported proteins are similar in both prokaryotic and eukaryotic cells (2, 8, 10, 22). In prokaryotic cells, the amino acid residues at positions −3 and −1 are important for cleavage site recognition by the signal peptidases when signal peptides are cleaved between positions −1 and +1, with position −1 being particularly important (30). On the basis of the amino acid sequences of signal peptides reported by Watson (31) and Perlman and Halvorson (23), we compared the amino acid residues at positions −3 and −1 in 34 exported proteins of prokaryotic cells, excluding lipoproteins, whose signal peptides are cleaved by signal peptidase II (6). Eighty percent (27 of 34) of the amino acid residues at position −1 consisted of Ala, 44% (15 of 34) of the amino acid sequences at positions −3 and −1 were Ala-X-Ala, and 9% (3 of 34) of the sequences were Val-X-Ala and Thr-X-Ala. The sequence Ala-X-Ala predominates at positions −3 and −1 of the signal peptide cleavage site in precursors of exported proteins.

Yamazaki et al. (34) predicted the amino acid sequence around the signal peptide cleavage site of *Bacillus subtilis* α-amylase to be Gly-27–Pro–Ala–Ala–Ala–Ser–Ala ↓ Glu–Thr-35. The cleavage site was confirmed as the peptide bond between Ala-33 and Glu-34 (Fig. 1a), on the basis of the analysis of the NH_2-terminal amino acid sequences of the intermediate and mature forms of α-amylase (28) and on the basis of the analysis of deletion mutations in the *B. subtilis* α-amylase gene (*amyE*) (25). The propeptide from Glu-34 to Glu-41 in the secreted precursor protein of AmyE was mainly cleaved by the action of extracellular alkaline protease (12).

In the signal peptide of AmyE, there were two Ala-X-Ala sequences at positions 29 to 31 and 31 to 33 near the signal peptide cleavage site. A cleavage site was found only at the peptide bond between Ala-33 and Glu-34 and not after Ala-31.

In order to analyze the structural requirements for signal peptide cleavage in *B. subtilis* and to compare the sites with those in a gram-negative bacterium, *Escherichia coli*, whose protein translocation apparatus has been extensively studied, we constructed artificial hybrid genes (*amyE'-'amyT*) for the *amyE* signal peptides of different lengths and the mature thermostable α-amylase (*'amyT*) of *Bacillus stearothermophilus*. The thermostable α-amylase does not contain a natural prosequence (26). DNA sequences encoding single and repeated Ala-X-Ala sequences and their modifications were introduced between *amyE'* and *'amyT*. The *amyE'-'amyT* hybrid genes were highly expressed in both *B. subtilis* and *E. coli* cells, although the expression of *amyE* in *E. coli* cells was only 3 to 5% of that in *B. subtilis*. The resulting thermostable α-amylases were rapidly purified in the presence of phenylmethylsulfonyl fluoride (PMSF). Signal peptide cleavage sites were determined by the NH_2-terminal amino acid sequences of the exported thermostable α-amylases from the hybrid genes. It has been shown that a possible secondary proteolysis in the α-amylase preparations did not occur or was negligible under the present culture conditions with the protease-deficient *B. subtilis* strain and *E. coli* HB101 as the host cells and during the subsequent purification steps under hydrophobic conditions (11, 12). The processing rates of the signal peptides were evaluated by pulse-chase experiments.

In this article, we demonstrate that the length of the signal peptides is an important factor for their efficient cleavage in addition to the presence of an Ala residue at the −1 amino acid position of the signal peptide cleavage sites and that the cleavage sites are changeable.

* Corresponding author.

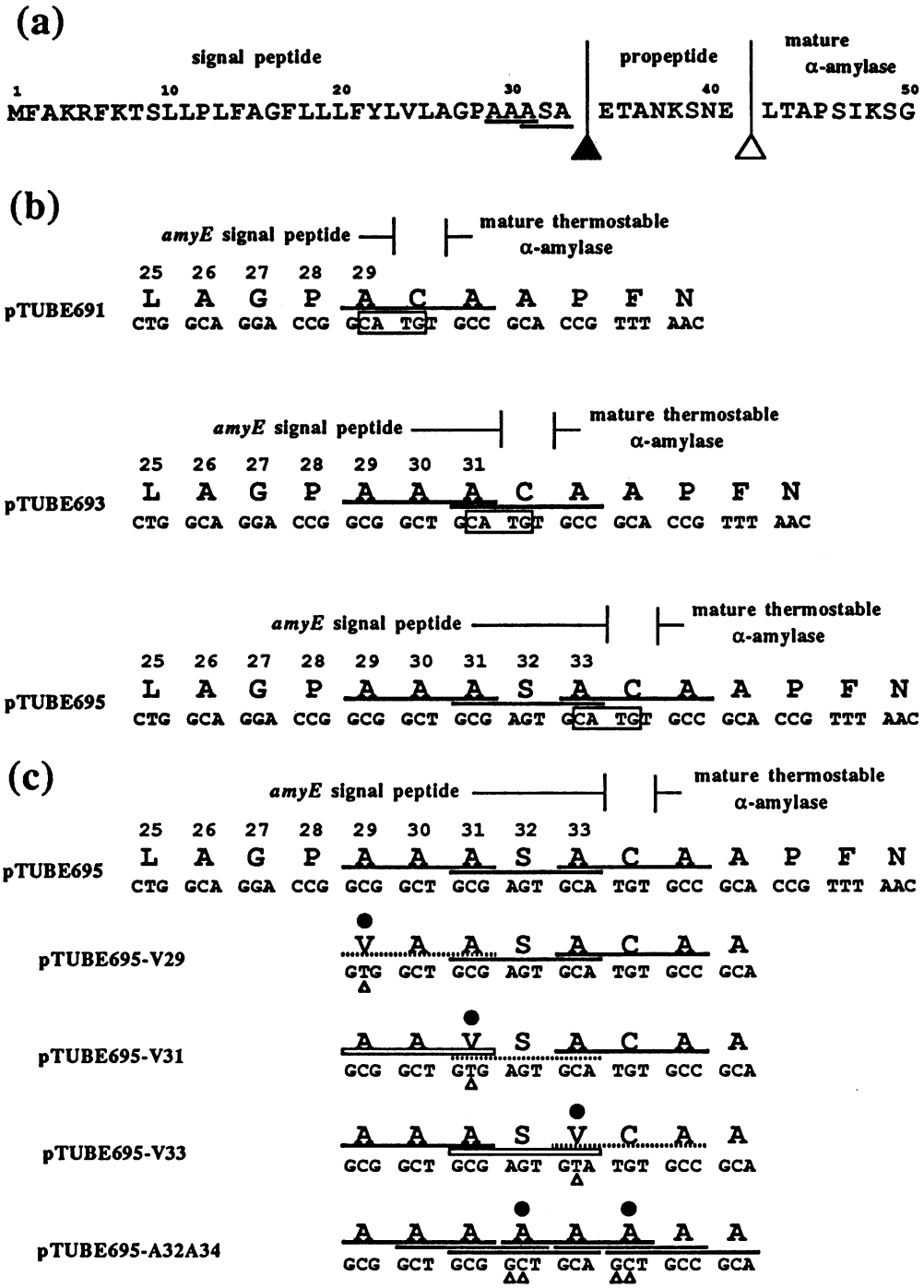

FIG. 1. Predicted amino acid sequences around the signal peptide cleavage sites in the wild-type and mutant precursor proteins containing one or repeated Ala-X-Ala sequences and in their mutations. (a) Predicted amino acid sequence of the signal peptide and propeptide of *B. subtilis* α-amylase. Numbers above the amino acid residues indicate the positions from the translation initiator, Met. Ala-X-Ala sequences are underlined. Symbols: ▲, signal peptide cleavage site; △, final cleavage site for formation of the extracellular mature α-amylase of *B. subtilis*. (b) Nucleotide and predicted amino acid sequences around the junction regions of *B. subtilis* α-amylase signal peptide (*amyE'*) and the mature thermostable α-amylase (*'amyT*) of *B. stearothermophilus* A631 in plasmids pTUBE691, pTUBE693, and pTUBE695, in which one, two, and three Ala-X-Ala sequences were inserted, respectively. The nucleotides derived from the *Hin*dIII linker DNA are boxed. (c) Nucleotide and amino acid replacements in mutated plasmids pTUBE695-V29, -V31, -V33, and -A32A34, which were prepared from pTUBE695. Symbols: △, replaced nucleotides; ●, replaced amino acid residues; ▬, Ala-X-Ala sequence; ·····, Val-X-Ala sequence; ▭, Ala-X-Val sequence.

MATERIALS AND METHODS

Bacterial strains. An α-amylase- and protease-deficient strain of *B. subtilis*, 104HLA (*amyE15 nprE18 ΔaprA3 hisA1 leuA8* Str^r), which was derived from a protease-deficient strain, *B. subtilis* BD104 (13), and *E. coli* HB101 (F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$) were used as the host cells. These strains harboring plasmids were cultured in L broth containing 10 μg of kanamycin per ml under conditions described previously (33).

Construction of *E. coli-B. subtilis* shuttle plasmids pTUBE691, pTUBE693, and pTUBE695 containing single and repeated Ala-X-Ala sequences. To construct the pTUBE695 plasmid containing three Ala-X-Ala sequences around the signal peptide cleavage site, an *E. coli-B. subtilis* shuttle plasmid, pTUBE219-5 (25), was used, in which a DNA sequence (*amyE'*) encoding the 33 amino acids (only the first nucleotide in the codon for Ala-33) from the translation initiator Met of *amyE'* followed by *Hin*dIII linker DNA (5'CAAGCTTG3') was present. The 2.2-kb *Hin*dIII-*Bgl*II fragment of pTUBE219-5 was replaced with a 5.5-kb *Hin*dIII-*Bgl*II fragment from pTUB616 (26), with the DNA encoding the mature enzyme of *'amyT* with 1 bp (TA) added at its 5' terminus after the *Hin*dIII linker. The constructed plasmid was cleaved at the unique *Hin*dIII site, treated with mung bean nuclease, and religated. By this treatment, 4 bp of the *Hin*dIII cohesive ends were deleted and the *amyE'* signal peptide was able to fuse with *'amyT* in a correct reading frame, although the Cys residue (TGT) remained at the junction of *amyE'* and *'amyT* (Fig. 1b). By the same strategy, pTUBE691 and pTUBE693 containing one or two Ala-X-Ala sequences were constructed from *E. coli-B. subtilis* shuttle plasmids pTUBE219-1 and pTUBE219-3, with DNA sequences encoding the 29 (only the first nucleotide in the codon for Ala-29) and 31 (only the first nucleotide in the codon for Ala-31) amino acids from the Met of *amyE'*, respectively. The DNA nucleotide sequences of the constructed plasmids were ascertained by the method of Sanger et al. (24), and the predicted amino acid sequences in the plasmids are indicated in Fig. 1b.

Site-directed mutagenesis and construction of mutagenized plasmids pTUBE695-V29, -V31, -V33, and -A32A34. Four appropriate mutagenic oligonucleotides (18-mer to 22-mer) were synthesized on an automated DNA synthesizer (model 380A; Applied Biosystems). The oligonucleotides were phosphorylated and subjected to site-directed mutagenesis on pTUBE695 according to the method of Kunkel (15). The resulting plasmids were designated as pTUBE695-V29 (Ala-29→Val-29), pTUBE695-V31 (Ala-31→Val-31), pTUBE695-V33 (Ala-33→Val-33), and pTUBE695-A32A34 (Ser-32–Cys-34→Ala-32–Ala-34) (Fig. 1c).

Transformation of *E. coli* and *B. subtilis* cells. Transformation of *B. subtilis* 104HLA by the plasmids was performed by the protoplast transformation method of Chang and Cohen (4). Transformation of *E. coli* HB101 was accomplished by the method of Hanahan (9).

Purification of thermostable α-amylases from *B. subtilis* and *E. coli*. To determine the NH_2-terminal amino acid sequences of the exported thermostable α-amylases, the plasmid-encoded enzymes were purified from the culture supernatants of *B. subtilis* and the periplasm of *E. coli* strains in the presence of 1 mM PMSF. All purification procedures were performed at 4°C or on ice. To prepare the thermostable α-amylases from the transformants of the protease-deficient *B. subtilis* strains, the cells were grown in 2 to 5 liters of L broth containing 10 μg of kanamycin per ml at 37°C for 24 h. The cells were removed by centrifugation (6,000 × *g* for 10 min) and the proteins in the supernatants, containing 3×10^5 to 5×10^5 U of α-amylases, were precipitated by the addition of solid ammonium sulfate to reach 85% saturation. The pellets were dissolved in 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM PMSF, and, after adjustment with ammonium sulfate to 25% saturation by the addition of a saturated solution, they were loaded onto Toyopearl HW55 columns (1.2 by 5 cm) that had been equilibrated with ammonium sulfate (25% saturation) in 10 mM Tris-HCl, pH 7.5. The α-amylases were eluted with a 300-ml linear gradient of ammonium sulfate (25 to 0% saturation) in 10 mM Tris-HCl buffer, pH 7.5. They were eluted at 15 to 12% saturation. The amylases in the eluates were precipitated by the addition of solid ammonium sulfate to 85% saturation.

To prepare the α-amylases from the periplasm of *E. coli* strains, the enzymes were extracted from the cells by the osmotic-shock method of Chan et al. (3) in the presence of 1 mM PMSF after cultivation of the cells at 37°C for 12 h. Then proteins containing 5×10^5 U of α-amylases were precipitated by the addition of solid ammonum sulfate and α-amylases were purified with the same columns and under the same conditions as described above.

The purity of the thermostable α-amylases in the preparations ranged from 80 to 90% on the basis of sodium dodecyl sulfate (SDS)–8.5% polyacrylamide gel electrophoresis (16) followed by staining with Coomassie brilliant blue. The specific activities (units per milligram of protein) of the thermostable α-amylase preparations from both the periplasm of *E. coli* and the culture supernatants of *B. subtilis* were 1.8×10^4 U/mg of protein on the basis of the starch-degrading activity assayed by the blue value method of Fuwa (7).

Amino acid sequencing. For the determination of the NH_2-terminal amino acid sequences of the thermostable α-amylases, 100 μg of amylase proteins, which were estimated on the basis of their specific activities in each purified α-amylase preparation, were boiled for 3 min in a 1% SDS–1% mercaptoethanol solution. Then each sample was subjected to SDS–8.5% polyacrylamide gel electrophoresis. The bands corresponding to the amylases were blotted onto a polyvinylidene difluoride membrane by the method of Matsudaira (17). Then the NH_2-terminal amino acid sequences of the blotted enzymes were determined by an automatic Edman degradation sequencer (470A sequencer; Applied Biosystems).

Pulse-chase experiments to analyze the signal peptide processing rates in *B. subtilis* and *E. coli*. The *B. subtilis* and *E. coli* strains containing each plasmid were grown in modified L broth and M9 media, respectively. When the cultures reached a reading of 300 in a Klett colorimeter, the formation of the precursor and mature forms of the thermostable α-amylases was examined by pulse-labeling of the cells with 100 μCi of [^{35}S]methionine (DuPont-New England Nuclear) for 30 s at 37°C followed by a chase with 200 μg of nonradiolabeled L-methionine per ml. The labeled cultures (300 μl) of *B. subtilis* were withdrawn at the indicated periods, immediately mixed with an equal volume of ice-cold 10% trichloroacetic acid, and kept on ice for 30 min. Precipitates were collected by centrifugation, washed with acetone, dried, and dissolved in 30 μl of 50 mM Tris-HCl buffer, pH 8.0, containing 2 mM EDTA and 1% glucose. Then the samples were incubated with 5 mg of egg white lysozyme per ml at 37°C for 10 min and boiled for 10 min in the presence

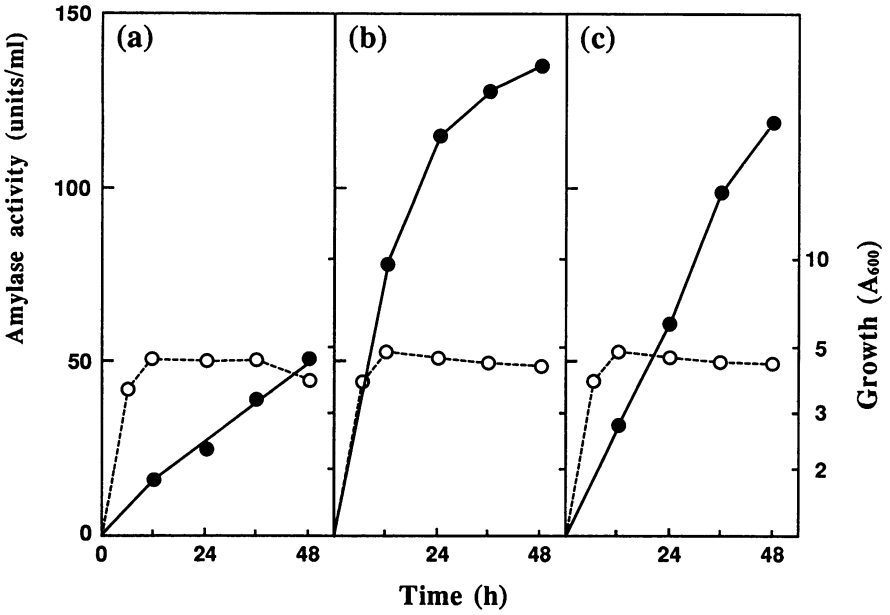

FIG. 2. Activity of thermostable α-amylases in culture supernatants of *B. subtilis* 104HLA transformants carrying pTUBE691 (a), pTUBE693 (b), and pTUBE695 (c). The cells were cultured in L broth containing 10 μg of kanamycin per ml at 37°C, and the cells were removed by centrifugation at 6,000 × *g* for 10 min. Symbols: ●, amylase activity at 60°C; ○, growth.

of 0.1% SDS. The thermostable α-amylases were precipitated with antiserum against AmyT of *B. stearothermophilus*. The labeled cultures of *E. coli* were boiled for 3 min in the presence of 1% SDS and 1 mM EDTA (20). The thermostable α-amylases were precipitated with anti-AmyT serum. The immunoprecipitated materials were washed, boiled for 3 min in the presence of 1% SDS and 1% mercaptoethanol, and subjected to SDS–10% polyacrylamide gel electrophoresis to obtain autoradiographs. The densities of the bands for the precursor and mature forms of the thermostable α-amylases were quantitated with a densitometer (Bio-Image; Millipore Co., Ann Arbor, Mich.).

RESULTS

Expression of thermostable α-amylases from pTUBE691, pTUBE693, and pTUBE695 in *B. subtilis*. Similar amounts of the enzymes (125 and 115 U/ml after 48 h of cultivation) were produced in the culture supernatants of *B. subtilis* strains harboring pTUBE693 or pTUBE695 (Fig. 2b and c). The activity in the strain harboring pTUBE691 (Fig. 2a), however, was approximately 40% (48 U/ml) of those of the former two. In the three *B. subtilis* strains, more than 85% of the amylase activity was located in the culture supernatants and less than 10 U of the activity per ml was found in the cells. The molecular masses of the extracellular α-amylases from the three plasmids were estimated to be 61 kDa, the same as that of the enzyme produced by *B. stearothermophilus*.

Processing rates of signal peptides in the precursors from the three plasmids. The processing of the signal peptides of the extracellular α-amylases from the three plasmids was analyzed by pulse-chase experiments (Fig. 3). In the *B. subtilis* strain harboring pTUBE691, a large amount of the precursor accumulated and approximately 50% of the proteins still remained as the precursor after 4 min of chase. However, the precursors in the strains harboring pTUBE693 and pTUBE695 were efficiently converted into their mature forms during 4 min of chase. To estimate the processing rate of each signal peptide, the bands for the precursor and mature proteins in the autoradiographs shown in Fig. 3 were quantitated densitometrically and the half-life of each precursor protein was calculated from Fig. 4. The results are summarized in Fig. 7. The signal peptide of pTUBE691

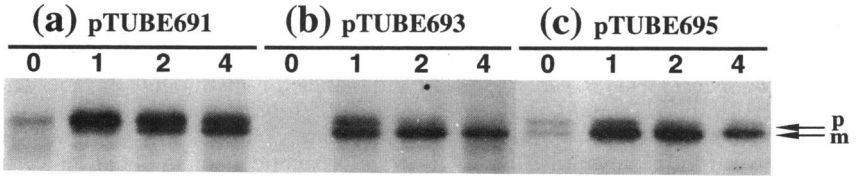

FIG. 3. Autoradiographs of SDS-polyacrylamide gels resolving immunoprecipitates with rabbit anti-AmyT serum from transformants of *B. subtilis*. *B. subtilis* strains containing pTUBE691 (a), pTUBE693 (b), and pTUBE695 (c) were pulse-labeled with [^{35}S]methionine for 30 s and chased with nonlabeled methionine for 0, 1, 2, and 4 min. Samples were precipitated with the serum after the cells were lysed, and then samples were analyzed by SDS-polyacrylamide gel electrophoresis. p, precursor proteins of the thermostable α-amylases from the plasmids; m, mature forms.

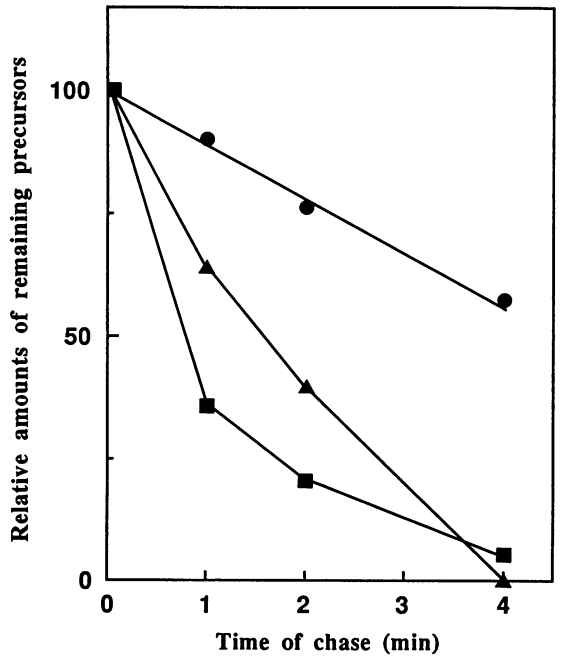

FIG. 4. Kinetics of the processing of the precursor proteins of the thermostable α-amylases in transformants of *B. subtilis*. Autoradiograms in Fig. 3 were quantitated densitometrically. The proportions of the densities of the bands for the precursor proteins against the total densities of the bands for the precursor and mature α-amylases were calculated. The calculated proportions at 0 min of chase were expressed as 100, and relative proportions at 1, 2, and 4 min of chase were plotted for each α-amylase. Relative amounts of the precursor proteins from pTUBE691 (●), pTUBE693 (▲), and pTUBE695 (■) are indicated. The half-lives (in minutes) of each precursor protein were obtained, and they are summarized in Fig. 7.

α-amylase does not seem to be a suitable peptide in *B. subtilis*. Its half-life was approximately three and six times longer than those of the precursors from pTUBE693 and pTUBE695, respectively.

The conversion of the precursor proteins to the mature forms was completely inhibited by the addition of 25 and 50 mM carbonyl cyanide *m*-chlorophenylhydrazone as reported for the processing of signal peptides of exported proteins in *E. coli* (5) and *B. subtilis* (18) cells (data not shown).

NH_2-terminal amino acid sequences of the thermostable α-amylases from pTUBE691, pTUBE693, and pTUBE695. The NH_2-terminal amino acid sequence of the extracellular α-amylase from pTUBE691 was Ala-Pro-Phe-Asn-Gly--. This indicates that the precursor proteins from pTUBE691 were cleaved at the peptide bond between Ala-31 and Ala-32 (Fig. 5a). The Ala residues at the NH_2 terminus corresponded to the second amino acid of the mature thermostable α-amylase when it was produced by the parental strain, *B. stearothermophilus* A631, and by the parental plasmid (pTUB607) in *B. subtilis* (26). No cleavage between Ala-29 and Cys-30 was observed.

The same NH_2-terminal amino acid sequence, Ala-Pro-Phe-Asn--, starting from position 34 was observed in the pTUBE693 α-amylase, whereas another NH_2-terminal amino acid sequence, Cys-Ala-Ala-Pro-Phe--, starting at position 34 was detected in the pTUBE695 α-amylase (Fig. 5b and c). These results indicated that the precursor proteins from pTUBE693 and pTUBE695 were cleaved at the peptide bond between Ala-33 and Ala-34 and between Ala-33 and Cys-34, respectively.

The processing rate of the signal peptide in the pTUBE691 α-amylase was reduced to one-third to one-sixth of those of the precursors from pTUBE693 and pTUBE695, and the extracellular production of α-amylase in the strain harboring pTUBE691 was decreased to 40% of the amounts found in the strains harboring pTUBE693 and pTUBE695. Therefore, the 33-amino-acid signal peptides in the precursors from pTUBE693 and pTUBE695 were more efficient for secretion of the α-amylases than the 31-amino-acid signal peptide of pTUBE691.

Effect of the replacement of Ala with Val around the signal peptide cleavage site. To analyze the changes of the signal peptide cleavage sites and the processing rate in the precursor protein from pTUBE695, Ala residues at positions 29, 31, and 33 were replaced with Val as shown in Fig. 1c. By these replacements, it became possible to analyze the effect of the presence of Val-X-Ala and Ala-X-Val sequences on the cleavage sites.

The extracellular production of the α-amylases by the *B. subtilis* transformants harboring pTUBE695-V29, -V31, and -V33 was quite similar to that of the parental strain harboring pTUBE695. The processing rates of the precursor proteins from the three plasmids, which were calculated from the pulse-chase experiments (Fig. 6), were also similar to the value found for the precursor from pTUBE695.

The NH_2-terminal amino acid sequences of the α-amylases from the mutant plasmids were analyzed, and the cleavage sites were determined (Fig. 7). An NH_2-terminal amino acid sequence, Cys-Ala-Ala-Pro-Phe--, starting from position 34 was detected in the α-amylase preparations from plasmids pTUBE695-V29 and -V31, while another NH_2-terminal amino acid sequence, Ala-Pro-Phe-Asn-Gly-Thr-Met--, starting from position 36 was observed in pTUBE695-V33 α-amylase. These amino acid sequences indicated that the precursor proteins from pTUBE695-V29 and -V31 were cleaved at the peptide bond between Ala-33 and Cys-34 and that the cleavage site in the precursor from pTUBE695-V33 was shifted to the peptide bond between Ala-35 and Ala-36. No cleavage at the peptide bond between Ala-31 and Ser-32 was observed.

These experiments indicated the following. (i) The signal peptides were cleaved at the peptide bonds after Ala-X-Ala and Val-X-Ala at equal rates in *B. subtilis* cells. (ii) The bond after Ala-X-Val in the precursor was not cleaved and the cleavage site was able to shift to another peptide bond after an Ala residue. (iii) A longer signal peptide was selected for the efficient cleavage and production of extracellular α-amylase in *B. subtilis*.

Signal peptide cleavage in *E. coli* cells. To compare the signal peptide cleavage in *B. subtilis* with that in *E. coli*, the cleavage sites and processing rates in the precursor proteins from the six plasmids in *E. coli* cells were also analyzed (Fig. 7). The signal peptides of pTUBE691, pTUBE693, and pTUBE695 functioned extremely well in *E. coli* cells, and amounts of each α-amylase approximately 10 times higher than those in *B. subtilis* were produced in their periplasm. The precursor proteins from the three plasmids were cleaved preferentially at the peptide bond between Ala-31 and amino acid X at position 32. The half-lives of the precursors were less than 0.5 min.

However, the processing rates in the modified precursor proteins from pTUBE695-V29, -V31, and -V33 were less than that of the precursor from pTUBE695, with half-lives of 2.1, 5.0, and 1.0 min, respectively. The peptide bond after

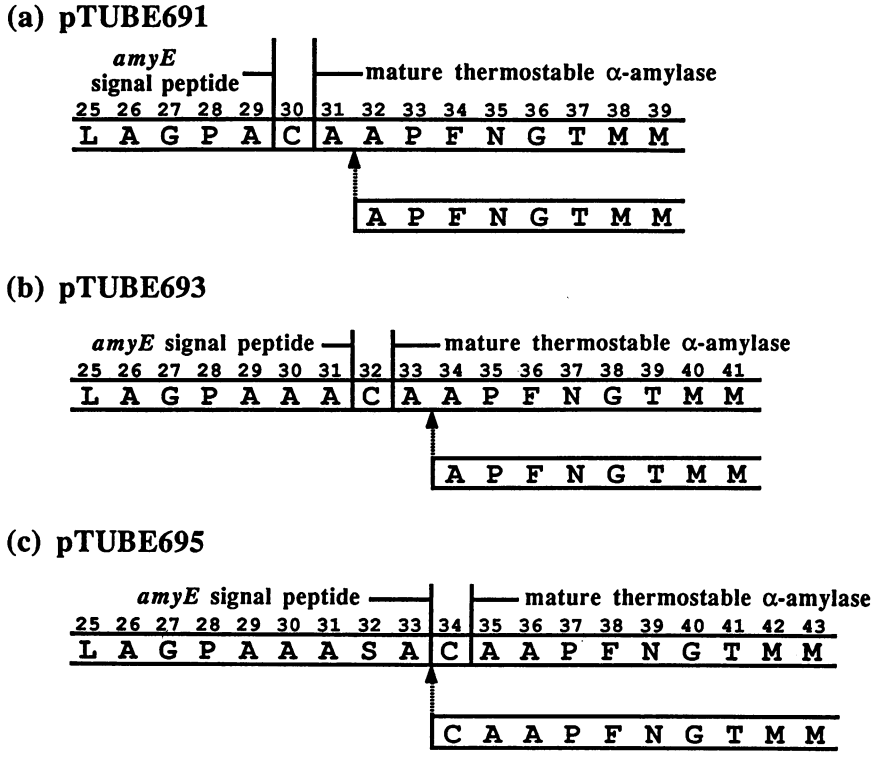

FIG. 5. NH_2-terminal amino acid sequences of extracellular α-amylases from *B. subtilis* transformants which harbor pTUBE691 (a), pTUBE693 (b), and pTUBE695 (c). Numbers above the amino acid residues indicate the amino acid positions from the initiator, Met. ▲, cleaved peptide bonds.

Val-X-Ala at positions 29 to 31 does not seem to be a suitable bond for cleavage. The processing rate of the precursor from pTUBE695-V29 was less than one-fourth that of the parental precursor from pTUBE695, with cleavage at the two peptide bonds between Ala-31 and Ser-32 and between Ala-35 and Ala-36 at a ratio of 1:1.

The signal peptide cleavage at the peptide bond between amino acid positions 31 and 32 disappeared when Ala-31 was replaced with Val. The cleavage site in pTUBE695-V31 was shifted to the peptide bond between Ala-35 and Ala-36 but not to the peptide bond between Ala-33 and Cys-34 or between Ala-29 and Ala-30. The processing rate was reduced to less than 1/10 that of pTUBE695. The production of the α-amylase in the periplasm of pTUBE695-V31 was decreased to approximately half the amount in the strain harboring pTUBE695. The peptide bond between Ala-33 and Cys-34 was almost never cleaved in *E. coli* cells, like in the pTUBE695-V29 protein, but the peptide bond after Ala-33 was cleaved well when Cys-34 in pTUBE695-V31 was replaced with Ala. In the mutant precursor protein, the ratio of the cleavage at each peptide bond between Val-31 and Ser-32, between Ala-33 and Ala-34, between Ala-34 and Ala-35, and between Ala-35 and Ala-36 was 0:2.0:0:1.0.

Signal peptide cleavage sites in an Ala cluster. To determine the preferential signal peptide cleavage sites in *B. subtilis* and *E. coli* cells, pTUBE695-A32A34 was constructed, from which precursor protein an Ala cluster consisting of eight Ala residues from positions 29 to 36 is present and six repeating Ala-X-Ala sequences are contained (Fig. 1c). The peptide bond between positions 33 and 34 was cleaved by *B. subtilis*. In contrast, the peptide bonds between positions 31 and 32 and positions 33 and 34 were cleaved at a ratio of 5:1 in *E. coli* cells (Fig. 7). In *E. coli*, it is possible to observe cleavage at the peptide bond between Ala-32 and Ala-33. However, the cleavage at this site was less than 1/10 that at

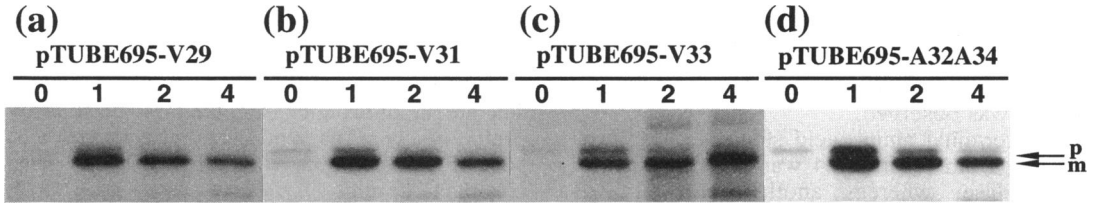

FIG. 6. Autoradiographs of SDS-polyacrylamide gels resolving immunoprecipitates with rabbit anti-AmyT serum from *B. subtilis* strains containing pTUBE695-V29 (a), pTUBE695-V31 (b), pTUBE695-V33 (c), and pTUBE695-A32A34 (d). The cells were pulse-labeled with [^{35}S]methionine for 30 s and analyzed under the conditions described in the legend of Fig. 3. The half-life of each mutant precursor was calculated from the density of the bands under the conditions indicated in the legend of Fig. 4.

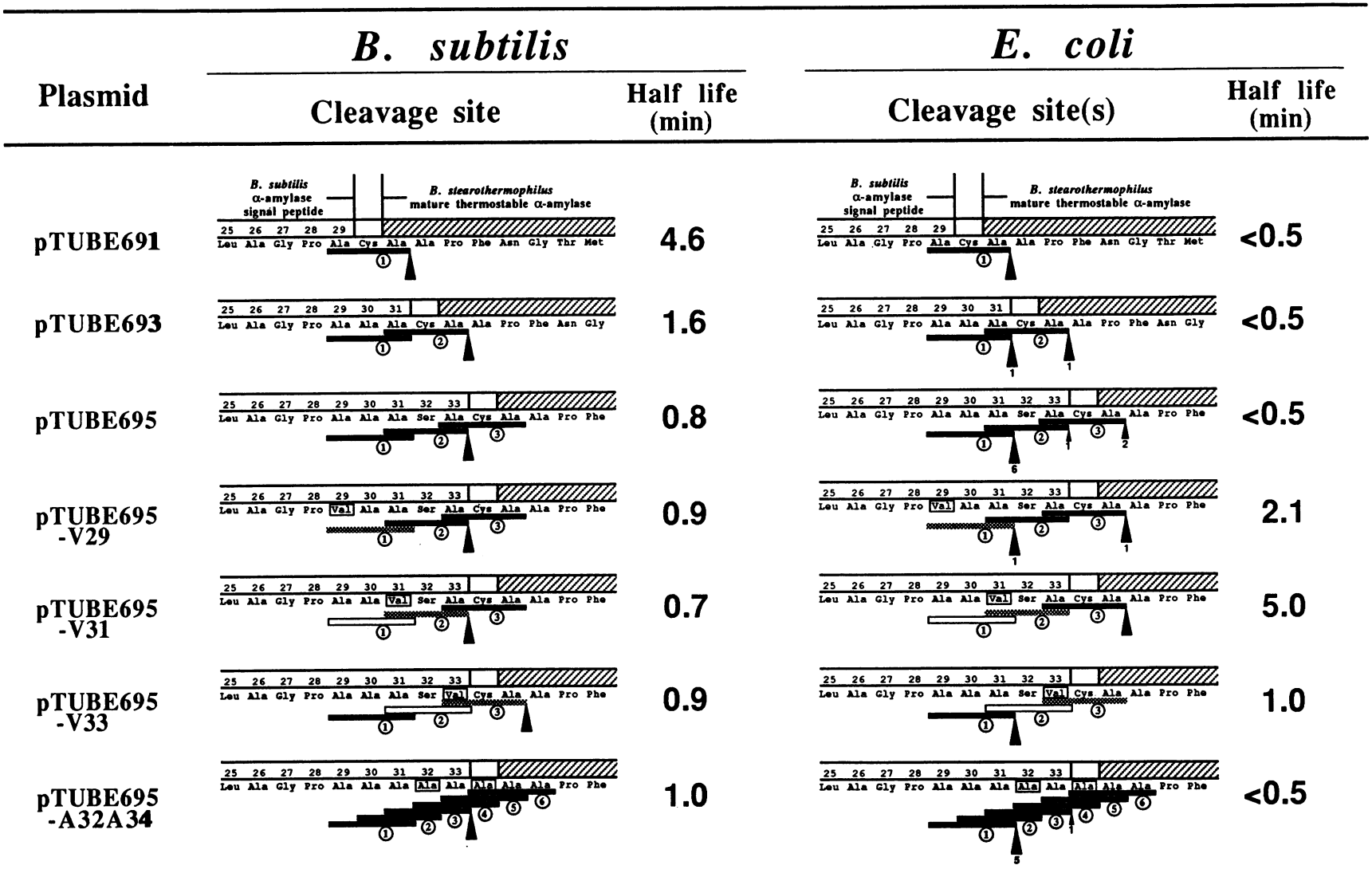

FIG. 7. Signal peptide cleavage sites and processing rates of the precursor proteins from the *amyE'-'amyT* hybrid genes in *B. subtilis* and *E. coli* cells. ▲, signal peptide cleavage sites determined from the NH_2-terminal amino acid sequences of the purified extracellular or periplasmic thermostable α-amylases; ▭, region of *B. subtilis* α-amylase signal peptide; ▨, region for the mature thermostable α-amylase of *'amyT*; □, Cys residue introduced during the construction of the *amyE'-'amyT* hybrid genes; ▬, Ala-X-Ala sequences; ▬, Val-X-Ala sequences; ▭, Ala-X-Val sequences. Replaced amino acid residues are boxed. Numbers above the amino acid residues indicate the amino acid positions in the *B. subtilis* α-amylase signal peptide region from the initiator, Met. Circled numbers indicate the numbers of repeated Ala-X-Ala sequences and their derivatives. Numbers below triangles in the cases of *E. coli* indicate the rates of cleavage at each peptide bond estimated from the molecular ratios of the NH_2-terminal amino acid sequences.

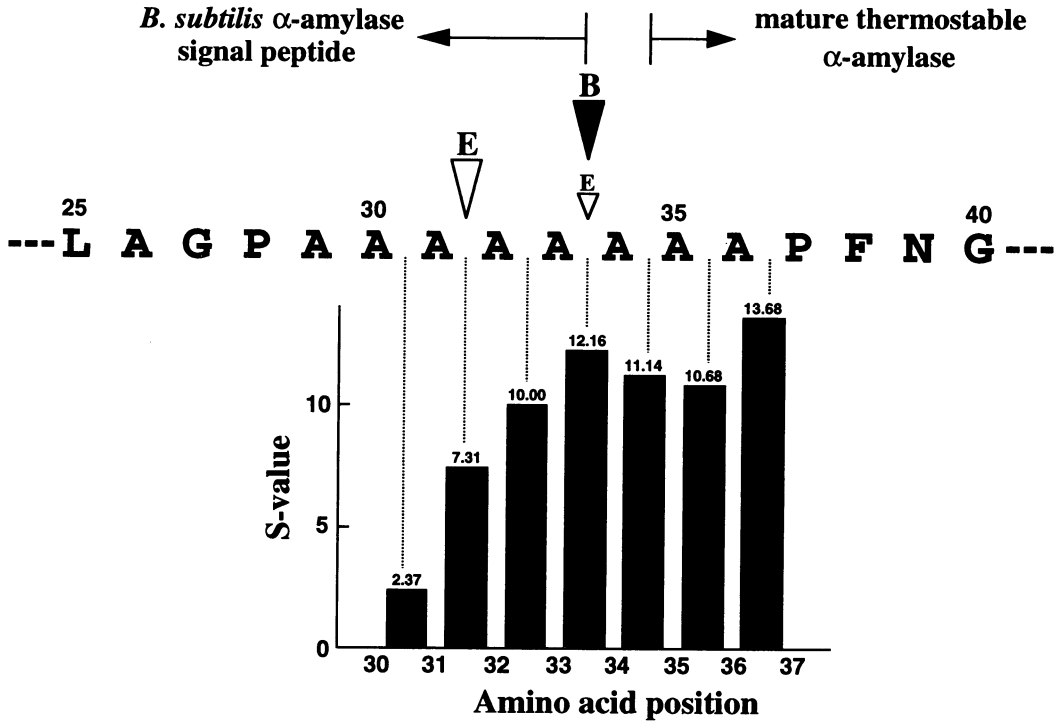

FIG. 8. S values for each peptide bond in the Ala cluster region of the precursor protein from pTUBE695-A32A34 and the signal peptide cleavage sites in it. $\overset{B}{\blacktriangledown}$, cleavage site in *B. subtilis*; $\overset{E}{\triangledown}$, cleavage sites in *E. coli*. S values were calculated by the method of von Heijne (30). Numbers above the amino acid residues indicate the amino acid positions from the initiator, Met.

the peptide bond between positions 31 and 32. The half-lives of the precursor proteins from pTUBE695-A32A34 in the two microorganisms were similar to that of the precursor protein from pTUBE695 (Fig. 6d and 7).

DISCUSSION

To analyze the structural requirements for the appropriate cleavage of the *B. subtilis* α-amylase signal peptide, the lengths of the *amyE'* signal peptides and amino acid sequence near the cleavage site were modified and then the cleavage positions and processing rates in *B. subtilis* and *E. coli* were examined.

A single cleavage site at the peptide bond between Ala-33 and Cys-34 was found in the precursor protein from pTUBE695 in *B. subtilis*. This cleavage site was identical with that of the intact *amyE* signal peptide expressed in *B. subtilis*. For pTUBE693, the precursor protein was also cleaved at the peptide bond after Ala-33 although the processing rate was somewhat decreased. This cleavage site was located at the NH_2 terminus and second amino acid of the mature thermostable α-amylase. Therefore, it is suggested that the cleavage site can be shifted into the NH_2-terminal region of the mature protein when the length of the signal peptide is shortened to less than 33 amino acids.

On the other hand, the site in the precursor from pTUBE691 was shifted to the peptide bond between Ala-31 and Ala-32. No cleavage was observed at the peptide bond between Ala-32 and Pro-33. The processing rate of pTUBE691 signal peptide was approximately one-sixth that at the peptide bond between Ala-33 and Cys-34 of pTUBE695 on the basis of the half-lives of pTUBE691 and pTUBE695 precursor proteins (Fig. 7). Moreover, the production of the extracellular thermostable α-amylase in *B. subtilis*(pTUBE691) was decreased to 40% of the value recorded for the *B. subtilis* strains harboring pTUBE693 and pTUBE695. In the case of pTUBE691, there is only one Ala-X-Ala sequence at positions 29 to 31 in the signal peptide and the amino acid at position 33 is a Pro residue. The peptide bond between Pro-33 and Phe-34 in the precursor from pTUBE691 was not cleaved, although the length of the signal peptide is appropriate. Pro rarely appears at the −1 position of the cleavage site in statistical studies of many secretory proteins whose structures have been elucidated. This is consistent with the idea that a peptide bond between Pro-33 and Phe-34 cannot be recognized as the signal peptide cleavage site and consequently the peptide bond after only one Ala-X-Ala sequence was cleaved, with a great reduction in the processing rate. In contrast, in the case of pTUBE695-V33, in which Ala-33 was replaced with Val, the cleavage site was shifted to the peptide bond between Ala-35 and Ala-36 without a considerable reduction of the processing rate or of the production of extracellular α-amylase. The longer signal peptide seemed to be more functional than the shorter one in *B. subtilis*, as previously reported (19).

In the reduced production of pTUBE691 α-amylase in *B. subtilis*, it is possible to consider that the decreased rate of processing of the signal peptide directly affected the production of the enzyme but it is also possible that the shortened signal peptide affected the expression of the α-amylase gene.

In *E. coli*, in contrast, the major cleavage site in the precursor proteins was the peptide bond after Ala-31 for all cases examined here, except for the replacement of Ala-31 with Val (Fig. 7). Therefore, it is suggested that signal peptides of different organisms may have particular lengths. This speculation is well supported by the results from the

analyses of pTUBE695-A32A34. There is an Ala cluster composed of eight Ala residues from positions 29 to 36, encompassing the putative cleavage site in the precursor from pTUBE695-A32A34. Figure 8 shows the cleavage sites of the precursor in *B. subtilis* and *E. coli* and S values at each peptide bond calculated by the weight-matrix method of von Heijne (30). The S value is considered to be a good index for estimating the signal peptide cleavage site. The values at the peptide bond from Ala-32 to Ala-36 ranged from 10 to 13. Although the values are considered to be approximately the same, the most abundant cleavage site in *B. subtilis* is the peptide bond between Ala-33 and Ala-34. In contrast, the peptide bond between Ala-31 and Ala-32 was the major cleavage site in *E. coli*. The S value at the peptide bond was 7.31 and was smaller than those found at the peptide bond from Ala-32 and Ala-36. On the basis of these results, we point out that the length of the signal peptides is a prerequisite for appropriate and efficient processing in vivo, in addition to the presence of Ala.

The average length of the signal peptides of exported proteins in *E. coli* was approximately 22 amino acids, as in the case of alkaline phosphatase (21 amino acids) (14) and maltose-binding protein (26 amino acids) (1). The length was 8 to 10 amino acids shorter than that of the extracellular enzymes of bacilli. The average length of the signal peptides of extracellular enzymes of bacilli is 30 amino acids, including the *B. subtilis* alkaline protease (29 amino acids) (32) and *B. amyloliquefaciens* α-amylase (31 amino acids) (21). However, the lengths of the hydrophobic cores in the signal peptides of the two microorganisms are similar, 10 to 12 amino acids. The distance from the hydrophobic cores to the cleavage sites is one of the major differences in the signal peptides of the two. Therefore, it is possible to consider the possibility that this difference in the distance may reflect the difference in the cleavage sites of the tested *amyE'* signal peptides in *B. subtilis* and *E. coli*. It remains to be studied, however, why are such large differences are present in the lengths of the native signal peptides between *B. subtilis* and *E. coli* proteins.

In this article, we have shown that *amyE'* signal peptides functioned extremely well in *E. coli* cells but that the preferential cleavage site in the precursors was different from that in *B. subtilis*. Recently, the *sipS* gene for *B. subtilis* signal peptidase was cloned in *E. coli* and SipS was found to be active in *E. coli* cells. The predicted amino acid sequence contained 27.5 to 39.1% identity in three noncontiguous regions of the leader peptidases of *E. coli* and *Salmonella typhimurium* (29). Suominen et al. (27) reported that, in *E. coli*, a large fraction (40%) of *B. stearothermophilus* α-amylase appeared to be processed at a site different from that in *B. stearothermophilus*. These data suggest that, notwithstanding the functional similarity, the signal peptidases of gram-positive and gram-negative bacteria are quite distinct in specificity of substrate recognition. However, another possibility to be studied is whether there is any difference in the properties of the membranes of the two bacteria, in which the signal peptidases are embedded.

ACKNOWLEDGMENTS

We thank Kenji Takase (National Institute of Agrobiological Resources) for encouragement during the early stage of this work and R. H. Doi (University of California at Davis) for helpful discussions.

This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Bedouelle, H., P. J. Bassford, Jr., A. V. Fowler, I. Zabin, J. Beckwith, and M. Hofnung.** 1980. Mutations which alter the function of the signal sequence of the maltose-binding protein of *Escherichia coli*. Nature (London) **285**:78–81.
2. **Blobel, G., and B. Dobberstein.** 1975. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**:835–851.
3. **Chan, S. J., J. Weiss, M. Konrad, T. White, C. Bahl, S.-D. Yu, D. Marks, and D. F. Steiner.** 1981. Biosynthesis and periplasmic segregation of human proinsulin in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **78**:5401–5405.
4. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
5. **Daniels, C. J., D. G. Bole, S. C. Quay, and D. L. Oxender.** 1981. Role for membrane potential in the secretion of protein into the periplasm of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **78**:5396–5400.
6. **Dev, I. K., and P. H. Ray.** 1990. Signal peptidases and signal peptide hydrolases. J. Bioenerg. Biomemb. **22**:271–290.
7. **Fuwa, H.** 1954. A new method for microdetermination of amylase activity by the use of amylose as the substrate. J. Biochem. **41**:583–603.
8. **Gierasch, L. M.** 1989. Signal sequences. Biochemistry **28**:923–930.
9. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
10. **Inouye, M., and S. Halegoua.** 1980. Secretion and membrane localization of proteins in *Escherichia coli*. Crit. Rev. Biochem. **7**:339–371.
11. **Itoh, Y., K. Kanoh, K. Nakamura, K. Takase, and K. Yamane.** 1990. Artificial insertion of peptides between signal peptide and mature protein: effect on secretion and processing of hybrid thermostable α-amylases in *Bacillus subtilis* and *Escherichia coli*. J. Gen. Microbiol. **136**:1551–1558.
12. **Itoh, Y., M. Sumi, K. Nakamura, and K. Yamane.** 1990. Processing of an NH_2-terminally extended thermostable α-amylase by *Bacillus subtilis* alkaline protease. J. Biochem. **108**:954–959.
13. **Kawamura, F., and R. H. Doi.** 1984. Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. J. Bacteriol. **160**:442–444.
14. **Kikuchi, Y., K. Yoda, M. Yamasaki, and G. Tamura.** 1981. The nucleotide sequence of the promoter and the amino-terminal region of alkaline phosphatase structural gene (*phoA*) of *Escherichia coli*. Nucleic Acids Res. **9**:5671–5678.
15. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82**:488–492.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262**:10035–10038.
18. **Murén, E. M., and L. L. Randall.** 1985. Export of α-amylase by *Bacillus amyloliquefaciens* requires proton motive force. J. Bacteriol. **164**:712–716.
19. **Nakamura, K., Y. Fujita, Y. Itoh, and K. Yamane.** 1989. Modification of length, hydrophobic properties and electric charge of *Bacillus subtilis* α-amylase signal peptide and their different effects on the production of secretory proteins in *B. subtilis* and *Escherichia coli*. Mol. Gen. Genet. **216**:1–9.
20. **Nakamura, K., H. Takamatsu, Y. Akiyama, K. Ito, and K. Yamane.** 1990. Complementation of the protein transport defect of an *Escherichia coli secY* mutant (*secY24*) by *Bacillus subtilis secY* homologue. FEBS Lett. **273**:75–78.
21. **Palva, I., R. F. Pettersson, N. Kalkkinen, P. Lehtovaara, M. Sarva, H. Söderlund, K. Takkinen, and L. Kääriäinen.** 1981. Nucleotide sequence of the promoter and NH_2-terminal signal peptide region of the α-amylase gene from *Bacillus amylolique-*

faciens. Gene **15**:43–51.
22. **Perara, E., and V. Lingappa.** 1988. Transport proteins into and across the endoplasmic reticulum membrane, p. 3–47. *In* R. C. Das and P. W. Robbins (ed.), Protein transfer and organelle biogenesis. Academic Press, Inc., New York.
23. **Perlman, D., and H. O. Halvorson.** 1983. A putative signal peptidase recognition site and sequence in eukaryotic and prokaryotic signal peptides. J. Mol. Biol. **167**:391–409.
24. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
25. **Sasamoto, H., K. Nakazawa, K. Tsutsumi, K. Takase, and K. Yamane.** 1989. Signal peptide of *Bacillus subtilis* α-amylase. J. Biochem. **106**:376–382.
26. **Sohma, A., T. Fujita, and K. Yamane.** 1987. Protein processing to form extracellular thermostable α-amylases from a gene fused in a *Bacillus subtilis* secretion vector. J. Gen. Microbiol. **133**: 3271–3277.
27. **Suominen, I., M. Karp, J. Lautamo, J. Knowles, and P. Mantsala.** 1987. Thermostable alpha amylase of *Bacillus stearothermophilus*: cloning, expression, and secretion by *Escherichia coli*, p. 129–137. *In* J. Chaloupka and V. Krumphanz (ed.), Extracellular enzymes of microorganisms. Plenum Press, New York.
28. **Takase, K., H. Mizuno, and K. Yamane.** 1988. NH_2-terminal processing of *Bacillus subtilis* α-amylase. J. Biol. Chem. **263**: 11548–11553.
29. **van Dijl, J. M., A. de Jong, J. Vehmaanpera, G. Venema, and S. Bron.** 1992. Signal peptidase I of *Bacillus subtilis*: patterns of conserved amino acids in prokaryotic and eukaryotic type I signal peptidases. EMBO J. **11**:2819–2828.
30. **von Heijne, G.** 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. **133**:17–21.
31. **Watson, M. E. E.** 1984. Compilation of published signal sequences. Nucleic Acids Res. **13**:5145–5164.
32. **Wong, S.-L., C. M. Price, D. S. Goldfarb, and R. H. Doi.** 1984. The subtilisin *E* gene of *Bacillus subtilis* is transcribed from a σ^{37} promoter *in vivo*. Proc. Natl. Acad. Sci. USA **81**:1184–1188.
33. **Yamane, K., Y. Takeichi, T. Masuda, H. Kawamura, and H. Saito.** 1982. Construction and physical map of a *Bacillus subtilis* specialized transducing phage ρ11 containing *B. subtilis lys*$^+$ gene. J. Gen. Appl. Microbiol. **28**:417–428.
34. **Yamazaki, H., K. Ohmura, A. Nakayama, Y. Takeichi, K. Otozai, M. Yamasaki, G. Tamura, and K. Yamane.** 1983. α-Amylase genes (*amyR2* and *amyE*$^+$) from an α-amylase-hyperproducing *Bacillus subtilis* strain: molecular cloning and nucleotide sequences. J. Bacteriol. **156**:327–337.