Requirement of Pro-sequence for the Production of Active Subtilisin E in *Escherichia coli*

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Subtilisin E, an alkaline serine protease of *Bacillus subtilis* 168, is first produced as a precursor, pre-pro-subtilisin, which consists of a signal peptide for protein secretion (pre-sequence) and a peptide extension of 77 amino acid residues (pro-sequence) between the signal peptide and mature subtilisin. When the entire coding region for pre-pro-subtilisin E was cloned into an *Escherichia coli* expression vector, active mature subtilisin E was secreted into the periplasmic space. When the pre-sequence was replaced with the *E. coli* OmpA signal peptide, active subtilisin E was also produced. When the OmpA signal peptide was directly fused to the mature subtilisin sequence, no protease activity was detected, although this product had the identical primary structure as subtilisin E as a result of cleavage of the OmpA signal peptide and was produced at a level of approximately 10% of total cellular protein. When the OmpA signal peptide was fused to the 15th or 44th amino acid residue from the amino terminus of the pro-sequence, active subtilisin was also not produced. These results indicate that the pro-sequence of pre-pro-subtilisin plays an important role in the formation of enzymatically active subtilisin. It is proposed that the pro-sequence is essential for guiding appropriate folding of the enzymatically active conformation of subtilisin E.

Subtilisin E is an alkaline serine protease produced by *Bacillus subtilis*. There are several other subtilisins produced by various species of *Bacillus*: subtilisin BPN′ from *B. amylooliquefaciens*, subtilisin Carlsberg from *B. licheniformis* and *B. pumilis*, and subtilisin Amylosacchariticus from *B. amylosacchariticus* (1). Because of extensive enzymatic studies as well as x-ray crystallographic studies on subtilisin (1, 2), this enzyme has provided an ideal model system for protein engineering (3–8).

DNA sequence analysis of the gene for subtilisin E has revealed the existence of a long peptide (pro-sequence) consisting of 77 amino acid residues between the signal peptide (pre-sequence) and the mature protease (9, 10). This is also the case for the other subtilisin genes (11–13). It has been shown that subtilisin E is first synthesized as a pre-pro-form (3). Although it has not yet been identified in vivo, pro-subtilisin is thought to be first secreted across the cytoplasmic membrane with the aid of the signal peptide. Subsequently, the pro-sequence is removed either autocatalytically or by pre-existing active subtilisin to produce active mature subtilisin, which is then released into the medium (3).

Such pro-sequences have been found in many secretory proteins in various Gram-positive bacteria, although the lengths of the pro-sequences are widely different from protein to protein. For example, 8 amino acid residues are found in the penicillinase from *B. licheniformis* (14), 19 residues in nuclease A from *Staphylococcus aureus* (15), and nearly 190 residues in the neutral protease from *B. subtilis* (16). The function of the pro-sequence has not been well established. The pro-sequence may be required for the association of the pro-enzyme with the cell before the release of the mature active enzyme into the medium and/or for guiding the protein to the appropriate folding for the active conformation.

In our earlier study on staphylococcal nuclease, we have shown that, when the signal peptide for OmpA, a major *Escherichia coli* outer membrane protein, is directly fused to the mature nuclease, active nuclease is efficiently secreted into the *E. coli* periplasmic space (17). This result indicates that, at least in the case of staphylococcal nuclease, the pro-sequence is not required for the folding of the active enzyme.

In the present study, we have performed a similar construction with subtilisin E and found that, in contrast to staphyloccocal nuclease, active subtilisin is not produced when the OmpA signal peptide is directly fused to mature subtilisin in spite of the fact that a large amount of a polypeptide having the identical primary structure to subtilisin E is secreted into the periplasmic space. From these results, we propose that the subtilisin pro-sequence plays an essential role in guiding the proper folding of the protein to give active subtilisin.

**EXPERIMENTAL PROCEDURES**

**Materials**—The chromosomal DNA of *B. subtilis* 168 was prepared by the method of Marmur (18). An *E. coli* strain JA221 (hisD* trpE5 leuB6 lacY* recAl/F* lacI* pro*) (19) was used as host cell. The isopropyl β-D-galactopyranoside (IPTG)-inducible pNI-III-ompA vector (20) was used for the expression and secretion of subtilisin E. All the restriction enzymes used were from New England Biolabs, Bethesda Research Laboratories, International Biotechnologies (IBI), or Boehringer Mannheim and used as recommended by the suppliers. Streptomycin subtilisin inhibitor (21–23) was a gift from Dr. S. Sato at the Biochemical Research Laboratory of Toto Joso Ltd., Shizuoka, Japan.

**Construction of Plasmids**—Cloning of the subtilisin E gene was performed as shown in Fig. 1 by ligating the 2.5-kilobase pair KpnI-EcoRI fragment from the total chromosomal DNA of *B. subtilis* 168 directly to pUC18 cloning vector (IBI) on the basis of the DNA sequence of the subtilisin gene (9). A clone of the subtilisin E gene was isolated by the colony hybridization technique using a synthetic oligonucleotide, 5'−AAAGGTTTATCACC−3' as a probe. Various

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FIG. 1. Restriction map of subtilisin gene and plasmid construction. The restriction map of PIN-III-ompA is according to Ghrayeb et al. (20). DNA fragments cloned with PIN-III-ompA were obtained by various restriction enzyme digestions of the cloned gene for subtilisin E: AccI-XmnI digestion for pH1126, DraI-XmnI for pH1102, HpaI-XmnI for pH1103, FspI-XmnI for pH1100, and NaeI-XmnI for pH1114. These fragments are shown by lines with arrows, at the bottom. The number at the left-hand side for each line is the residue number from the amino terminus of mature subtilisin (+1). The plasmids pH1212, 214, and 215 were derived from pH1126 by oligonucleotide-directed site-specific mutagenesis. In the linear restriction map, the solid box represents the coding region for mature subtilisin, the shaded box for the pro-sequence, and the empty box for the pre-sequence.

DNA fragments obtained by restriction enzyme digestions from this clone were inserted into the unique HindIII site of the PIN-III-ompA expression vector (20).

The AccI-XmnI fragment of the subtilisin E gene was inserted into a PIN-III-ompA vector after the vector plasmid was digested with HindIII and treated with DNA-polymerase Klenow fragment. As shown in Fig. 1 and Fig. 2, the resulting plasmid (pH1126) contains the entire coding sequence of subtilisin plus 48 extra bases from the 5′ uncoding region including its own Shine-Dalgarno sequence after the coding region for the OmpA signal peptide. As a result, synthesis of a peptide starting from the OmpA signal peptide terminates at residue -108 (see Fig. 2). However, in this plasmid (pH1126), protein synthesis can be independently initiated from the subtilisin signal peptide at residue -106. Similarly, the DraI-XmnI fragment and the HpaI-XmnI fragment were inserted into a PIN-III-ompA vector, yielding pH1102 and pH1103, respectively (Fig. 1). The plasmid pH1100 was prepared by ligating the FspI-XmnI fragment to the large linear fragment of the PIN-III-ompA vector resulting from the digestion of the vector with EcoRI and HindIII followed by treatment with DNA-polymerase Klenow fragment. The NaeI-XmnI fragment was also inserted into the PIN-III-ompA vector like pH1126. The resulting plasmid was digested with EcoRI, treated with DNA-polymerase Klenow fragment, and then religated to adjust its reading frame, yielding pH1114.

In order to construct pH1212, pH1214, and pH1215, site-specific mutagenesis was carried out on pH1126 as described later.

Site-specific Mutagenesis—Oligonucleotides were synthesized on a Systec Microsysyn 1450 DNA synthesizer using phosphoamidite chemistry (24). Monomers for the synthesis were purchased from American BioNuclear. Oligomers were purified by polyacrylamide gel electrophoresis. Site-specific mutagenesis was carried out according to the method of Inouye and Inouye (25) directly on the plasmids and the mutation was confirmed by DNA sequencing.

In order to delete a DNA sequence between the -366th and -250th base from pH1126 (see Fig. 2), a synthetic oligonucleotide, oligomer-a (Fig. 3), was used for site-specific mutagenesis. As a result of this deletion, the OmpA signal peptide was fused to the -83rd residue from the pre-sequence cleavage site (or the -6th residue from the pro-sequence cleavage site; see Fig. 2) to generate plasmid pH1212 (see also Fig. 1). Similarly, oligomer-b and -c (Fig. 3) were used in order to delete DNA sequences from the -366th to -238th base, and from the -366th to -232nd base, respectively (see Fig. 2). As a result of the former deletion, the OmpA signal peptide was fused to the -79th amino acid residue from the pro-sequence cleavage site (or the
Fig. 2. Partial DNA sequence of the plasmid pH1126 around the translation initiation site and the following amino-terminal region of pre-pro-subtilisin, with the amino acid sequences derived from the DNA sequence. Numbers indicate the base positions (above the DNA sequence) and amino acid positions (under the amino acid sequence, in parentheses) from the translation initiation site and the following amino-acid sequences at the fusion site for pH1100 and pH1700 are shown by arrows. The clean type of the subtilisin pro-sequence is shown by a filled arrow. The sites with the OmpA signal peptide were fused are shown by arrows with casamino acids (2%). At a Klett reading of 50 with a blue filter, 1 unit was defined as 1 pmol of p-nitroaniline.

FIG. 3. DNA sequence of synthetic oligonucleotides used for site-specific mutagenesis.

Table:

| Oligomer | Sequence |
|----------|----------|
| oligomer-a | 5'-GCGAAGGCGAAGTGCAGTGGG-3' |
| oligomer-b | 5'-GCGAAGGCGAAGTGCAGTGGG-3' |
| oligomer-c | 5'-GCTACGGAAGGCGGAGTGG-3' |
| oligomer-d | 5'-GCTACGGAAGGCGGAGTGG-3' |
| oligomer-e | 5'-GCGAAGGCGAAGTGCAGTGGG-3' |

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The latter deletion resulted in direct fusion of the OmpA signal peptide to the mature subtilisin sequence, yielding pH1214. The amino acid sequences at the fusion site for pH1100 and pH1700 are shown in Fig. 4C. In the case of pH1700, the OmpA signal peptide was directly fused to the mature subtilisin sequence.

Expression of the Subtilisin E Gene—Expression of the subtilisin E gene was performed at 37 °C in M9 medium (26) supplemented with casamino acids (2%). At a Klett reading of 50 with a blue filter, IPTG was added to the culture medium to a final concentration of 2 mM sodium citrate (pH 5.0) to the starting cell culture (1 unit was defined as 1 pmol of p-nitroaniline of p-nitroaniline after 2-h induction). The activity was calculated as unit/ml of the resulting cell culture (1 unit was defined as 1 μmol of p-nitroaniline per h).

Purification of Subtilisin—Subtilisin E from B. subtilis 168 was purified according to the method described by Estell et al. (7) using a
CM52 column. In the case of active subtilisin E produced in *E. coli*, the periplasmic fraction of induced cells was applied to a CM52 column, since most of the activity was found in the periplasmic space as described later.

**RESULTS**

**Production of Active Subtilisin in *E. coli*—**When the plasmid pH126 was constructed as an intermediate for the following mutageneses (see Fig. 1), it was found that the cells carrying this plasmid produced a small amount of active subtilisin in the presence of IPTG, and the cell fractionation experiment showed that active subtilisin was secreted into the periplasmic space (data not shown). Since the subtilisin gene can be expressed only from its own initiation codon at amino acid residue −106 (see Fig. 2) by this plasmid, we infer that the subtilisin signal sequence is functional in *E. coli* and that pro-subtilisin can be processed to active subtilisin in the *E. coli* periplasmic space.

In order to examine whether the subtilisin signal peptide can be replaced with the OmpA signal peptide, the OmpA signal peptide was fused to pro-subtilisin at three different sites within the pre-pro-subtilisin signal sequence. This gave rise to three plasmids, pH1212, 214, and 215, as shown in Fig. 1. The amino acid sequences of these proteins at the fusion regions are shown in Fig. 4B. In all cases, when induced with IPTG, protease activity was detected in both the culture medium and the soluble fraction of the sonicated cells. No significant differences in the total activity were found among the three constructions described. When cells carrying pH1212 were induced for 2 h with 2 mM IPTG at 37 °C, the total activity per milliliter of culture was 69 units. These results indicate that active enzyme was produced from these fusion proteins.

When total cellular protein from the cells described were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 5, *lanes 2–4*, a few new bands were observed. In all cases, a new band was observed at the same position as authentic subtilisin E (*lane 1*). In the case of cells carrying pH1212 (*lane 2*), in addition to the mature subtilisin band, two new major bands were seen, a band migrating a little slower and the one a little faster than the band for elongation factor Tu. The apparent molecular masses of the upper and the lower bands were estimated to be 44 and 42 kDa, respectively. From these molecular masses, the upper band is deduced to be the full-length OmpA-pro-subtilisin from pH1212, and the lower band to be pro-subtilisin resulting from the cleavage of the OmpA signal peptide. Judging from the densities of these bands, approximately half of OmpA-pro-subtilisin appears to be processed to pro-subtilisin. In the case of pH1214 and pH1215 (*lanes 3 and 4*, respectively), the upper bands migrated almost at the same position as elongation factor Tu and pro-subtilisin migrated a little faster than pro-subtilisin from pH1212 (*lane 2*), as expected from the structure shown in Fig. 1. In all cases, the plasmid-derived proteins (the upper band plus the lower band) became the major products of the cells.

It was not possible to determine the cellular location of the OmpA-subtilisin hybrid proteins owing to their insolubility. It should be noted that, in all cases, cells carrying these plasmids were unable to form colonies on plates containing 0.5 mM IPTG. In liquid cultures, cells lysed when grown in the presence of IPTG. This lysis is probably a major reason for the protease activity found in culture medium.

When the cells were induced by lower concentrations of IPTG or at lower temperatures, this lysis occurred much later, and less activity was found in culture medium. When the cells carrying pH1212 were induced with 0.005 mM IPTG at 23 °C for 3 h, the total activity per milliliter of culture increased to 1100 units from 69 units, which was obtained at 37 °C with 2 mM IPTG. Under these conditions, 89% of the total activity was recovered in the periplasmic fraction of the induced cells. Furthermore, lesser amounts of the precursor forms were detected when these cells were analyzed by SDS-polyacrylamide gel electrophoresis (data not shown).

The active enzyme produced in *E. coli* harboring pH1212, 214, and 215 was identified as subtilisin E since the protease activity was inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride (1) and a specific subtilisin inhibitor, streptomyces subtilisin inhibitor (21–23). The SDS-polyacrylamide gel electrophoresis of purified enzyme from the cells harboring pH1212 is shown in Fig. 6.

Since subtilisin activity is thought to be required for maturation of subtilisin in *B. subtilis* (3), a mutation abolishing that activity was constructed on pH1212. In this mutant plasmid, pH1216, Asp-32, which is known to be essential for protease activity, was replaced by Asn-32. Both precursor bands produced by pH1212 were observed in pH1216-bearing strains (Fig. 5, *lane 5*); however, no mature band was seen. Induction of subtilisin from pH1216 was also performed at 23 °C. Although this temperature favors production of mature subtilisin from strains harboring pH1212, 214, and 215, no mature subtilisin was observed (data not shown). Therefore, these results indicate that the production of mature subtilisin in *E. coli* is absolutely dependent on subtilisin activity.

**Direct Fusion of OmpA Signal Peptide to Mature Subtilisin—**In the plasmid pH1100 the OmpA signal peptide was directly fused to mature subtilisin except that there are 2 extra amino acid residues (Glu-Leu) between Ala-1 and Gln-2 as shown in Fig. 4C. Assays of lysates from induced cells harboring pH1100 did not show any detectable subtilisin activity, despite the fact that SDS-polyacrylamide gel electrophoresis of the induced cultures showed a large amount of a protein with a mobility close to that of subtilisin (Fig. 5, *lane
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8). This product accounted for as much as 10% of total cellular protein after a 2-h induction. Like the major products of pH1212, 214, 215, and 216, the pH1100 product was insoluble and was easily purified from a low-speed centrifuge pellet. Amino acid sequencing from the amino terminus to the 10th residue of this product by Edman degradation confirmed the processing of the OmpA signal peptide.

The lack of detectable activity in the pH1100 product could be due to the 2 extra amino acid residues inserted in the amino-terminal region of mature subtilisin. Therefore, these 2 residues were deleted by site-specific mutagenesis (see Fig. 4C), giving a fusion in which the OmpA signal sequence is fused directly to a sequence encoding authentic mature subtilisin. The cells harboring the new plasmid pH700 again showed no detectable subtilisin activity, as was the case with pH1100. This is despite the fact that these cells produced an amount of authentic mature subtilisin equivalent to 10% of total cellular protein. The product was again insoluble, as was the pH1100 product, and migrated slightly faster than the pH1100 product, as expected from the fact that the pH700 product is shorter by 2 amino acid residues (see Fig. 7). Thus, this product is considered to have the identical sequence as mature subtilisin.

These results indicate that the pH700 product does not have the same three-dimensional structure as a native subtilisin E. Since pH1212, 214, and 215 are able to produce active subtilisin, it appears that the pro-sequence is essential for folding of subtilisin into its native structure.

A similar result was obtained with plasmid pH1114 (see Figs. 2 and 4D). This plasmid is able to produce a polypeptide of which the amino-terminal 14 amino acid residues of the mature subtilisin are replaced with a hexapeptide, Gly-Ile-Asn-Ser-Lys-Leu, due to the plasmid construction procedure. No subtilisin activity was detected. The product was again produced in a large amount in an insoluble form and migrated at 31 kDa as shown in Fig. 5, lane 9.

OmpA Subtilisin Fusion Proteins with Shortened Pro-sequences—In order to examine further the role of the pro-sequence for the production of active subtilisin, two plasmids, pH1102 and pH1103, were constructed, in which the OmpA signal peptide was fused to the -63rd (pH1102) and -33rd (pH1103) residue in the mature subtilisin sequence (see Fig. 2). The resulting product of pH1102 has the carboxyl-terminal 63 amino acid residues of the pro-sequence with 5 extra amino acids (Ala-Glu-Phe-Gln-Ala) at its amino terminus, as shown in Fig. 4D. The product of pH1103 has the carboxyl-terminal 33 amino acids of the pro-sequence and 5 extra amino acids (Ala-Glu-Phe-Gln-Ala) at its amino terminus, also shown in Fig. 4D. No subtilisin activity was detected from cells harboring either of these plasmids. SDS-polyacrylamide gel electrophoresis showed that pH1102 produced a major product of apparent molecular mass 41 kDa (Fig. 5, lane 6) when induced with IPTG. This product was found to be insoluble and could be pelleted by low-speed centrifugation. Besides this major product, a few new bands are observed as shown by dots. However, none of these bands corresponds to mature subtilisin. All the other minor bands are probably derived by degradation of the major product. Similarly, pH1103 produced a major product of apparent mass 37 kDa (Fig. 5, lane 7). This product was also insoluble, and no band corresponding to mature subtilisin was seen. The differences of these apparent molecular masses from that of mature subtilisin on SDS-polyacrylamide gel electrophoresis were in good agreement with the actual molecular mass differences between mature subtilisin and the products after OmpA signal peptide cleav-
age. Inductions of 23 °C with 0.005 mM IPTG gave the same results.

**DISCUSSION**

When the subtilisin signal peptide (pre-sequence) was replaced with the OmpA signal peptide, as in the OmpA-prosubtilisin fusions pH1212, 214, and 215, active mature subtilisin was found in the periplasmic space, as well as insoluble and inactive pro-subtilisin. The processing to the mature, enzymatically active subtilisin from OmpA pro-subtilisin can be either before or after the cleavage of OmpA signal peptide. However, since longer incubation did not increase the amount of active subtilisin, the pro-subtilisin which accumulated in large amount appeared to be defective for further processing to active subtilisin. It is interesting to note that lower temperatures (23 °C) and/or reduced concentrations (0.005 mM) of IPTG were found to be effective in increasing the production of active subtilisin with less accumulation of pro-subtilisin. This result suggests that a lower rate of synthesis and lower temperature may improve the processing of pro-subtilisin.

The activation of pro-subtilisin in *E. coli* occurred in the case of pH1212. By contrast, no band corresponding to the mature subtilisin was observed in the case of pH215 having a mutation at the active site. This result suggests that at least initial activation of pro-subtilisin (cleavage of the pre-sequence) is likely to be an autocatalytic process.

The importance of the pre-sequence for the production of active subtilisin was clearly demonstrated by the product of pH700 in which the OmpA signal peptide was directly fused to pHT700. In the case of subtilisin E, this region of the pro-sequence (see Fig. 4). These regions may be important for the proper conformation necessary for activity. The analysis of peptide sequences derived from DNA coding sequences (13) of subtilisins from different species of *Bacillus* has shown the existence of a highly homologous region in the carboxyl-terminal part of the pre-sequence, and a common sequence, Tyr-Ile-Val-Gly-Phe-Lys, in the amino-terminal region of the pre-sequence. In the case of subtilisin E, this sequence is found from residue 68 to residue 62 in the pre-sequence (see Fig. 4). These regions may be important for the production of active subtilisin. Furthermore, the predicted secondary structure and the hydrophobic residue distribution of the pre-sequence (13) show a high degree of similarity among all subtilisins, even in regions where their primary structures are quite different. This suggests the notion that the secondary structure of the pre-sequence plays an important role for the production of active subtilisin. In addition, it is interesting to note that there are exceptionally large numbers of charged residues in the pre-sequence (23 out of 77 in the case of subtilisin E). This unique feature may also be important for the function of the pre-sequence of subtilisin.

Restoring the carboxyl-terminal 33 amino acid residues of the pre-sequence (pH1103) did not allow the production of active subtilisin. Furthermore, restoring the carboxy-terminal 63 amino acid residues (pH1102) was still not enough to produce active subtilisin. The latter construction lacks only 15 residues from the amino terminus of the pre-sequence including 5 residues from the common sequence discussed above. The results indicate that almost the entire pre-sequence is important for its function.

From the tertiary structure obtained from X-ray crystallography of subtilisin, one can find that charged residues are unevenly distributed on the surface of the molecule. Especially along the cleft in which the active site is located, only a few charged residues are found. On the other hand, the pre-sequence is structurally well conserved among the various subtilisins and it is highly enriched with charged residues. Therefore, it is feasible to speculate that the pre-sequence interacts in a very specific manner with the mature portion of subtilisin, and this interaction is essential for folding of subtilisin into the enzymatically active conformation. In this regard, it is of great interest to purify pro-subtilisin and to study its crystallographic structure. Currently, purification of pro-subtilisin from pH1215 is in progress.

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**REFERENCES**

1. Marklund, F. S., Jr., and Smith, M. L. (1971) in *The Enzymes*, (Boyer, P. D., ed) Vol. 3, pp. 561-608, Academic Press, New York
2. Kraut, J. (1971) in *The Enzymes*, (Boyer, P. D., ed) Vol. 3, pp. 547-560, Academic Press, New York
3. Powers, S. D., Adams, R. M., and Wells, J. A. (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 3096-3100
4. Bryan, P., Pantoliano, M. W., Quil, S. G., Hsiao, H. Y., and Poulos, T. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 83, 3743-3745
5. Wells, J. A., Vasser, M., and Powers, D. B. (1985) *Gene (Amst.*) 34, 315-323
6. Thomas, P. G., Russell, A. J., and Fersht, A. J. (1985) *Nature* 318, 375-376
7. Estell, D. A., Graycar, T. P., and Wells, J. A. (1985) *J. Biol. Chem.* 260, 6518-6521
8. Wells, J. A., and Powers, D. B. (1986) *J. Biol. Chem.* 261, 6564-6570
9. Stahl, M. L., and Ferrari, E. (1984) *J. Bacteriol.* 158, 411-418
10. Wong, S.-L., and Doi, R. H. (1986) *J. Biol. Chem.* 261, 10176-10181
11. Wells, J. A., Ferrari, D., Henmer, D. J., Estell, D. A., and Chen, E. Y. (1983) *Nucleic Acids Res.* 11, 7911-7925
12. Vasantha, N., Thompson, L. D., Rhodes, C., Banner, C., Nagle, J., and Filipula, D. (1984) *J. Bacteriol.* 159, 811-819
13. Jacobs, M., Elisson, M., Uhlbn, M., and Flock, J. I. (1985) *Nucleic Acids Res.* 13, 8913-8922
14. Chong, C. N., Nielsen, J. B. K., Iai, K., Blobel, G., and Lampen, J. O. (1982) *J. Biol. Chem.* 257, 4340-4344
15. Shortle, M. (1983) *Gene (Amst.*) 22, 181-189
16. Yang, M. Y., Ferrari, E., and Henmer, D. J. (1984) *J. Bacteriol.* 160, 15-21
17. Takahara, M., Hibiya, D. W., Barr, P. J., Gerlt, J. A., and Inouye, M. (1985) *J. Biol. Chem.* 260, 2670-2674
18. Marmur, J. (1961) *J. Mol. Biol.* 3, 208-218
19. Nakamura, K., Masui, Y., and Inouye, M. (1982) *J. Mol. Appl. Genet.* 1, 289-299
20. Ghayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y., and Inouye, M. (1984) *EMBO J.* 3, 2437-2442
21. Murao, S., and Sato, S. (1972) *Agric. Biol. Chem.* 36, 160-183
22. Sato, S., and Murao, S. (1973) *Agric. Biol. Chem.* 37, 1067-1074
23. Sato, S., and Murao, S. (1974) *Agric. Biol. Chem.* 38, 2227-2233
24. Siba, N. D., Biernat, J., McManus, J., and Köster, H. (1984) *Nucleic Acids Res.* 12, 4595-4597
25. Inouye, M., and Inouye, S. (1987) in *Synthesis of DNA, RNA, and Their Application* (Naranj, S., ed), Academic Press, New York, in press
26. Miller, J. H. (1972) *Experiments in Molecular Genetics*, pp. 431-432, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27. Strongin, A. Y., Izotova, L. S., Abramov, Z. T., Gorodetsky, D. I., Ermakov, L. M., Baratova, L. A., Belyanova, L. P., and Stepanov, V. M. (1978) *J. Bacteriol.* 133, 1401-1411