Subtilosin A, a New Antibiotic Peptide Produced by
Bacillus subtilis 168: Isolation, Structural Analysis,
and Biogenesis

Katsuhiko BABASAKI, Toshifumi TAKAO, Yasutsugu SHIMONISHI,
and Kiyoshi KURAHASHI

Institute for Protein Research, Osaka University,
Yamadaoka, Suita, Osaka 565

Received for publication, April 3, 1985

Subtilosin A, a new antibiotic produced by Bacillus subtilis 168, was extracted from
culture medium with n-butanol and purified to homogeneity by a combination of
gel filtration and thin-layer chromatography. The yield was 5.5 mg from a liter
of culture. It had bacteriocidal activity against some gram-positive bacteria. Amino
acid analysis and mass spectrometry showed that it was a peptide with a molecular
weight of 3398.9, consisting of 32 usual amino acid and some non-amino acid resi-
dues. Its amino- and carboxyl-termini were blocked. By analysis of the fragments
obtained by partial acid hydrolysis, as well as by chymotryptic and thermolysin
digestions of reduced and S-carboxymethylated samples and Achromobacter protease
I digestion of performic acid-oxidized samples, the amino acid sequence was deter-
mined to be as follows: X-Gly-Leu-Gly-Leu-Trp-Gly-Asn-Lys-Gly-Cys-Ala-Thr-
Cys-Ser-Ile-Gly-Ala-Ala-Cys-Leu-Val-Asp-Gly-Pro-Ile-Pro-Asp-Glx-Ile-Ala-Gly-Ala.
The analyses of cross-linking structures revealed that there were linkages between
the amino- and carboxyl-termini and between the Cys-19 and the Glx-28 residues
through an unknown residue with a residue weight of 163. Consequently, sub-
tilosin A was deduced to be a cyclic peptide antibiotic with a novel cross-linking
structure.

The production of subtilosin A begins at the end of vegetative growth and
finishes before spore formation. Studies on the correlation between the production
of subtilosin A and spore formation with decoyinine in the original strain and in
asporogenous mutants of B. subtilis 168 suggested that there was no close correla-
tion between the two phenomena. The production of subtilosin A was repressed

1 This study was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and
Culture of Japan.

Abbreviations: HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DTNB, 5,5'-dithio-
bis(2-nitrobenzoic acid); DABITC, 4-N,N-dimethylamino-azobenzene-4'-isothiocyanate; PITC, phenyl isothiocya-
nate; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; RCM, reduced and S-carboxymethylated; NSM, nutrient
sporulation medium; MIC, minimum inhibitory concentration; FAB, fast atom bombardment; N-, amino-; C-,
carboxyl-; m, molecular weight; z, electric charge; MES, 2-(N-morpholino)ethanesulfonic acid monohydrate.
by inhibitors of protein and RNA synthesis in contrast to that of many other anti-
bacterial peptides, suggesting that it is synthesized by the mechanism of usual protein
synthesis.

A number of antibacterial and antifungal sub-
stances produced by various strains of Bacillus
subtilis have been isolated and characterized (1-3). However, there is little information about such
substances produced by B. subtilis 168, although
it is the most well-characterized strain, genetically
and biochemically, among B. subtilis and has been
used extensively for the study of sporulation of
bacterial cells (4-6). A close correlation between
antibiotic production and spore formation in the
Marburg strain of B. subtilis has been reported by
Balassa et al. (7), Schmitt and Freese (8), and
Spizizen (9). Schaeffer (10) reported that in the
crude filtrate of the Spo+ culture three distinct
antibiotics were present. Schmitt and Freese (8)
observed that there was no antibiotic activity dur-
ing exponential growth, but that it greatly in-
creased during the developmental period. They
distinguished three major and two minor antibiotic
components by thin-layer chromatography and
column chromatography on Sephadex LH-20.
However, none of them has been isolated or
characterized further.

The correlation between antibiotic formation
or its physiological function and sporulation in
other strains of B. subtilis or other species of bacilli has also been studied by many workers (cf.
3, 11), using well-defined antibiotics such as baci-
tracins, mycobactin, gramicidin S, tyrocidines, or
gramicidin A. In some cases, results that favor
a function of antibiotics in sporulation were ob-
tained, but in others, the results indicated that
there was no direct cause-and-effect relationship
between antibiotics and sporulation, though there
seems to be a closely related regulating mechanism
for both antibiotic production and sporulation.

We have attempted to isolate and characterize
an antibiotic produced by B. subtilis 168 and to
study the correlation between its production and
sporulation. This paper describes the isolation and characterization of a new antibiotic peptide,
subtilosin A. It was found, however, that this
antibiotic has no correlation with sporulation of
this organism.

MATERIALS AND METHODS

Materials—The sources of materials used in
this work were as follows: nutrient broth, tryptone,
yeast extract, vitamin assay casamino acids, and
Bacto-agar from Difco Laboratories; pre-coated
TLC plates (silica gel; 5715 and 5745) from E.
Merck; analytical TLC precoated plates KC18 from
Whatman Inc.; Sephadex LH-20 (40-120 μm) and
SP-Sephadex C-25 (40-120 μm) from Pharmacia
Fine Chemicals, Inc.; EKICRODISC 13 from
Gelman Sciences Japan, Ltd.; morpholinopropane
sulfonate and 2-(N-morpholino)ethanesulfonic acid
monohydrate (MES) from Dojindo Laboratories;
fluorescamine from Hoffmann-La Roche Inc.;
rifamycin SV sodium salt (B grade) from Calbio-
chem-Behringer; chloramphenicol from Sankyo,
Co., Ltd.; proline-specific endopeptidase from
Seikagaku Kogyo Co., Ltd.; α-chymotrypsin and
carboxypeptidase A-DFP from Sigma Chemical
Co.; thermolysin from Nakarai Chemicals, Ltd.;
dansyl chloride, phenyl isothiocyanate (PITC) and
5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) from
Wako Pure Chemical Industries; hydrazine (an-
hydrous), aminopeptidase M and 4-N,N-dimethyl-
aminoazobenzene-4'-isothiocyanate (DABITC)
from Pierce Chemical Co.; carboxypeptidase Y
from Oriental Yeast Co., Ltd.; polyamide layer
sheets from Cheng Chin Trading Co., Ltd.; liquid
chromatography columns, Yanapak ODS-T (4×
250 mm, 10 μm) from Yanagimoto Mfg. Co., Ltd.
and Chemcopak (4.6×250 mm, C8, 7 μm, and C18,
5 μm) from Chemco Scientific Co., Ltd. Deoxy-
nine U-7984 was a gift from Dr. Joseph E. Grady
of the Upjohn Co. and Achromobacter protease I
was supplied by Dr. Kazuo Fujikawa of the Uni-
versity of Washington. All other chemicals were
obtained commercially. Solvents for sequencing
were of sequencing grade. Acetonitrile and 2-
propanol were of high-performance liquid chro-
matography (HPLC) grade.

Bacterial Strains—B. subtilis 168 obtained
from Dr. S. Okubo was used as a producer organ-

J. Biochem.
ism of subtilosin A. Other B. subtilis sporulation mutants tested for the production of subtilosin A were obtained from Drs. J. Spizizen and P. Schaeffer as described previously (12).

**Media**—Nutrient sporulation medium (NSM) is the 2 × SG medium of Korch and Doi (13) except that it contained double strengths of nutrient broth and glucose. S6C medium is the same as that described by Freese et al. (14) except for the omission of methionine. S6CG medium contained 1% glucose. LB medium contained 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl supplemented with 1 g of glucose per liter, the pH being adjusted to 7.0 with NaOH. LB agar and soft agar were supplemented with 1.5% and 0.7% agar, respectively.

**Thin-Layer Chromatography and Detection of Subtilosin A**—Subtilosin A was chromatographed on the following thin-layer plates with solvent systems A and B.

1. Silica gel plates (Merck 5715 or 5745, 200×200 mm) with Solvent System A (CHCl₃ : CH₃OH : H₂O = 60 : 25 : 4, v/v)
2. Reverse-phase plate (Whatman KC₁₈, 200×200 mm) with Solvent System B (CH₃OH : H₂O = 70 : 30, v/v)

Subtilosin A on plates was detected by UV, fluorescamine, Ehrlich's reagent, and by bioautography with Bacillus amyloliquefaciens H as an indicator organism according to the method of Schmitt and Freese (8). The Rₚ values were found to be around 0.5 and 0.2 on silica gel plates and the reverse-phase plates, respectively.

**Quantitative Analysis of Subtilosin A**—An appropriate amount of culture removed at intervals was mixed with one-fourth volume of n-butanol and vortexed for a few minutes. The organic phase was removed and centrifuged. One milliliter of the supernatant solution was evaporated to dryness. The residue was dissolved in 1 ml of 20% acetonitrile in 0.1% trifluoroacetic acid (TFA) and filtered through an EKICRODISC 13. The filtrate was subjected to HPLC (Yanaco liquid chromatograph L-4000W) on a reverse-phase column (C₁₈, 10 µm, 4×250 mm) equilibrated with 0.1% TFA. The elution was carried out with 40 ml of a linear gradient of 55% to 65% acetonitrile in 0.1% TFA at a flow rate of 1.5 ml/min and monitored with a Yanaco UV detector S-310A at 280 nm. Subtilosin A was eluted at 58% acetonitrile in 0.1% TFA. The amount of subtilosin A was estimated from the absorbance at 280 nm, using the molecular extinction coefficient of tryptophan (5690), because subtilosin A contains one residue of tryptophan per mol.

**Fast Atom Bombardment (FAB) Mass Spectrometry**—The molecular weight of subtilosin A was determined in a double focusing mass spectrometer (Jeol JMS-HX100) equipped with an FAB ion source and a mass data analysis system (Jeol JMA-3100 or DA-5000). Typical experiments were carried out with a xenon atom beam source at 7 keV accelerating potential. Mass assignment was made by using a mixture of CsI and KI as a mass reference. A sample solution containing 15–40 µg of subtilosin A was loaded on a stainless steel plate and mixed with glycerol and α-thioglycerol on the plate.

**Analyses of Amino Acid Composition and Sequence**—(1) Amino acid analyses: Amino acid analyses of peptides were performed on an amino acid analyzer (Hitachi 835-S) after hydrolysis with constant-boiling HCl containing 0.2% phenol in evacuated, sealed tubes at 110°C for 24 h. No corrections were made for the destruction of amino acids during acid hydrolysis. Cysteine residues were determined as cysteic acid after oxidation of a sample with performic acid (15). Tryptophan residues were determined after hydrolysis of a sample in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (16).

(2) Methylamine treatment, reduction, and carboxyymethylation: Because sufficient reduction and S-carboxymethylation of subtilosin A could not be achieved by the standard method of Crestfield et al. (17), the following modification was devised. Subtilosin A was dissolved in methanol containing 40% methylamine and kept at room temperature for 1.5 h. After evaporation of the solvent, the method of Crestfield et al. (17) was followed. Salt was removed by gel filtration on Sephadex G-10 with 0.1% formic acid as a solvent.

(3) Partial acid hydrolyses: Reduced and S-carboxymethylated (RCM) subtilosin A (2.55 mg) was dissolved in 1 ml of 50% acetic acid containing 30 mM HCl and hydrolyzed at 105°C for 12 h. Intact subtilosin A (3 mg) was dissolved in 0.3 ml of iron-free concentrated HCl and hydro-
lyzed at 37°C for 17 h.

(4) Digestion of RCM-subtilosin A with proteases: Samples, 1.5 mg and 0.2 mg, were dissolved in 1 ml and 0.2 ml of 0.1 M NH₄HCO₃ containing 10 mM CaCl₂, pH 8.1, and digested with 50 µg of chymotrypsin at 37°C for 6 h and with 10 µg of thermolysin at 60°C for 1 h, respectively.

(5) Digestion of performic acid-oxidized subtilosin A with Achromobacter protease I: Two samples (1.4 mg each) were dissolved in 0.14 ml of 0.1 M NH₄HCO₃ containing 10 mm CaCl₂. One sample was digested with 28 µg of Achromobacter protease I at 37°C for 2 h and the other with 3.5 µg of the enzyme at 37°C for 45 min.

(6) Peptide isolation: The digests or the hydrolyzates were subjected to HPLC on reverse phase columns (C8, 7 µm; C18, 10 µm or 5 µm) and peptide fragments were eluted with concentration gradients of acetonitrile in 0.1 % TFA or a 7 : 3 mixture of 2-propanol and acetonitrile in 0.1 % TFA. The aminoterminal fragments yielded by chymotryptic or thermolysin digestion were isolated as flow-through fractions of ion-exchange chromatography on an SP-Sephadex C₂₅ column (12 x 50 mm) with 25 mm phosphoric acid as a solvent, and were purified by HPLC.

(7) Sequence determination: The dansyl-Edman procedure was performed as described by Chen (18) and Kimura (19). The DABITC/PITC double coupling method of Chang et al. (20) as modified by Allen (21) was used. Digestion with carboxypeptidase A was performed on about 100 nmol of performic acid-oxidized samples in 100 µl of 0.2 M N-ethylmorpholine acetate buffer, pH 8.5. Carboxypeptidase A was used at a weight ratio of enzyme to substrate of 1 : 23, and incubation was at 37°C. Digestion with carboxypeptidase Y was performed on samples dissolved in 20 mM sodium phosphate buffer, pH 6.4, at a concentration of 0.1% (w/v). The weight ratio of enzyme to substrate was 1 : 50 and incubation was at 37°C. After lyophilization, the digests were directly applied to an amino acid analyzer.

(8) Titration of thiol groups: Dried subtilosin A was dissolved in anhydrous hydrazine, then the solution was left at room temperature for 5 min, evaporated to dryness and titrated with DTNB according to the method of Kortt and Liu (22).

RESULTS

Isolation of Subtilosin A—One milliliter aliquots of logarithmically growing preculture cells of B. subtilis 168 at 170 Klett units were transferred into 2-liter Erlenmeyer flasks containing prewarmed 350 ml of NSM. The culture was incubated at 37°C on a New Brunswick rotatory shaker Model 25 with vigorous shaking until 5 h after the end of vegetative growth, when the level of subtilosin A reached the maximum. Then one-fourth volume of n-butanol was added to the culture. The mixture was emulsified vigorously for 1 h and allowed to stand overnight at room temperature. The butanol layer was removed and processed as shown by the diagram in Fig. 1. As shown in Fig. 2, subtilosin A, eluted in the void fractions of the first Sephadex LH-20 column chromatography, was separated from most other UV-absorbing materials and antibiotics. The antibiotic substances still contaminating subtilosin A were removed by the following TLC and the second LH-20 gel filtration steps. Subtilosin A thus obtained was homogeneous in HPLC on a reverse-phase column as shown in Fig. 3 and in mass spectrometry (data not shown). The concentration of subtilosin A in the culture was about 8 mg per liter, and the overall yield was 5.5 mg from one liter of culture or about 70%.

Properties of Subtilosin A—Subtilosin A is a hydrophobic peptide as evidenced by its elution at 58% acetonitrile in 0.1% TFA on a reverse-phase column (C₁₈) in HPLC (Fig. 3). It is soluble in methanol, glacial acetic acid, 70% formic acid, and dimethylsulfoxide, but not in non-polar organic solvents such as ether or hexane. It is soluble in alkaline solutions, but is labile and decomposes gradually even in a mild alkaline solu-

Structure—Intact subtilosin A (1 mg) was suspended in 1.6 ml of 0.1 M MES buffer, pH 6.5, containing 10 mM CaCl₂, and digested with 255 µg of thermolysin at 37°C for 75 h. The fragments with a cross-linking structure were isolated from the digests by reverse-phase HPLC, and then dissolved in 50 µl of 50 mM sodium phosphate buffer, pH 6.5, and subjected to additional digestion with 60 µg of proline-specific endopeptidase at 37°C for 17 h. After digestion, fragments were isolated by reverse-phase HPLC.
Subtilosin A, a new antibiotic peptide from B. subtilis 168

Fig. 1. Flow diagram of isolation procedures of subtilosin A. All procedures were carried out at room temperature. Samples were stored in methanol.

<table>
<thead>
<tr>
<th>Whole culture (3.5 l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction with 1/4 vol. of n-butanol</td>
</tr>
<tr>
<td>Butanol extract (ca. 800 ml)</td>
</tr>
<tr>
<td>Concentrated in vacuo</td>
</tr>
<tr>
<td>Water layer discarded</td>
</tr>
<tr>
<td>Residue</td>
</tr>
<tr>
<td>Dissolved in methanol (&lt;6 ml)</td>
</tr>
<tr>
<td>Gel filtration on a Sephadex LH-20 column (2 x 47 cm):</td>
</tr>
<tr>
<td>Elution performed with methanol at a flow rate of 120 ml/h</td>
</tr>
<tr>
<td>Fractions of 3 ml collected</td>
</tr>
<tr>
<td>Elute monitored by change of absorbance at 230 nm</td>
</tr>
<tr>
<td>Void fractions</td>
</tr>
<tr>
<td>Concentrated in vacuo</td>
</tr>
<tr>
<td>Thin-layer chromatography on a silica gel plate (Merck 5745)</td>
</tr>
<tr>
<td>with the solvent system A</td>
</tr>
<tr>
<td>Extraction with methanol</td>
</tr>
<tr>
<td>Concentrated in vacuo</td>
</tr>
<tr>
<td>Crude subtilosin A (32 mg)</td>
</tr>
<tr>
<td>Thin-layer chromatography on three reverse-phase plates</td>
</tr>
<tr>
<td>with the solvent system B</td>
</tr>
<tr>
<td>Extraction with methanol</td>
</tr>
<tr>
<td>Concentrated in vacuo</td>
</tr>
<tr>
<td>Subtilosin A fraction</td>
</tr>
<tr>
<td>Sephadex LH-20 gel filtration in the same manner as above</td>
</tr>
<tr>
<td>Purified subtilosin A (19.4 mg)</td>
</tr>
</tbody>
</table>

The solubility of subtilosin A in 0.1 M NH₄HCO₃ containing 10 mM CaCl₂ was 98.5 μg/ml. Subtilosin A gives positive reactions with fluorescamine and Ehrlich's reagent, and shows bacteriocidal activity against gram-positive bacteria such as Bacillus megaterium, B. amyloliquefaciens, and Streptococcus faecium as shown in Table I. It is also effective on B. subtilis 168, which is the producer organism of subtilosin A, but not on the fungi tested.

Structural Analysis of Subtilosin A—Table II shows the amino acid composition of subtilosin A. It consists of 32 amino acid residues. Methionine, tyrosine, phenylalanine, histidine, and arginine are missing. No unusual ninhydrin-positive peaks were found in amino acid analyses. D-Amino acids were not detected in acid hydrolysates of subtilosin A with D-amino acid oxidase (data not shown) (25). The FAB mass spectrum of subtilosin A gave an intense signal at m/z 3399.9 as shown in Fig. 1S (m and z denote molecular weight and electric charge, respectively). Because the mass values of intact peptides were observed as [m+H]⁺ in the positive mass spectra, the mo-
Fig. 2. (A) Sephadex LH-20 gel filtration of a butanol extract. The butanol extract from 3.5 liters of culture was subjected to Sephadex LH-20 gel filtration as shown in Fig. 1. Closed circles, absorbance at 230 nm; a solid bar indicates the subtilosin A fractions pooled; and $V_o$ and $V_t$ indicate the positions of the void and total volumes of the column, respectively. (B) Bioautography of the eluate for antibiotic activity. A portion (25 $\mu$l) of every other fraction was chromatographed on a silica gel plate (Merck 5715) with solvent system A followed by bioautography as described in "MATERIALS AND METHODS." The numbers of fractions correspond to those of fractions in (A). The hatched area indicates subtilosin A ($R_f = 0.48$) and the areas marked by lines the other antibiotics.

Molecular weight of subtilosin A was estimated to be 3398.9. This is larger by 369.5-373.4 than the theoretical values (3025.5-3029.4) calculated from the amino acid composition. From this it was inferred that subtilosin A possesses other constituents than amino acids.

Figure 4 summarizes the strategy of amino acid sequencing of the peptide portion. Because preliminary experiments indicated that the N-terminal residue of this antibiotic was blocked, we first obtained fragments with free amino groups by partial acid hydrolysis of an intact sample with concentrated HCl, digestion of RCM-samples with chymotrypsin or thermolysin, partial acid hydrolysis of an RCM-sample with 0.03 M HCl in 50% aqueous acetic acid, or digestion of a performic acid-oxidized sample with Achromobacter protease I. The resultant fragments were purified by HPLC on a reverse-phase column. The N-terminal fragments were independently isolated by HPLC from the flow-through fractions, which were obtained by ion-exchange chromatography of the chymotryptic and thermolysin peptides of RCM-samples on a SP-Sephadex C-25 column. Details of the isolation of fragments are described in the legends to Fig. 11S through 18S. Table III shows the amino acid compositions of the fragments used for sequencing. Sequencing of these fragments by the dansyl-Edman method, the DABITC/PITC double coupling method and the analysis with carboxypeptidase Y was repeated at least twice. The carboxyl (C)-terminal sequence was also determined by digestion with carboxypeptidase Y of performic acid-oxidized subtilosin A (Fig. 2S). As shown in Fig. 4, the complete amino acid sequence of subtilosin A was constructed from the above results. It is a single peptide with an N-blocked glycine residue at the N-terminus and with an alanine residue at the C-terminus. The $\gamma$-carboxyl group of the Glx residue at position 28 is substituted as will be described below. There-
SUBTILOSIN A, A NEW ANTIBIOTIC PEPTIDE FROM B. subtilis 168

TABLE I. Antibiotic spectrum of subtilosin A. Cells of each microorganism except for Lactobacillus casei were grown on LB soft agar plates containing 0.2% Brij-58, and their sensitivities to subtilosin A were examined by a paper disc method (23). After incubation at 37℃ for 18 h, the diameters of the zone of inhibition, which was formed around paper discs (13 mm) containing 27 μg of subtilosin A, were measured. The sensitivity was designated as follows: — means no halo, + <16 mm, ++ <18 mm, +++ <20 mm, and ++++ ≥ 20 mm. The minimum inhibitory concentration (MIC) was determined by an agar dilution method (23). L. casei was grown on a 0.7% agar plate of Lactobacilli medium (24) containing 0.2% Brij-58.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Sensitivity</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus amyloliquefaciens H</td>
<td>++</td>
<td>13.5</td>
</tr>
<tr>
<td>Bacillus brevis ATCC 9999</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bacillus licheniformis ATCC 10716</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bacillus megaterium IFO 12108</td>
<td>++++</td>
<td>1.7</td>
</tr>
<tr>
<td>Bacillus natto IFO 13169</td>
<td>++</td>
<td>27</td>
</tr>
<tr>
<td>Bacillus polymyxa ATCC 10401</td>
<td>+</td>
<td>&gt;108</td>
</tr>
<tr>
<td>Bacillus subtilis 168</td>
<td>+</td>
<td>&gt;108</td>
</tr>
<tr>
<td>Bacillus subtilis ATCC 6633</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Lactobacillus casei IFO 3435</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Staphylococcus aureus IFO 12732</td>
<td>+</td>
<td>54</td>
</tr>
<tr>
<td>Streptococcus faecium IFO 3181</td>
<td>+++</td>
<td>3.4</td>
</tr>
<tr>
<td>Escherichia coli K-12 HB 101</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aspergillus niger IFO 6341a</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mucor javanicus IFO 4570a</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

a Grown at 28℃.

TABLE II. Amino acid composition of subtilosin A. Amino acid analyses were carried out as described in "MATERIALS AND METHODS."

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>3.0 (3) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Thr</td>
<td>0.7 (1)</td>
</tr>
<tr>
<td>Ser</td>
<td>0.8 (1)</td>
</tr>
<tr>
<td>G1x</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Pro</td>
<td>1.9 (2)</td>
</tr>
<tr>
<td>Gly</td>
<td>7.1 (7)</td>
</tr>
<tr>
<td>Ala</td>
<td>5.0 (5)</td>
</tr>
<tr>
<td>Cys b</td>
<td>2.5 (3)</td>
</tr>
<tr>
<td>Val</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Ile</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Leu</td>
<td>3.0 (3)</td>
</tr>
<tr>
<td>Lys</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Trp c</td>
<td>0.5 (1)</td>
</tr>
</tbody>
</table>

Total residues (32)

a Values are expressed as residues per mol. Numbers in parentheses are residue values based on the sequence.
b Cysteine was determined as cysteic acid. The S-carboxymethyl-cysteine contents were determined to be 2.0 and 0.8 mol/mol on RCM-subtilosin A with and without pretreatment with methylamine, respectively.
c Tryptophan content estimated from the absorbance at 280 nm in methanol was 1.1-1.2 mol/mol.

Therefore, we could not determine whether this residue was derived from glutamic acid or glutamine. Preliminary analyses of possible modifications of side chains of amino acid residues showed that serine and threonine residues were not substituted, because they were sensitive to oxidation with chromic acid (26). Dansyl chloride reacted with only the ε-amino group of the lysine residue. Two of the three Asx residues were concluded to be aspartic acid by analysis of a sample that was esterified with methanol-HCl followed by reduction with sodium borohydride (27). These results were also confirmed by sequencing of the fragments containing the relevant residues. As shown in Table II, subtilosin A has three cysteine residues (determined as cysteic acids), but no thiol groups were titrated with DTNB, whereas they all were alkylated with iodoacetic acid after reduction (Table III, CH-2). When the total thiol groups of this antibiotic were titrated with DTNB after treatment with anhydrous hydrazine, it was found that there were three thiol groups, as shown in Fig. 3S. These results suggest that this antibiotic contains a modified cysteine residue in addition to cystine.

To locate the modified cysteine residue, we...
digested intact subtilosin A with thermolysin at pH 6.5. Five peptide fragments (Fig. 4S), which were designated as Fragments I–V, were obtained from the digests by reverse-phase HPLC (Fig. 5S). On the basis of the results of amino acid analysis (Table 1S) and sequencing (Fig. 4S), Fragments I and II were found to have a novel cross-linking structure through the cysteine residue at position 19, whereas Fragments III and IV indicated the possibility of linking between the N- and C-termini. Fragment V consisted of three peptide fragments that were linked to each other through the two cross-linking structures mentioned above.

The FAB mass spectrum of Fragment I gave two intense signals (Fig. 6S): a parent ion signal at $m/z$ 1362 and a fragment ion signal around $m/z$ 1101. We shall below consider this fragmentation in FAB mass spectrometry. The molecular weight of Fragment I was estimated to be 1361 from the parent ion signal. This value was 145–146 larger than what was accounted for by the amino acid composition, suggesting that another constituent in addition to amino acids was present. We designated this unknown residue as Xu.

To analyze the cross-linking structure in Fragment I and to locate Xu, further digestion of Fragment I with proline-specific endo-peptidase at pH 6.5 was carried out. We obtained a peptide from the digests by reverse-phase HPLC, and designated it as Fragment U (Fig. 7S). On the basis of the results of amino acid analysis and sequencing, Fragment U was concluded to consist of two fragments as shown in Fig. 5: the fragment with the sequence from residue 17 through residue 19, Ala-Ala-Cys (chain A), and the fragment with the sequence from residue 25 through residue 29, Ile-Pro-Asp-Glx-Ile (chain B). The FAB mass spectrum of Fragment U (Fig. 8S, A) again showed the same fragmentation as demonstrated in the case of Fragment I, and gave a parent ion signal at $m/z$ 994 and a fragment ion signal around $m/z$ 733. From the parent ion signal the molecular

---

**Fig. 4.** Summary of the strategy of amino acid sequencing of subtilosin A. Amino acid residues are indicated by one-letter notation. CH, chymotryptic peptides of RCM-samples; TH, thermolysin peptides of RCM-samples; AP, digests of performic acid-oxidized samples with Achromobacter protease I; dAH, hydrolyzates of RCM-samples with dilute acid; cAH, hydrolysates of intact samples with concentrated HCl. ———, ———, ———, and ——— indicate sequencing by digestion with carboxypeptidase A, with carboxypeptidase Y, dansyl-Edman degradation, and the DABITC/PITC double coupling method, respectively.
### TABLE III. Amino acid compositions of fragments used for sequencing. Notation of fragments is indicated in the legend to Fig. 4.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>CH-1</th>
<th>CH-2</th>
<th>CH-3</th>
<th>CH-4</th>
<th>CH-5</th>
<th>CH-6</th>
<th>CH-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>dAH-1</td>
<td>2.0(1)</td>
<td>0.1(1)</td>
<td>0.1(1)</td>
<td>0.1(1)</td>
<td>0.1(1)</td>
<td>0.1(1)</td>
<td>0.1(1)</td>
</tr>
<tr>
<td>dAH-2</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
</tr>
<tr>
<td>dAH-3</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
</tr>
<tr>
<td>dAH-4</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
</tr>
<tr>
<td>dAH-5</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
<td>0.2(1)</td>
</tr>
<tr>
<td>CH-1</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
</tr>
<tr>
<td>CH-2</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
</tr>
<tr>
<td>CH-3</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
</tr>
<tr>
<td>CH-4</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
</tr>
<tr>
<td>CH-5</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
</tr>
<tr>
<td>CH-6</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
</tr>
<tr>
<td>CH-7</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
</tr>
</tbody>
</table>

**Yield (%)**

<table>
<thead>
<tr>
<th>Fragment</th>
<th>cAH-1</th>
<th>cAH-2</th>
<th>cAH-3</th>
<th>cAH-4</th>
<th>AP-1</th>
<th>AP-2</th>
<th>TH-1</th>
<th>TH-2</th>
<th>TH-3</th>
<th>TH-4</th>
<th>TH-5</th>
<th>TH-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>1.9(2)</td>
<td>1.8(2)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>1.1(1)</td>
<td>2.0(2)</td>
<td>1.0(1)</td>
<td>2.0(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr</td>
<td>0.8(1)</td>
<td>0.8(1)</td>
<td>0.8(1)</td>
<td>0.8(1)</td>
<td>1.1(1)</td>
<td>1.1(1)</td>
<td>1.1(1)</td>
<td>1.1(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>0.8(1)</td>
<td>0.8(1)</td>
<td>0.8(1)</td>
<td>0.8(1)</td>
<td>1.1(1)</td>
<td>1.1(1)</td>
<td>1.1(1)</td>
<td>1.1(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>1.4(1)</td>
<td>1.2(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>2.9(3)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>2.7(3)</td>
<td>2.7(3)</td>
<td>2.7(3)</td>
<td>2.7(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cys</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td>2.0(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td>1.0(1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Yield (%)**

| Fragment | 8.1 | 5.0 | 9.0 | 12.3 | 14.8 | 6.8 | 16.3 | 4.4 | 16.3 | 7.2 | 25.4 | 11.4 |

---

*a* Values are expressed as residues per mol. Numbers in parentheses are residue values based on the sequence. *b* Determined as S-carboxymethyl-cysteine. *c* Determined as cysteic acid. N.D., not determined.
Fig. 5. The upper diagram indicates the structure of Fragment U with the cross-linking structure between Cys-19 and Glx-28. Isolation of fragment U was described in “MATERIALS AND METHODS.” The amino acid composition of Fragment U was as follows: Asx1.0, Glx1.1, Pro1.0, Ala2.0, Cys0.1(1), Ile2.1. The residue weights of each amino acid and the unknown constituent (Xu) are as follows: Ala, 71; Cys, 103; Ile, 113; Pro, 97; Asp, 115; Glx, 128 or 129; Xu, 163; and Glx(Xu), 275. The molecular weight of fragment U is 993. The molecular weights of chain A (Ala-Ala-Cys) minus H and chain B (Ile-Pro-Asp-Glx-Ile) minus OH or NH, are 262 and 568, respectively. → indicates sequencing by the DABITC/PITC double coupling method. The lower diagram indicates the structure of a fragment with the cross-linking structure between the N- and C-termini. The fragment was derived from fraction ‡V or ‡W of the hydrolysates of intact subtilosin A with concentrated HCl (see Fig. 12S). The amino acid composition of the fragment was as follows: Gly2.0, Ala1.0, Leu1.3. → indicates amino-terminal analysis by the dansylation method. The yield of this fragment was about 2%.

Figure 10S shows the FAB mass spectrum of the digest of Fragment U with aminopeptidase M in 0.1 N H4HCO3 (pH 7.9) overnight. Intense signals are present at m/z 229, 344, 406, 521, 538, 636, and 731, but the parent ion signal at m/z 994 and the signals at m/z 343 and 748 are absent. The signals at m/z 731, 521, and 406 are considered to be fragment ions produced during FAB mass spectrometry, formed by splitting of the bond between Cys-19 and Xu, and to correspond to [Ile-Pro-Asp-Glx(Xu)-Ile]+, [Asp-Glx(Xu)-Ile]+, and [Glx(Xu)-Ile]+ ions, respectively, whereas the signals at m/z 229 and 344 are thought to represent [Ile-Pro+H]+ and [Ile-Pro-Asp+H]+, respectively. These results, especially the presence of signals at m/z 344 and 406, indicate that Xu with a residue weight of 163 is present between Cys-19 and Glx-28 as shown in Fig. 5.
gested to link with the C-terminus, though the possibility that the C-terminus does not link with the N-terminus but with the Cys-10 or -13 residue cannot be excluded. However, from the concentrated HCl-hydrolysates of intact subtilosin A, a fragment that had a Gly residue at the N-terminus and consisted of four residues as shown in Fig. 5 was obtained, though the yield of the fragment was only about 2%. From the amino acid composition and the N-terminal analysis, this fragment was considered to be composed of the peptide with the N-terminal sequence of residues 1 and 2 and the C-terminal sequence of residues 31 and 32. Therefore, we concluded that the N-terminus of subtilosin A is linked with the C-terminus. That the C-terminus of intact subtilosin A was substituted was also suggested, because no amino acids were detected when C-terminal analysis of intact subtilosin A was performed by the hydrazine method (29).

From the above results the primary structure of subtilosin A as shown in Fig. 6 was deduced, excluding the structures of the two unknown residues, Xu and X. The residue weight of the substituent X at the N-terminus was calculated to be 246.

Production of Subtilosin A in Relation to Sporulation—Since many reports on the correlation between the production of antibiotics and sporulation in members of the genus Bacillus have appeared (cf. 3, 4, 11, 30), we attempted to examine the correlation between the production of subtilosin A and sporulation in B. subtilis 168. The time course of the production of subtilosin A in relation to cell growth and spore formation in the nutrient sporulation medium is shown in Fig. 7. The synthesis of this antibiotic began at 1 h after the cessation of vegetative growth. The antibiotic content increased linearly with time for the first 2 h and reached a plateau, followed by a gradual decrease. The maximal concentration was about 8 mg per liter of culture. The antibiotic produced was mostly secreted into the medium. Heat-resistant spores appeared at 8 h after the end of vegetative growth, increased gradually in number and reached 20% of viable cells at 22 h. The production of subtilosin A had finished before the appearance of refractile spores.

Like spore formation, the production of antibiotics is known to be sensitive to catabolite repression (31). To determine whether the production of subtilosin A is controlled by catabolites, glucose at a concentration of 10 g per liter was added to the culture at the end of vegetative growth. As shown in Fig. 8, the addition of excess glucose resulted in promotion of cell growth and repression of both antibiotic production and
Fig. 8. Effect of glucose on production of subtilosin A. Cells were grown in two 2-liter Erlenmeyer flasks containing 350 ml of NSM on a New Brunswick rotary shaker at 37°C. The contents of the two flasks were mixed and divided equally at 10 min before the addition of glucose. Glucose was added to one flask at a concentration of 10 g per liter at the end of vegetative growth. Cell growth and the production of subtilosin A were followed as described in the legend to Fig. 7. Open circles and triangles, cell growth and the amount of subtilosin A, respectively, in the control culture; closed circles and triangles, cell growth and amounts of subtilosin A, respectively, in the culture with excess glucose; and the arrow indicates the time of glucose addition.

Freese and his associates (14, 32) discovered that decoyinine, a specific inhibitor of GMP synthesis, is able not only to induce spore formation even in the presence of excess catabolites such as glucose and ammonium ions, but also to lead some asporogenous mutants to initiate spore formation. To determine whether the production of subtilosin A as well as spore formation is induced by this drug in the presence of glucose, sporulation was induced by adding decoyinine and the production of subtilosin A was followed under conditions similar to those described by Freese et al. (14). Decoyinine indeed induced sporulation to a normal level in the presence of glucose, but no production of subtilosin A was detected in either experiment (with or without decoyinine) as shown in Table IV, suggesting that the mechanism of repression of subtilosin A production and sporulation by glucose may be different and that the two phenomena have no correlation with each other.

Spore formation is a temporally ordered process (5, 6); consequently, if subtilosin A is indispensable for spore formation, it would not be produced by sporulation-negative mutants, such as spoOA and spoOB, which are blocked at the earliest stage of sporulation, whereas the mutants,

TABLE IV. Effects of decoyinine on the production of subtilosin A and sporulation. Cells were grown in two 2-liter Erlenmeyer flasks containing 350 ml of S6CG on a rotary shaker at 37°C. When cells had grown to 170 Klett units, decoyinine (0.6 mg/ml) was added to one flask. Cell growth and subtilosin A production were followed hourly as described in the legend to Fig. 7. The numbers of spores and viable cells were determined at 10 h after the addition of the drug as described in the legend to Fig. 7.

<table>
<thead>
<tr>
<th>Viable cells (V) *</th>
<th>With</th>
<th>Without</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 × 10⁸</td>
<td>1.2 × 10⁸</td>
<td></td>
</tr>
<tr>
<td>Spores (S) *</td>
<td>1.0 × 10⁶</td>
<td>4.0 × 10⁶</td>
</tr>
<tr>
<td>S/V × 100</td>
<td>&lt;0.01</td>
<td>33</td>
</tr>
</tbody>
</table>

* Values were expressed as numbers per ml of culture.

TABLE V. Production of subtilosin A by asporogenous mutants of B. subtilis 168. Cells of each mutant were grown under the same conditions as those described in the legend to Fig. 7. Subtilosin A was determined as described in "MATERIALS AND METHODS."

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Antibiotic production *</th>
<th>Subtilosin A production (µg/ml) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>spo&quot;</td>
<td>+</td>
<td>8.0</td>
</tr>
<tr>
<td>SR22</td>
<td>spoOA12</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td>6Z</td>
<td>spoOB6Z</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td>9V</td>
<td>spoOC9V</td>
<td>+</td>
<td>5.0</td>
</tr>
<tr>
<td>94UL</td>
<td>spoIII94U</td>
<td>+</td>
<td>0.0</td>
</tr>
<tr>
<td>11T</td>
<td>spoIV11T</td>
<td>+</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Production of antibiotics other than subtilosin A determined by bioautography. b Determined at 5 h after the end of vegetative growth.

J. Biochem.
such as spoIII and spoIV, which are blocked at the later stages and are known to produce some antibiotics, would be expected to produce subtilosin A. Table V summarizes the production of subtilosin A and other unidentified antibiotics by various asporogenous strains of B. subtilis 168. Of the strains tested only wild type and spoOC9V, in which the mutation is located within the spoOA gene (Ikeuchi, T., Kudoh, J., & Kurahashi, K., manuscript submitted) and which is less pleiotropic than spoOA mutations, produced both subtilosin A and other antibiotics. As expected, spoOA and spoOB mutants did not produce subtilosin A or other antibiotics, whereas spoIII and spolV mutants failed to produce subtilosin A, but produced other antibiotics.

On the basis of these results we conclude that subtilosin A is not involved in sporulation, though the time course of the production of subtilosin A coincides with spore formation.

Effects of Inhibitors of Protein and RNA Synthesis on the Production of Subtilosin A—Most of the peptide antibiotics so far studied have been reported to be synthesized by the multienzyme thiotemplate mechanism without the participation of either RNAs or ribosomes (cf. 33). In contrast to usual protein synthesis, inhibitors of protein and RNA synthesis have little effect on their syntheses. To determine whether the synthesis of subtilosin A follows a similar mechanism or not, we examined the effects of inhibitors of protein and RNA synthesis on the production of subtilosin A. Figures 9 and 10 show the effects of chloramphenicol and rifamycin SV, respectively. When chloramphenicol was added to the culture at a concentration of 20 μg/ml at 1 h after the onset of the production of subtilosin A, it completely repressed the production without marked effect on cell growth, and the level remained constant thereafter. The results suggest that subtilosin A

![Fig. 9](image_url)  
**Fig. 9.** Effect of chloramphenicol on subtilosin A production. Cell culture and determination of subtilosin A were carried out as described in the legend to Fig. 8, but chloramphenicol (20 μg/ml) instead of glucose was added at two and one-third hours after the end of vegetative growth. Open circles and triangles, cell growth and amounts of subtilosin A, respectively, in the absence of the inhibitor; closed circles and triangles, cell growth and amounts of subtilosin A, respectively, in the presence of the inhibitor. The arrow indicates the time of inhibitor addition.

![Fig. 10](image_url)  
**Fig. 10.** Effect of rifamycin SV on subtilosin A production. The cultivation of cells was carried out under the same conditions as those described in the legend to Fig. 9 except that rifamycin SV (30 μg/ml) was added in place of chloramphenicol. Because rifamycin SV overlapped subtilosin A in the HPLC system for quantitative analysis of subtilosin A as described in "MATERIALS AND METHODS," the method of subtilosin A estimation was modified as follows. Subtilosin A eluted from the HPLC system was converted to a fluorescamine-adduct, which was subjected to HPLC on the same column with a 40 ml linear gradient of 60% to 75% acetonitrile in 0.1% TFA. The amount of subtilosin A was calculated from the absorbance of the fluorescamine-adduct. Open circles and triangles, cell growth and amounts of subtilosin A, respectively, in the absence of the inhibitor; closed circles and triangles, cell growth and amounts of subtilosin A, respectively, in the presence of the inhibitor; the arrow indicates the time of inhibitor addition.
synthesis is ribosome-dependent and that its degradation is also prevented by the inhibitor. A similar result was obtained with kanamycin (data not shown). When rifamycin SV was used at a concentration of 30 μg/ml with similar timing, it also inhibited the production (Fig. 10), but the level of subtilosin A increased at a lower rate for the first 2 h after the addition of the inhibitor until it reached a plateau and remained constant thereafter. In this strain the minimum inhibitory concentration (MIC) of rifamycin SV was 3 μg/ml. In the above experiments the drug was used at 10 times higher concentration than the MIC. Therefore, the reduced rate of production of subtilosin A observed after the addition of rifamycin SV is considered to be due to the synthesis of subtilosin A using the preformed subtilosin A mRNA, which may have a longer half life than other prokaryotic mRNAs.

DISCUSSION

We found that B. subtilis 168 produced more than 10 antibiotics in the sporulation medium. Subtilosin A reported here is one of major antibiotics produced in the early stages of sporulation. We also found that B. subtilis ATCC 6633 and Bacillus natto IFO 13169 produced a subtilosin A-like antibiotic, which had the same retention time on a reverse-phase column in HPLC and the same amino acid composition as that of subtilosin A. B. megaterium and B. amyloliquefaciens H did not produce such a substance.

Subtilosin A consists of 32 amino acid residues of usual amino acids and two non-amino acid residues. The N-terminus is blocked by an unknown residue X. No sugars were detected in subtilosin A by the phenol-sulfuric acid reaction (34). There are unique cross-linking structures between the cysteine residue at position 19 and the Glx residue at position 28 through an unknown residue Xu, and between the N- and C-termini (Fig. 5). The linkage between cysteine 19 and Xu may be a thioester bond because of its lability in alkali treatment and performic acid oxidation, while the linkage between the Glx-28 and Xu may be an O-ester bond. Because performic acid-oxidized subtilosin A had a free α-carboxyl group as evidenced by the digestion with carboxypeptidase A-DFP, the linkage between the N- and C-termini is not an amide bond. To determine whether this antibiotic has S- and/or O-ester bonds, intact subtilosin A was treated with alkaline hydroxylamine (35). The presence of 1.3 equivalent/mol of ester bonds was detected, confirming the above postulated cross-linking structures. Such a cross-linking structure between a Cys residue and a Glx residue has already been reported in human complement 3 (36) and α2-macroglobulin (37), but the linkage is directly formed between the thiol group of the Cys residue and the γ-carboxyl group of the Glx residue in contrast to that of subtilosin A.

Of the antibiotic peptides produced by species of Bacillus for which the structures and synthetic mechanisms have been clarified, only subtilin is similar to subtilosin A in size and synthetic mechanism. Subtilin is a peptide antibiotic produced by B. subtilis ATCC 6633, and consists of 32 amino acid residues including unusual amino acids, such as methyllanthionine, lanthionine, dehydroalanine, and dehydrobutyric acid. It was shown to be formed by processing of precursor proteins (24). Since subtilosin A was shown to be synthesized by a mechanism dependent on mRNA-ribosomes and it had no methionine residues, it may well be synthesized as a larger precursor protein.

Ray and Bose (38) and Haavik and Thomassen (39) showed that the nonproducer mutants of mycobacillin and bacitracin were able to sporulate normally, suggesting that these antibiotics were dispensable for spore formation. Our results showed that subtilosin A seems to have no direct correlation with sporulation.

Further studies are required to elucidate the complete structure of subtilosin A. The blocking group at the N-terminus and the cross-linking structure between cysteine at residue 19 and Glx at residue 28 are being studied.

We are indebted to Miss Y. Yagi and to Drs. F. Sakiyama and S. Tsunasawa of this Institute for amino acid analyses and for helpful discussion, respectively. We are very grateful to Dr. J.E. Grady of the Upjohn Co. and Dr. K. Fujikawa of the University of Washington for generous gifts of decoyinine U-7984 and Achromobacter protease I, respectively. We thank Drs. S. Okubo, P. Schaeffer, J. Spizizen, and H. Araki for supplying strains of bacteria.
REFERENCES

Table IS. Amino acid compositions of Fragments I-V. I-V correspond to Fragments I-V, respectively.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>2.1 (2)</td>
<td>2.3 (2)</td>
<td>1.2 (1)</td>
<td>1.3 (1)</td>
<td>3.1 (3)</td>
</tr>
<tr>
<td>Thr</td>
<td>1.0 (1)</td>
<td>1.0 (1)</td>
<td>1.0 (1)</td>
<td>1.0 (1)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Ser</td>
<td>0.1</td>
<td>0.1</td>
<td>0.9 (1)</td>
<td>0.9 (1)</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Glx</td>
<td>1.2 (1)</td>
<td>1.3 (1)</td>
<td>1.3 (1)</td>
<td>1.3 (1)</td>
<td>1.3 (1)</td>
</tr>
<tr>
<td>Pro</td>
<td>2.3 (2)</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>Gly</td>
<td>1.1 (1)</td>
<td>1.4 (1)</td>
<td>4.7 (5)</td>
<td>6.0 (6)</td>
<td>6.9 (7)</td>
</tr>
<tr>
<td>Ala</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>Cysb</td>
<td>0.9 (1)</td>
<td>0.9 (1)</td>
<td>1.6 (2)</td>
<td>1.7 (2)</td>
<td>2.5 (3)</td>
</tr>
<tr>
<td>Val</td>
<td>1.0 (1)</td>
<td>1.1 (1)</td>
<td>0.9 (1)</td>
<td>0.9 (1)</td>
<td>0.9 (1)</td>
</tr>
<tr>
<td>Ile</td>
<td>2.0 (2)</td>
<td>2.0 (2)</td>
<td>1.0 (1)</td>
<td>2.8 (3)</td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>0.7 (1)</td>
<td>2.0 (2)</td>
<td>2.1 (2)</td>
<td>2.1 (2)</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>1.0 (1)</td>
<td>1.1 (1)</td>
<td>1.1 (1)</td>
<td>1.1 (1)</td>
<td>1.1 (1)</td>
</tr>
<tr>
<td>Trp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.D. (1)</td>
</tr>
</tbody>
</table>

a Values are expressed as residues per mol. Numbers in parentheses are residue values based on the sequence.

b Cysteine was determined as cysteic acid after performic acid oxidation.

N.D., Not determined.

LEGENDS TO SUPPLEMENTAL FIGURES

Fig. 1S. FAB mass spectrum of intact subtilosin A. The highest peak is at m/z 3401.8, but the peak at m/z 3399.9 is thought to correspond to [m+H]+ ion, if one takes the presence of isotopes into consideration.

Fig. 2S. Digestion of performic acid oxidized-subtilosin A with carboxypeptidase A-DFP. Amino acids released are expressed as follows: open and closed circles, Gly and Ala, respectively; open and closed triangles, Glu and Ile, respectively. Digestion with carboxypeptidase Y instead of carboxypeptidase A gave the following results. Amino acids released (mol/mol) were Ala (1.62), Gly (0.83), Ile (0.65), Asp (0.16), Glu (0.07), Leu (0.06) after incubation for 5 h; and Ala (1.96), Gly (1.17), Ile (1.16), Pro (0.50), Asp (0.48), Leu (0.23), Val (0.21), Glu (0.16) after 18 h. Both conditions are described in "Materials and Methods".

Fig. 3S. Titration of the thiol groups released by the treatment with anhydrous hydrazine of subtilosin A, bovine insulin and thermolysin. Dried samples were dissolved in anhydrous hydrazine, and allowed to stand at room temperature. At various times, portions (16 nmol in 25 μL) of the solution were removed, evaporated to dryness and titrated with DTNB according to the method of Kortt and Liu (22). Thermolysin and bovine insulin, which contain no and three cystine residues, respectively, were used as references. Closed triangles, circles, and squares indicate the amounts of thiol groups of bovine insulin, subtilosin A, and thermolysin titrated, respectively.

J. Biochem.
SUBTILOSIN A, A NEW ANTIBIOTIC PEPTIDE FROM B. subtilis 168

Isolation of peptides was described in the legend to Fig. 5S. The amino acid sequence of each peptide is given in one-letter notation. ~ indicates sequencing by the dansyl-Edman method. X and Xu are unknown residues.

Fig. 5S. Reverse-phase HPLC of thermolysin peptides of intact subtilosin A. I-V correspond to the structures of Fragments I-V, respectively. Digestion was performed as described in "Materials and Methods". After excluding precipitates from the digests by centrifugation, the supernatant was applied to a reverse-phase HPLC column (Chemcopak, C18). Elution was performed with a 40 ml linear gradient of 10% to 70% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min, and monitored at 215 nm. Each peak was further purified in the same manner as above, but with shallower gradients of acetonitrile. The fragments derived from peaks I-V were designated as Fragments I-V, respectively.

Fig. 6S. FAB mass spectra of Fragments I, III, and IV. I, III, and IV show the FAB mass spectra of Fragments I, III, and IV, respectively. From the observed mass values of the parent ion signal, the molecular weights of Fragments I, III, and IV were estimated to be 1361, 1808.9, and 1978.8, respectively. In the FAB mass spectrum of Fragment I, the structure of each signal is shown above the signal by the one-letter notation for amino acids.

Fig. 7S. Reverse-phase HPLC of the thermolysin digests of Fragment I for isolation of Fragment U. Fragment I was digested as described in "Materials and Methods". The digests were subjected to HPLC on a reverse-phase column (Chemcopak, C18 and eluted with 40 ml of a linear gradient of 0% to 35% acetonitrile in 0.1% TFA. Eluates were monitored at 215 nm. The bar and asterisk indicate the positions of Fragments U and I, respectively.
Fig. 8S. FAB mass spectrum of Fragment U in the range from 200 to 1200 atomic mass units. The structure corresponding to each signal is shown above the signal by the one-letter notation for amino acids.

Fig. 9S. FAB mass spectrum in the range from 200 to 1100 atomic mass units of fragment U after incubation in 0.1% NH₄HCO₃ (pH 7.9) at 37°C for 4 h.

Fig. 10S. FAB mass spectrum in the range from 200 to 1100 atomic mass units of the digests of fragment U with aminopeptidase M. Digestion was carried out in 0.1% NH₄HCO₃ (pH 7.9) at 37°C overnight. The inset is the FAB mass spectrum enlarged three-fold in the range from 350 to 750 atomic mass units.

Fig. 11S. Reverse-phase HPLC of dilute acid hydrolyzate of RCM-subtilosin A. Hydrolysis of the sample with dilute acid was carried out as described in "Materials and Methods". A half of the hydrolyzate was applied to a reverse-phase column (Yanapak ODS-T). Elution was performed with 80 ml of a 0 to 60% linear gradient of a 7 : 3 mixture of 2-propanol and acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. Eluates were monitored at 215 nm. The underlined fractions were further purified to homogeneity by HPLC. Peptides dAH-1 through -5 in Table III were derived from fractions I through V, respectively.

Fig. 12S. Reverse-phase HPLC of concentrated HCl-hydrolyzates of intact subtilosin A. The sample (13 mg) was hydrolyzed as described in "Materials and Methods". The hydrolyzates were chromatographed by HPLC on a reverse-phase column (Yanapak ODS-T) with 80 ml of a 0 to 60% linear gradient of acetonitrile in 0.1% TFA at a flow rate of 0.8 ml/min. Eluates were monitored at 215 nm. The underlined fractions were further purified to homogeneity by HPLC. Peptides cAH-1 through -4 in Table III were derived from fractions I through VI, respectively. Peptides with a cross-linking structure between the N- and C-termini were obtained from fractions V and VI.

Fig. 13S. Reverse-phase HPLC of the first digests of performic acid-oxidized subtilosin A with Achromobacter protease I. The sample (1.4 mg) was digested with 28 μg of the protease in 0.14 ml of 0.1 M NH₄HCO₃ containing 10 mM CaCl₂ at 37°C for 2 h. The digests were subjected to reverse-phase HPLC on a Yanapak ODS-T column. Elution was carried out with 80 ml of a linear gradient of 0 to 40% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. Eluates were monitored at 215 nm. Peptide AP-I in Table III was obtained from the fractions marked by a bar that had been purified to homogeneity by HPLC.

J. Biochem.
SUBTILOSIN A, A NEW ANTIBIOTIC PEPTIDE FROM B. subtilis 168

Fig. 14S. Reverse-phase HPLC of the second digests of performic acid-oxidized subtilosin A with Achromobacter protease I. All procedures (except that the sample was digested with 3.5 μg of Achromobacter protease I at 37°C for 45 min) were performed as described in the legend to Fig. 13S. Peptide AP-2 in Table III was derived from the fraction marked by a bar that had been purified to homogeneity by HPLC.

Fig. 15S. Reverse-phase HPLC of chymotryptic peptides of RCM-subtilosin A. Digestion was carried out as described in “Materials and Methods”. The digests were chromatographed on a reverse-phase HPLC column (Chrompack, C18) with 60 ml of a linear gradient of 0 to 65% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. Eluates were monitored at 215 nm. The underlined fractions were further purified to homogeneity by HPLC. Peptides CH-2 through -7 in Table III were obtained from fractions I through VII, respectively.

Fig. 16S. Reverse-phase HPLC of thermolysin peptides of RCM-subtilosin A. Digestion was performed as described in “Materials and Methods”. A half of the digest was applied to a reverse-phase HPLC column (Chrompack, C18), and eluted with 40 ml of a linear gradient of 0 to 65% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. The eluates were monitored at 215 nm. The underlined fractions were further purified to homogeneity by HPLC. Peptides CH-2 through -6 in Table III were obtained from fractions I through VI, respectively.

Fig. 17S. Isolation of the N-terminal fragments from chymotryptic peptides of RCM-subtilosin A by reverse-phase HPLC. RCM-subtilosin A (1.53 mg) was digested with 75 μg of α-chymotrypsin in 0.2 M N-ethylmorpholine acetate buffer, pH 8.5, at 37°C for 11 h. The digests were applied to a SP-Sephadex C-25 column (12 x 50 mm) equilibrated with 25 mM phosphoric acid, pH 2.0, and then washed thoroughly with the same solvent. The washings were chromatographed by reverse-phase HPLC on a Chrompack C18 column with 40 ml of linear gradient of 30 to 50% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. Eluates were monitored at 215 nm. Peptide CH-1 in Table III was obtained from the fraction indicated by a bar that had been purified to homogeneity by HPLC.

Fig. 18S. Isolation of the N-terminal fragments from thermolysin peptides of RCM-subtilosin A by reverse-phase HPLC. RCM-subtilosin A (3 mg) was digested with 300 μg of thermolysin in 0.1 M NH4HCO3 containing 10 mM CaCl₂ at 60°C for 3 h. The digests were subjected to ion-exchange chromatography and reverse-phase HPLC in the same manner as described in the legend to Fig. 17S. Peptide TH-1 in Table III was obtained from the fraction marked by a bar that had been purified to homogeneity by HPLC.