Biosci. Biotechnol. Biochem., **69** (4), 700–704, 2005

JSBA

Enhancement of Linear Gramicidin Expression from *Bacillus brevis* ATCC 8185 by Casein Peptide

Tomonori Nakai,† Daisuke Yamauchi, and Kou Kubota

University of Hyogo, 2167 Shosha, Himeji, Hyogo 671-2201, Japan

Received September 13, 2004; Accepted January 3, 2005

***Bacillus brevis* (*Brevibacillus parabrevis*) ATCC 8185 synthesizes two kinds of antibiotic peptides, cyclopeptide tyrocidine and linear gramicidin. The production of linear gramicidin can be induced by the standard method (using a skim milk medium for pre-culture and beef broth for the main culture) employed for the induction of tyrocidine. In this study, we tried to determine the optimal growth medium for *B. brevis* ATCC 8185 for synthesizing linear gramicidin. The yield of linear gramicidin produced by the standard method was 3.11 μg/ml. When beef broth was used both as the pre-medium and the main medium, the yield of the antibiotic was only 0.59 μg/ml. To confirm the influence of skim milk, the strain was grown in a 1% skim milk medium. As a result, the amount of linear gramicidin produced reached 20.3 μg/ml. These findings show the importance of skim milk in the production of linear gramicidin. In the skim milk medium, the cells produced an extracellular protease 2 h before the linear gramicidin was expressed. The 1% skim milk medium pretreated by this protease did not allow the induction of linear gramicidin into the cells, and protease activity was not detected in the supernatant of the culture. When the cells were cultivated in a 1% egg albumin medium, protease activity from the supernatant of the culture was detected, but production of linear gramicidin was not observed. Therefore, a 1% casein medium was used for production of linear gramicidin. As a result, the yield of linear gramicidin produced in the medium reached 6.69 μg/ml. We concluded that a digested product of the extracellular protease from casein enhances linear gramicidin production.**

Key words: linear gramicidin; skim milk; protease; *Bacillus brevis*; expression

The gram-positive bacteria of genus *Bacillus* produce a variety of oligo-peptide antibiotics and lipo-peptides.[1] Among these, *Bacillus brevis* (*Brevibacillus parabrevis*) ATCC 8185 synthesizes two kinds of antibiotic peptides, cyclopeptide tyrocidine and linear gramicidin.[1,2] These antibiotics are produced simultaneously and accumulate in cells.[3]

Linear gramicidin forms transmembrane channels in a dimer structure in lipid bilayers.[4,5] These channels have selectivity for monovalent cations, and the effect of this is dissipated H^+ and K^+ gradients.[6] Hence linear gramicidin has been used to study the potential role of the plasma membrane.[7]

Linear gramicidin is produced *via* a non-ribosomal process, like tyrocidine and gramicidin S.[2] It is a terminally blocked pentadecapeptide with alternating L- and D-amino acids, having the following structure: fVal-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-Try-D-Leu-Trp-D-Leu-Trp-D-Leu-Trp-Etn (f = formyl, Etn = ethanolamine).[8] Synthesis of linear gramicidin starts with a formylated amino terminal. The initial formyl action of the thioester-attached valine takes place on the first multienzyme.[9–11] The carboxy-terminal is modified by ethanolamine.[12]

In early studies of linear gramicidin, Pschorn *et al.*, showed induction of sporulation by tyrocidine and linear gramicidin on *Bacillus*,[13] and Ristow *et al.* reported that the production level of linear gramicidin was affected by the nitrogen concentration in the culture medium.[3] But the induction factor of the oligopeptide is unclear in these reports. Although it has been reported that extracellular protease contributes to the induction of linear gramicidin,[14] the role of the protease remains to be elucidated.

In 1966, Fujikawa *et al.* reported a method for efficiently producing tyrocidine.[15] This method was used to produce linear gramicidin, but was troublesome because four kinds of media are needed from the plating to the main growth stage.[12,15,16] Although this method is very complicated, no one has attempted study of the growth media to optimize the synthesis, or of the factors contributing to the induction of linear gramicidin. Research on an inducer or induced mechanism of the peptide would contribute to the establishment of a mass production system, for many applications in medicine, agriculture, and biochemical research.

Here we report a method of growing *B. brevis* ATCC 8185 to synthesize linear gramicidin that is simpler and more efficient than the standard method proposed by Fujikawa *et al.*[15] Furthermore, we suggest that linear

† To whom correspondence should be addressed. Fax: +81-792-67-4920; E-mail: nakait@sci.u-hyogo.ac.jp

gramicidin is induced by a protease-digested product from a casein polypeptide.

Materials and Methods

Bacterial strain and media. Bacillus brevis ATCC 8185 was used in this study. This bacterium was grown on a nutrient agar plate (0.8% nutrient broth, Difco, 1.5% agar, pH 6.8), and stored at 4 °C until used. The strain stored at 4 °C was grown on a nutrient agar plate or skim milk agar plate (2% skim milk, Difco, 0.2% polypeptone, Nippon Seiyaku, 0.2% NaCl, 0.02% $MgSO_4$, 0.1% KH_2PO_4, pH 6.8) at 37 °C for 2 days. A beef broth (1% beef extract, Nakarai, 1.0% polypeptone, 0.1% NaCl, pH 6.8), a skim milk-yeast extract (SY) medium (1.0% skim milk, 0.1% yeast extract, 3.7 mM KH_2PO_4, 2.9 mM K_2HPO_4, 2.0 mM $MgSO_4 \cdot 7H_2O$, 0.17 mM NaCl, 0.05 mM $MnCl_2 \cdot 4H_2O$, 0.036 mM $FeSO_4 \cdot 5H_2O$, 0.15 mM $CaCl_2$, pH 6.8), or a medium containing only skim milk (1% skim milk medium) was used as the pre-cultivation medium, and each medium was incubated for 15 to 24 h at 37 °C on a rotary shaker (120 rpm). The pre-culture medium (3 ml) was inoculated into beef broth (60 ml), 1% skim milk medium (60 ml), 1% casein medium (60 ml), 1% egg albumin medium (1% egg albumin, Wako, pH 6.8), or protease-pretreated 1% skim milk medium, and the inoculated cultures were grown with shaking (120 rpm) for 10 to 24 h at 37 °C. The 1% casein medium was prepared by solubilizing casein powder (Wako) in 0.1 M NaOH at 60 °C and then adjusting to pH 6.8 with acetic acid.

To prepare a protease-pretreated 1% skim milk medium, the 1% skim milk medium was boiled for 15 min. A papain protease solution (1.0 mg/ml final concentration) or a crude extract (preparation method described below), containing the extracellular protease from ATCC 8185 with an activity corresponding to that of the 1.0 mg/ml papain solution, was added to the medium and incubated for 24 h at 37 °C. By autoclaving the protease-pretreated 1% skim milk medium at 115 °C for 15 min, the added protease was inactivated, and this medium was used as the protease-pretreated 1% skim milk medium. The papain solution or the crude extract at an appropriate concentration was filtrated and then added to the media. The papain protease was purchased from Wako.

Preparation of crude extract containing extracellular protease from a culture supernatant of B. brevis *ATCC 8185.* A supernatant of *B. brevis* ATCC 8185 culture in 1% skim milk medium (450 ml) was recovered by centrifugation (6,000 × *g*, 4 °C, 10 min; RA-1500 rotor, Kubota 6900). Ammonium sulfate was added to the resulting supernatant until 80% saturation. After shaking at 0 °C for 30 min, a pellet was recovered by centrifugation (15,000 × *g*, 4 °C, 30 min; RA-1500 rotor, Kubota 6900). The pellet was resuspended with 4 ml of 10 mM Tris–HCl (pH 6.8) and then dialyzed against 10 mM Tris–HCl (pH 6.8) at 4 °C. The resulting crude extract containing extracellular protease was stored at 4 °C until used.

Measurement of linear gramicidin. The amount of linear gramicidin was measured according to a modification of the procedure of Oyama and Kubota.[14] The growth culture of *B. brevis* was adjusted to pH 4.0 with 1 M HCl, and the cell pellet was recovered by centrifugation at 6,000 × *g* at room temperature for 10 min (RA-1500 rotor, Kubota 6900). The pellet was resuspended with 2 ml of ethanol and then heated at 95 °C for 3 min. The cell suspension was centrifuged at 6,000 × *g* at room temperature for 10 min, and the resulting supernatant was recovered. Linear gramicidin was purified from the supernatant using a basic alumina (active grade I, ICN) column (0.9 × 1.5 cm). The alumina column was equilibrated with 2 ml of 85% ethanol. H_2O (0.15 ml) was added to the supernatant (0.85 ml), and the mixture was applied to the alumina column. Elution from the column was performed by adding 2 ml of 85% ethanol to the column. To the resulting elution (0.5 ml) containing the antibiotic was added the same volume of 2.0% *p*-dimethylaminobenzaldehyde in 35% HCl, followed by 50 µl of 0.1% $NaNO_2$. After incubation for at least 15 min at room temperature, the absorbance (660 nm) was measured. For standardization, commercially available linear gramicidin (Sigma) was used. Linear gramicidin is shown as the amount contained in 1 ml of the growth culture.

Measurement of cell concentration. To measure cell concentration, the number of viable cells was determined by counting the number of colony-forming units (CFU) per ml of culture.

Measurement of protease activity. To determine protease activity in the supernatant of cultures, 1 ml of the supernatant was recovered from the culture by centrifugation at 15,000 rpm for 10 min at 4 °C (RT15A2 rotor, Himac CR 15D, Hitachi). A sampling tube containing 100 µl of the supernatant, 100 µl of 1% azocasein (Sigma), and 300 µl of 10 mM Tris–HCl (pH 6.8) was incubated at 37 °C for 2 h, and then 400 µl of 10% trichloroacetic acid was added. After incubation at 0 °C for 20 min, the tube was centrifuged at 15,000 rpm for 10 min at 4 °C. The optimal density of the resulting supernatant at 366 nm was measured.

Results and Discussion

Induction of linear gramicidin

The amount of linear gramicidin, produced by the standard method described by Fujikawa *et al.*, was 3.11 µg/ml[15] (Table 1). This was half of the value (6.20 µg/ml for 10 h) previously reported.[14] The yield of linear gramicidin in the growth medium cultivated for 24 h was 4.52 µg/ml (Table 1). In this experiment, the

Table 1. Amounts of Linear Gramicidin and Viable Cells in Different Media and Growth Times

Main medium	Growth time (h)	Linear gramicidin (μg/ml)**	Viable cells (CFU/ml)
Standard method*	10	3.11 ± 0.45	1.25×10^8
	24	4.52 ± 1.17	8.50×10^8
1% skim milk	10	4.25 ± 0.58	7.65×10^7
	24	20.3 ± 0.58	3.19×10^8
Beef broth	10	0.40 ± 0.10	1.23×10^9
	24	0.59 ± 0.22	9.36×10^9

*According to the method of Fujikawa *et al.*[15]
**Linear gramicidin is shown as the amount contained in 1 ml of growth culture.

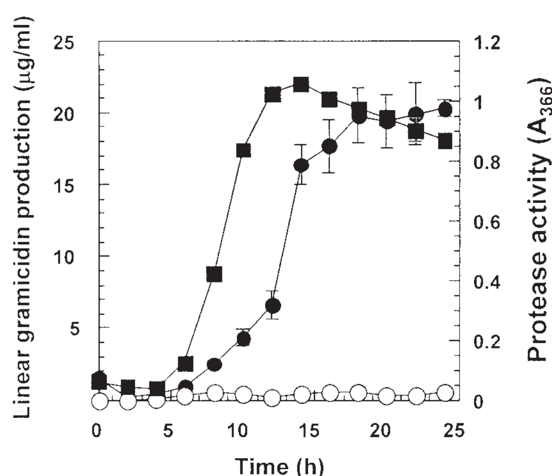

Fig. 1. Time Course of Linear Gramicidin Production and Extracellular Protease Activity.

Linear gramicidin is shown as the amount present in 1 ml of growth culture. The growth conditions were 1% skim milk medium both as pre-medium and main medium (closed circles), of beef broth both as pre-medium and main medium (open circles). Protease activity from the supernatant of the culture is shown as the value of the optical density at 366 nm when using azocasein as a substrate (closed squares). The growth condition was 1% skim milk medium both as pre-medium and main medium.

beef extract (Nakarai) present in the beef broth medium was different from that (Eurlich) used by Oyama and Kubota.[14] Because the contents were different in the two extracts, the value of linear gramicidin production in this study might be inconsistent with the reference value.[14]

B. brevis was grown on a nutrient agar plate and then, in beef broth only, the cells were cultivated. The attempt to induce linear gramicidin in the cells resulted in no detection (Fig. 1). Therefore, the beef broth was not appropriate for induction of the peptide.

Oyama and Kubota reported that the level of linear gramicidin productivity increased on the addition of a supernatant of skim milk medium culture and $MnCl_2$ to the main beef broth medium.[14] Additionally, a skim milk agar medium and skim milk liquid medium were used for pre-cultivation in the standard method.[15] Hence 1% skim milk liquid medium was used as the induction medium of linear gramicidin. Cells grown on the nutrient agar plate for 2 d were inoculated into the 1% skim milk medium and sub-cultivated for 24 h at 37 °C in a rotary shaker. Three ml of this sub-culture was inoculated into 60 ml of fresh 1% skim milk medium and the production level of linear gramicidin was monitored every 2 h. The cells grown for 10 h produced linear gramicidin as well as did those in the standard method (Table 1). An increase in the peptide was observed until 16 h, at which time the production of linear gramicidin reached 20.3 μg/ml (Table 1 and Fig. 1). When the beef broth was used as pre-cultivated medium instead of the 1% skim milk medium, the production of linear gramicidin in the main medium (1% skim milk medium) reached the same level (19.8 μg/ml) at 24 h. These results indicate that 1% skim milk medium is the most suitable medium for the induction of linear gramicidin, and that the omission of cell growth on the skim milk agar plate in the standard method does not affect linear gramicidin production.

The CFU of the 1% skim milk culture grown for 24 h was about 40% of that of the culture grown for 24 h by the standard method (Table 1). This means that the amount of linear gramicidin produced by cells grown in 1% skim milk medium increases twelve-fold compared to that by cells grown by the standard method. These results suggest that an inducer for linear gramicidin production might exist in skim milk medium.

The production of linear gramicidin was at the highest level at 24 h, regardless of the method used, and hence all cultures were cultivated for 24 h the experiments described below.

Contribution of an extracellular protease to the expression of linear gramicidin

Oyama and Kubota reported that the level of linear gramicidin productivity increased on the addition of a protease to the main beef broth medium.[14] To examine whether the cells secreted a protease in the growth medium, cells were grown in 1% skim milk medium and the protease activity in the supernatant of the culture was monitored. The activity appeared in the culture grown for 6 h, and reached a maximum level at 15 h (Fig. 1). The secretion of the protease was preceded by the expression of linear gramicidin. But when beef broth medium was used for cultivation, protease activity was not detected (Table 2). These results suggest that extracellular protease might contribute to the induction of linear gramicidin.

It has been reported that induction of linear gramicidin was observed in beef broth medium on the addition of certain proteases to a final concentration of 100 μg/ml.[14] To determine whether extracellular protease contributes directly to the expression of linear gramicidin, a crude extract that contained extracellular protease was prepared from the supernatant of the 1% skim milk growth culture by an ammonium sulfate precipitation.

Table 2. Effect of Protease in Beef Broth on Linear Gramicidin Production

Main medium	Supplement	Protease activity (A_{366})	Linear gramicidin (μg/ml)***	Viable cells (CFU/ml)
Beef broth	None	0.008 ± 0.005	0.59 ± 0.22	9.36×10^9
Beef broth	Papain*		0.62 ± 0.15	1.54×10^9
Beef broth	Crude extract**		0.43 ± 0.12	2.68×10^9

*Papain was adjusted to a 100 μg/ml final concentration.
**The crude extract contained extracellular protease with activity that corresponded to that of 100 μg/ml papain protease.
***Linear gramicidin is shown as the amount contained in 1 ml of growth culture.

The crude extract, the activity of which corresponded to the activity of 100 μg/ml papain protease (final concentration, $A_{366} = 0.767$), was applied to beef broth medium, and cells were grown in this medium. But linear gramicidin was not detected from the grown cells, and cells grown in beef broth medium that contained 100 μg/ml of papain protease (final concentration) also did not show linear gramicidin production (Table 2). When the extracellular protease with activity, which corresponded to the activity in the supernatant from the 1% skim milk culture grown for 12 h (final concentration, $A_{366} = 1.06$), was added to the beef broth, no production of linear gramicidin was observed (data not shown). Since a substrate necessary for inducing linear gramicidin production does not exist in beef broth medium, the addition of extracellular protease to beef broth medium might not have any effect on the induction of linear gramicidin production.

Importance of casein polypeptide in the expression of linear gramicidin

It has been reported that increased formation of L-ornithine by arginase accelerated the production of gramicidin S.[17] To investigate whether an amino acid that forms casein in skim milk is an important factor in linear gramicidin induction, skim milk was pretreated with a crude extract containing extracellular protease. The cells were cultivated with extracellular protease-pretreated 1% skim milk medium, and then the cell extract was obtained by boiling with ethanol. As for the result, the peptide contained in the cell extract decreased drastically (Table 3). When papain protease was added to 1% skim milk medium for pretreatment, to substitute for extracellular protease, the cells grown in the papain-pretreated 1% skim milk medium did not produce linear gramicidin. There is a possibility that the casein was digested in amino acids and short peptides, such as dipeptide or tripeptide, by the proteases, and that these amino acids and short peptides did not have any effect on linear gramicidin expression (Table 3). These results suggest that a protease-digested oligopeptide from casein is an important factor in the induction of linear gramicidin. But the extracellular protease induced by casein may activate an inducer for the peptide, because

Table 3. Amount of Linear Gramicidin and Protease Activity in Protease-Pretreated 1% Skim Milk Medium

Main medium	Pretreatment	Protease activity (A_{366})	Linear gramicidin (μg/ml)***	Viable cells (CFU/ml)
1% skim milk	None	0.868 ± 0.023	20.3 ± 0.58	3.19×10^8
	Papain*	0.050 ± 0.001	0.35 ± 0.12	1.87×10^8
	Crude extract**	0.043 ± 0.002	1.14 ± 0.07	5.64×10^8

*Papain was adjusted to a 100 μg/ml final concentration.
**The crude extract contained extracellular protease with activity that corresponded to that of 100 μg/ml papain protease.
***Linear gramicidin is shown as the amount contained in 1 ml of growth culture.

Table 4. Amount of Linear Gramicidin and Protease Activity in Certain Polypeptide Media

Main medium	Protease activity (A_{366})	Linear gramicidin (μg/ml)*	Viable cells (CFU/ml)
1% skim milk	0.868 ± 0.023	20.3 ± 0.58	3.19×10^8
1% egg albumin	1.468 ± 0.039	1.48 ± 0.18	2.18×10^9
1% casein	0.088 ± 0.014	6.69 ± 1.63	5.91×10^8

*Linear gramicidin is shown as the amount contained in 1 ml of growth culture.

extracellular protease activity was not detected in the supernatant from this protease-pretreated culture (Table 3).

B. brevis ATCC 8185 was cultivated in 1% egg albmin medium for 24 h at 37 °C. Then the protease activity in the supernatant of the culture and the level of linear gramicidin produced were investigated. Protease activity was detected, and its level was higher than that in the supernatant of the 1% skim milk culture, but the level of linear gramicidin produced was 1/13-fold lower than that of the 1% skim milk culture (Table 4). These results suggest that an oligopeptide from a protein involved in skim milk is important in the induction of linear gramicidin.

To investigate the effect of casein peptide, *B. brevis* was cultivated in 1% casein medium for 24 h at 37 °C. The supernatant from the 1% casein culture had little protease activity, but linear gramicidin was detected at significant levels from the cells grown in 1% casein medium (Table 4). The casein was treated with sodium hydrate to make it soluble in water. Therefore, *B. brevis* might be able to incorporate the casein peptide partially digested by sodium hydrate, even if the cells do not secrete extracellular protease. However, there is a possibility that the casein solubilized by sodium hydrate was different from the products digested by extracellular protease. Therefore, *B. brevis* might not be able to produce linear gramicidin in 1% casein medium as effectively as in 1% skim milk medium.

Although extracellular protease activity was detected at significant levels in the 1% egg albumin medium, the production level of linear gramicidin was lower than that

in the 1% skim milk medium. This result confirms that extracellular protease does not directly induce linear gramicidin. Therefore, an oligopeptide from the casein polypeptide digested by the extracellular protease might be incorporated into the cells and induce expression of linear gramicidin. Recently, nucleotide sequences encoding genes to synthesize linear gramicidin have been identified, and the entire biosynthetic gene cluster was identified on four very large open reading frames covering a region of more than 60 kbp.[18] Although a gene regulatory region for synthetic antibiotic has yet to be identified, the product obtained by protease from casein might contribute to analysis of gene expression.

Acknowledgment

This work was supported by a grant from the Himeji Institute of Technology.

References

1) Zuber, P., Nakano, M. M., and Marahiel, M. A., Peptide antibiotics. In "*Bacillus subtilis* and Other Gram-Positive Bacteria", eds. Sonenshein, A. L., Hoch, J. A., and Losick, R., American Society for Microbiology, Washington, D.C., pp. 897–916 (1993).
2) Kleinkauf, H., and Döhren, H. von, Nonribosomal biosynthesis of peptide antibiotics. *Eur. J. Biochem.*, **192**, 1–15 (1990).
3) Ristow, H., Pschorn, W., Hansen, J., and Winkel, U., Induction of sporulation in *Bacillus brevis* by peptide antibiotics. *Nature*, **280**, 165–166 (1979).
4) Hladky, S. B., and Haydon, D. A., Ion transfer accross lipid membranes in the presence of gramcidin A. I. Studies of the unit conductance channel. *Biochim. Biophys. Acta*, **274**, 294–312 (1972).
5) Prasad, K. U., Alonso-Romanowski, S., Venkatachalam, C. M., Trapane, T. L., and Urry, D. E., Synthesis, characterization, and black lipid membrane studies of [7-L-alanine]gramicidin A. *Biochemistry*, **25**, 456–463 (1986).
6) Gómez-Puyou, A., and Gómez-Lojero, C., The use of ionophores and channel formers in the study of the function of biological membranes. In "Current Topics in Bioenergetics", ed. Sanadi, D. R., Academic Press, New York, pp. 221–257 (1977).
7) Schaller, A., and Frasson, D., Induction of wound response gene expression in tomato leaves by ionophores. *Planta*, **212**, 431–435 (2001).
8) Lipmann, F., On biosynthesis of the cyclic antibiotics gramicidin S and tyrocidine, and of linear gramicidin. In "Peptide Antibiotics Biosynthesis and Functions", eds. Kleinkauf, H., and Döhren, H. von, Walter de Gruyter, Berlin, pp. 23–45 (1982).
9) Akashi, K., and Kurahashi, K., Formylation of enzyme-bound valine and stepwise elongation of intermediate peptides of gramicidin A by a cell-free enzyme system. *Biochem. Biophys. Res. Commun.*, **77**, 259–267 (1977).
10) Akashi, K., Kubota, K., and Kurahashi, K., Biosynthesis of enzyme-bound formylvaline and formylvalylglycine: a possible initiation complex for gramicidin A biosynthesis. *J. Biochem.* (Tokyo), **81**, 269–272 (1977).
11) Akashi, K., and Kurahashi, K., Enzyme-bound formylvaline and forlylvalylglycine: an initiation complex for gramcidin A biosynthesis. *J. Biochem.* (Tokyo), **83**, 1219–1229 (1978).
12) Kubota, K., Membranous phosphoglyceride-linked biosynthesis of pentadeca peptide, linear gramcidin, by *Bacillus brevis* ATCC 8185. *Biochem. Biophys. Res. Commun.*, **144**, 203–209 (1987).
13) Pschorn, W., Paulus, H., Hansen, J., and Ristow, H., Induction of sporulation in *Bacillus brevis* 2. Dependence on the presence of the peptide antibiotics tyrocidine and linear gramicidin. *Eur. J. Biochem.*, **129**, 403–407 (1982).
14) Oyama, M., and Kubota, K., Induction of antibiotic production by protease in *Bacillus brevis* (ATCC 8185). *J. Biochem.* (Tokyo), **113**, 637–641 (1993).
15) Fujikawa, K., Suzuki, T., and Kurahashi, K., Incorporation of L-leucine-C^{14} into tyrocidine by a cell-free preparation of *Bacillus brevis* Dubos strain. *J. Biochem.* (Tokyo), **60**, 216–218 (1966).
16) Kubota, K., Generation of formic acid ethanolamine from serine in biosynthesis of linear gramicidin by a cell-free preparation of *Bacillus brevis* (ATCC 8185). *Biochem. Biophys. Res. Commun.*, **105**, 688–697 (1982).
17) Kanda, M., Ohgishi, K., Hanawa, T., and Saito, Y., Arginase of *Bacillus brevis* Nagano: purification, properties, and implication in gramicidin S biosythesis. *Arch. Biochem. Biophys.*, **344**, 37–43 (1997).
18) Kessler, N., Schuhmann, H., Morneweg, S., Linne, U., and Marahiel, M. A., The linear pentadecapeptide gramicidin is assembled by four multimodular nonribosomal peptide synthetases that comprise 16 modules with 56 catalytic domains. *J. Biol. Chem.*, **279**, 7413–7419 (2004).