Permeability of dormant spores of *Bacillus subtilis* to gramicidin S

Yasusuke Tanimoto, Yuko Ichikawa, Yoko Yasuda, Kunio Tochikubo *

Department of Microbiology, Nagoya City University Medical School, Mizuho-ku, Nagoya 467, Japan

Received 24 October 1995; revised 19 November 1995; accepted 1 December 1995

Abstract

Gramicidin S, dissolved in ethanol, penetrated into the inside of the dormant spores of *Bacillus subtilis*, had a partial inhibitory effect on L-alanine-initiated germination and completely inhibited their outgrowth and vegetative growth. The activity of particulate NADH oxidase of the antibiotic-treated dormant spores was also influenced significantly. Abnormal morphological changes were observed in germinated spores from gramicidin S-treated dormant spores. An immunoelectron microscopy method with colloidal gold-IgG complex showed that the penetration site of gramicidin S inside dormant spores was mainly the core region. These facts suggest that gramicidin S induces the damage of not only the outer membrane-spore coat complex but also the inner membrane surrounding the spore protoplast, and is able to penetrate into the core region of *B. subtilis* dormant spores.

Keywords: *Bacillus subtilis*; Dormant spores; Gramicidin S

1. Introduction

Bacterial spores are resistant to most antibiotics because they are dormant and become sensitive only after they germinate [1–4]. However, cyclic polypeptide antibiotics such as polymyxin B and colistin, and aminoglycoside antibiotics such as streptomycin, kanamycin and gentamicin combine with the dormant spores of *Bacillus subtilis*, inhibit outgrowth or vegetative growth after germination, and consequently act as sporocidal or sporostatic agents under certain limited conditions [5,6]. The results obtained from an immunoelectron microscopy using colloidal gold-immunoglobulin complex showed that polymyxin B and gentamicin are able to penetrate into the spore coat but not into the cortex or core, namely the permeability barrier to them exists in the outer membrane–spore coat complex [7,8]. This report describes the penetration of gramicidin S, a cyclic polypeptide antibiotic, into the core region of *B. subtilis* dormant spores and its effect on germination, outgrowth and vegetative growth.

2. Materials and methods

2.1. Treatment of dormant spores with gramicidin S and CaCl₂

The minimum inhibitory concentration (MIC) of gramicidin S (Meiji Seika, Tokyo, Japan) for *B.
_subtilis_ PCI219 was determined by using dormant spores. 0.2 ml of each of two-fold diluted gramicidin S solutions in ethanol, and 0.1 ml of a spore suspension were added to 19.7 ml of heart infusion broth (HIB, Eiken Chemical Co., Tokyo, Japan) (the final concentration of the spores was about $2.5 \times 10^8$ cells ml$^{-1}$), and incubated at 37°C for 24 h with shaking. The lowest concentration inhibiting visible growth after 24 h of incubation, 6.25 μg ml$^{-1}$, was determined as the MIC of gramicidin S. Dormant spores (about $10^9$ ml$^{-1}$) were incubated at 37°C for 2 h in ethanol containing $15 \times$ the MIC of gramicidin S, afterwards washed three times with 10 mM phosphate buffer (pH 7.2), and used as gramicidin S-treated dormant spores. Subsequently, they were incubated at 37°C for 2 h in 100 mM CaCl$_2$ in deionized water, washed three times with 10 mM phosphate buffer (pH 7.2), and used as the antibiotic and CaCl$_2$-treated dormant spores. Dormant spores treated with ethanol alone were also prepared.

2.2. Germination and growth conditions, and respiration measurement

Growth experiments were carried out in HIB as in [6], and germination was defined for the sake of convenience as the decrease in optical density at 650 nm (OD$_{650}$). Germination experiments were performed by suspending dormant spores (about $10^9$ ml$^{-1}$) in 50 mM phosphate buffer (pH 7.2) containing 1 mM L-alanine and incubating them at 37°C for 300 min. Germination was measured by a loss in turbidity (OD$_{650}$) for each spore suspension and the fractional OD$_{650}$ reduction calculated as OD decreased at each time divided by the initial OD. Simultaneously, respiration measurements were carried out as described previously [9] and the fractional O$_2$ consumption was expressed as percentage of the initial O$_2$ volume.

2.3. Preparation of particulate fraction and enzyme assay

The preparation of spore particulate fraction containing inner membrane and measurement of particulate NADH oxidase activity were carried out as previously described [10].

2.4. Electron microscopy for observing structure

Ultrastructure of gramicidin S-treated dormant and germinated spores was observed as described previously [11].

2.5. Antibody production and immunoelectron microscopy

The preparation of rabbit antiserum against gramicidin S and immunocytochemical staining were carried out as in [7], with the production of antibody confirmed by Ouchterlony immunodiffusion analysis (data not shown).

3. Results

3.1. Germination, outgrowth and vegetative growth of gramicidin S-treated dormant spores

Gramicidin S-treated dormant spores germinated in HIB but did not undergo outgrowth and vegetative growth (Fig. 1), the fact of which was also ascertained by a phase microscopy method (data not shown); subsequent treatment with CaCl$_2$ did not result in recovery of outgrowth and vegetative growth. The outgrowth and vegetative growth of ethanol-treated dormant spores were similar to those of untreated dormant spores (Fig. 1).

![Germination, outgrowth, and vegetative growth of untreated (), ethanol-treated (), gramicidin S-treated (■), and gramicidin S and CaCl$_2$-treated (□) dormant spores.](image)
3.2. Germination by L-alanine and respiration during germination of gramicidin S-treated dormant spores

The initial velocity of germination of gramicidin S-treated dormant spores was lower than that of untreated dormant spores and the extent of the germination showed only about 67% of the control even after 300 min (Fig. 2A); the germination curve of dormant spores treated with both gramicidin S and CaCl₂ was almost identical to that of gramicidin S-treated dormant spores. The germination of dormant spores of *B. subtilis* was affected by ethanol treatment and showed the lowest initial velocity, but the extent of germination after 300 min coincided with that of untreated dormant spores.

The oxygen consumption during germination by L-alanine of gramicidin S-treated dormant spores reached about 53% after 120 min, but a longer incubation period did not further increase O₂ consumption (Fig. 2B). A similar result was also obtained in gramicidin S and CaCl₂-treated dormant spores. Ethanol-treated dormant spores showed almost the same O₂ consumption curve as that of gramicidin S-treated dormant spores up to 120 min after incubation, but subsequently continued respiring and consumed about 97% of the O₂ after 300 min (Fig. 2B). Generally speaking, the patterns of germination and respiration were very similar for the various samples. The specific activities of NADH oxidase in the particulate fractions of untreated, gramicidin S-treated, and ethanol-treated dormant spores were 179.3 ± 4.6, 12.9 ± 2.3 and 16.8 ± 2.0 (mean ± S.D.), respectively.

Germination by L-alanine and the respiratory enzyme system of dormant spores of *B. subtilis* appears to be significantly affected by ethanol and to an additional extent by gramicidin S; however, the ethanol damage could be recovered during germination.

3.3. Morphological changes of gramicidin S-treated germinated spores

Morphological changes of dormant spores treated with gramicidin S were obscure (data not shown). On the other hand, a thin section of the germinated spores, which were obtained by incubation of gramicidin S-treated dormant spores for 2 h in L-alanine solution, showed an irregular core in form, a vacuole within the cytoplasm and detachment of a thin cell wall from the cytoplasmic membrane (Fig. 3B); ethanol treated germinated spores had a spore protoplasm with a clear roundish germ cell wall and granules like untreated ones (Fig. 3C).

3.4. Penetration site of gramicidin S within a dormant spore

Numerous gold particles were seen in the core regions of gramicidin S-treated, and gramicidin S and CaCl₂-treated dormant spores, whereas only a
Fig. 3. Electron micrographs of thin sections of untreated (A), gramicidin S-treated (B), and ethanol-treated (C) germinated spores. SC, spore coat; CX, cortex; CW, cell wall; CM, cytoplasmic membrane; M, mesosome; N, nucleoid. Bars represent 0.2 μm.

Few of them were observed in the other areas (Fig. 4C and D). Few gold particles were seen in untreated (Fig. 4B) and ethanol-treated dormant spores (data not shown). The rough distribution of gold--IgG particles was examined in 30 thin sections. The numbers of gold particles per 0.2 mm² in the core, cortex and coat regions of gramicidin S-treated dormant spores were 33.3 ± 16.1, 1.1 ± 1.5 and 2.0 ± 1.7 (mean ± S.D.), respectively, and those in the core, cortex and coat regions of gramicidin S and CaCl₂-treated dormant spores were 28.0 ± 11.6, 1.3 ± 1.8 and 1.1 ± 1.3, respectively; with untreated dormant spores the numbers of gold particles per 0.2 mm² in the core, cortex and coat regions were 4.3 ± 5.4, 1.4 ± 2.2 and 0.7 ± 1.1, respectively. From this it was concluded that gramicidin S penetrated into the core region of B. subtilis dormant spores and hence it was not released by the addition of CaCl₂.

4. Discussion

Gramicidin S is a cyclic polypeptide antibiotic with a molecular mass of 1002 which is produced by B. brevis. This antibiotic has an effect on the outgrowth of B. brevis spores, resulting in inhibition of their growth [13]. The outgrowth and vegetative growth of dormant spores of B. subtilis PCI219 treated with slightly dissolved gramicidin S in a mixture of a large quantity of phosphate buffer and a small quantity of ethanol were inhibited as reported previously [6]. In this paper the effect of gramicidin S completely dissolved in almost 100% ethanol on the dormant spores of the same strain was examined. Gramicidin S-treated dormant spores showed only about 67% OD₆₅₀ reduction value and about 53% O₂ consumption (Fig. 2), and did not undergo outgrowth and vegetative growth in HIB (Fig. 1). The specific activity of NADH oxidase in their particulate fraction was very low compared with untreated dormant spores and a core of their germinated spores was irregular in form (Fig. 3B). The inhibitory effect of gramicidin S could not be recovered by treatment with CaCl₂. Penetration of the antibiotic into the spore core was demonstrated by an immunoelectron microscopy method (Fig. 4C). Judging from these results gramicidin S, unlike polymyxin B, colistin and aminoglycoside antibiotics, seems to cause damage to the outer membrane--spore coat complex and the inner membrane and to be able to penetrate into the core region of B. subtilis dormant spores.

Ethanol also affected more or less the germination and the respiration of dormant spores (Fig. 2) and
lowered the activity of their particulate NADH oxidase, but its effect was reversible during germination. It has been reported that germination by L-alanine of the dormant spores of *B. subtilis* PC1219 strain is inhibited markedly in proportion to the concentration of ethanol between 0.1 and 0.5 M, and the inhibition is reversible by washing [14]. In this experiment, however, when the dormant spores treated with ethanol close to 100% were washed extensively, its partial inhibitory effect did not diminish (data not shown). These results suggest that ethanol does some damage to the L-alanine-germination apparatus and lowers the initial velocity of germination, but is broadly irrelevant to life or death of dormant spores.

**Acknowledgements**

We are grateful to Dr. Roy H. Doi (Section of Molecular and Cellular Biology, University of California, Davis, CA) for his review and valuable criticism of the manuscript.

**References**


---

Fig. 4. Typical electron micrographs of thin sections of dormant spores (A) fixed with glutaraldehyde and OsO$_4$ [11] and stained with uranyl acetate and Reynolds lead citrate [12], and untreated (B), gramicidin S-treated (C), and gramicidin S and CaCl$_2$-treated (D) dormant spores reacted with anti-gramicidin S IgG and then labelled with colloidal gold coated with anti-rabbit IgG. SC, spore coat; CX, cortex; CR, core. Bars represent 0.2 μm.


