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Interactions between gramicidin S and its producer, *Bacillus brevis*

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Gramicidin S (GS) inhibition of germination outgrowth of *Bacillus brevis* spores was reversed completely by a short pretreatment with sodium dodecyl sulfate, moderately by ethanol or by incubation at pH 10 but not by incubation at pH 4. Of five metal ions tested (Na^+, Mg^{2+}, Fe^{2+}, Cu^{2+}, Ca^{2+}), only Ca^{2+} reversed GS inhibition. When Ca^{2+} (but not the other four metal ions) was added to the growth medium, there was a considerable portion of the biosynthesized GS found in the extracellular fluid. These findings are interpreted in terms of the binding of GS to the external layers of the *B. brevis* spore.

Keywords: gramicidin S; *Bacillus brevis*; spore germination; calcium; germination outgrowth

Introduction

Gramicidin S (GS) is a cyclic decapeptide antibiotic produced by *Bacillus brevis* during late logarithmic and early stationary phases of growth. It is amphipathic and exists as a rigid β-pleated sheet with two hydrophilic L-ornithine residues on one side of the molecular plane; on the other side are two L-valine and two L-leucine residues giving hydrophobic properties. The rigid structure is thought to be maintained by two type-II' β-turns composed of D-phenylalanine-proline residues. GS acts predominantly on Gram-positive bacteria by binding to the liquid layer of the cytoplasmic membrane, disrupting membrane integrity and allowing efflux of K^+ from the cell. GS also inhibits the growth of Gram-negative bacteria, but higher concentrations are required.

GS does not inhibit vegetative growth of the producer, *B. brevis*, but acts as an inhibitor of the outgrowth stage of spore germination [8,15,17]. The antibiotic also imparts hydrophobicity to *B. brevis* spores [19]. GS is thought to inhibit spore germination in nature until environmental conditions become favorable for vegetative growth. However, there is only little information on the interaction between spores and GS and on the effect of exogenous GS on sporulation and GS formation. These matters are the subject of this paper.

Materials and methods

Strains

Bacillus brevis strain Nagano was used for this work. The bioassay of GS was done with *Bacillus subtilis* ATCC 6051 according to Bentzen *et al* [3].

Media

NBS medium (nutrient broth, salts) and LBA medium (peptone, yeast extract, L-arginine, L-phenylalanine, L-methionine, salts; formerly called LBS + 3 AA) were prepared according to Azuma *et al* [2]. NBS was used to prepare 'GS-poor' spores and LBA for 'GS-rich' spores [2].

Germination

Germination experiments were carried out in NBS medium. Spores were activated by incubating them in a water bath at 80°C for 15 min. The activated spores (0.025 ml) were inoculated into 5 ml NBS medium in test tubes containing various concentrations of GS and the course of germination occurring on a rotary shaker (250 rpm) at 37°C was followed spectrophotometrically at 660 nm.

Pretreatment of spores

GS-rich spore suspensions (30 μl of suspension containing approximately 10^9 CFU ml^{-1}) were mixed with 600 μl of various additives and incubated for 30 or 60 min (as described in the text) at 37°C. The suspensions were centrifuged and washed three times with sterile distilled water to remove unbound additive before addition of sterile distilled water to 30 μl. These spores were then used for germination studies as described above.

Growth, GS production and sporulation

A seed culture was developed by inoculating activated spores (as described under Germination) and grown in 5 ml NBS in a test tube which was shaken for 24 h on the rotary shaker at 250 rpm and 37°C. The seed was inoculated at 0.5% (v/v) into tubes of LBA medium with or without various amounts of GS. Growth was carried out at the same shaker speed and temperature and measured by absorbance at 660 μm using a Turner spectrophotometer (Model 330, Sequoia-Turner Co, Mountain View, CA, USA). Production of GS was measured by bioassay of extracted whole broth. Sporulation was measured by viable counts with and without a pasteurization treatment.

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Results

Quantitative effect of GS on germination

In order to determine the efficacy of GS as an inhibitor of germination outgrowth, GS-poor spores were activated, incubated with various concentrations of GS, and examined for germination by optical absorbance; GS-rich spores in the absence of exogenous GS served as positive control. As expected [17], GS delayed germination outgrowth (Figure 1a). Although 1 μg ml^{-1} had no effect, 10 μg exogenous GS per ml delayed germination of GS-poor spores to a degree similar to that of GS-rich spores in the absence of exogenous GS. When outgrowth time (the time at which OD began to increase) was plotted against exogenous GS (Figure 1b), it could be seen that saturation with exogenous GS occurred at 20 μg ml^{-1}. Since the spore concentration used was 7.2×10^7 spores ml^{-1} and the molecular weight of GS is 1141, the number of bound GS molecules needed to saturate each spore was calculated to be 1.5×10^8.

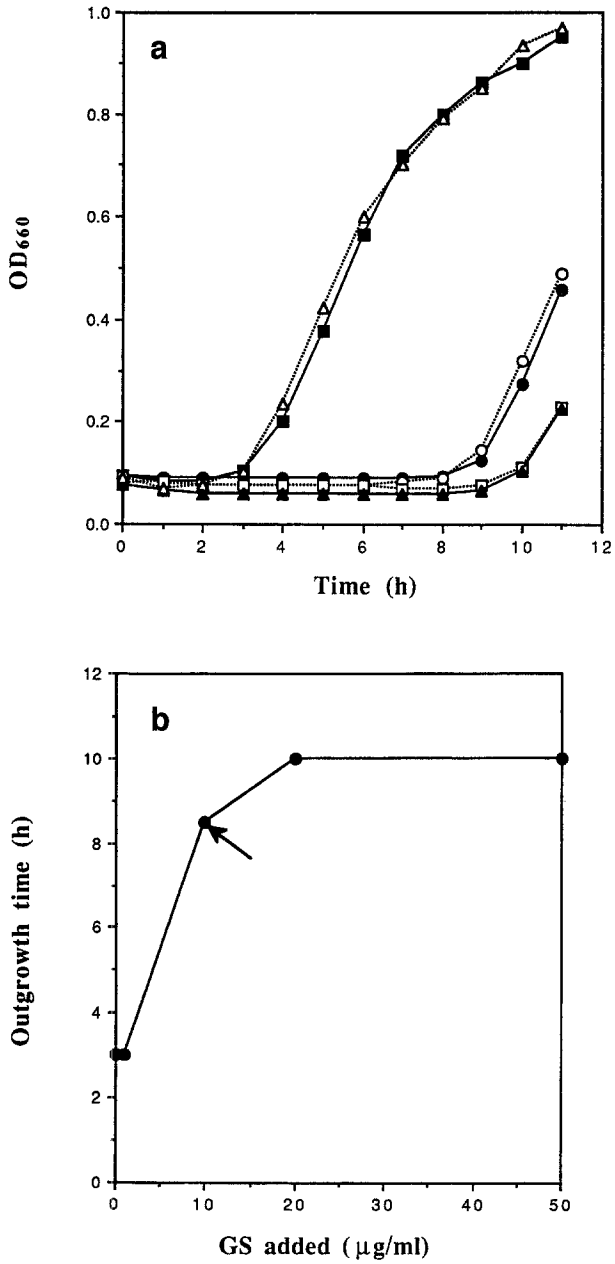

Figure 1 Effect of exogenous GS concentration (μg ml^{-1}) on germination (a) and outgrowth time (b) of GS-poor spores. (a) ■, 0; △, 1; ○, 10; □, 20; ▲, 50; ●, GS-rich spores with no exogenous GS. (b) The time taken for OD to increase is plotted *vs* GS added. Arrow, GS-rich spores with no exogenous GS.

Reversal of the GS effect on germination

In order to gain information on the type of interaction(s) occurring between spores and endogenous GS, various pretreatments were applied to GS-rich spores in order to remove GS and possibly reverse germination delay. From Figure 1b, it can be seen that such spores are approximately 50% saturated with GS and take about 8–9 h to start outgrowth. After pretreating and washing the spores, the germination course was followed; untreated GS-poor spores served as a negative control and untreated GS-rich spores as a positive control. Results depicted in Figure 2a show that pretreatment with 10% sodium dodecyl sulfate (SDS) for 30 min completely reversed germination delay and 1% SDS for 1 h achieved nearly the same effect. Ethanol pretreatment (Figure 2b) was less effective. Ten percent ethanol for 1 h showed a mild reversal effect as did incubation at pH 10 for 1 h (Figure 2c); incubation at pH 4 gave no reversal at all.

In the next experiment, a 1-h pretreatment of the GS-rich spores with the following salts was studied; 1% NaCl, 1% $MgSO_4 \cdot 7H_2O$, 1% $FeSO_4 \cdot 7H_2O$, 1% $CaCl_2 \cdot 2H_2O$ and 0.1% $CuSO_4 \cdot 5H_2O$; of these, only $CaCl_2$ was effective. A concentration of 1% calcium chloride showed a marked, although not complete, reversal of germination delay (Figure 3). A higher concentration (2%) was no more effective.

Effect of exogenous GS on vegetative cells

Although it is thought that GS does not affect growth of *B. brevis* vegetative cells [8], GS is known to bind to vegetative cells [11]. We were interested in determining its effect on sporulation and GS production. Various concentrations of GS were added to LBA medium which was inoculated with a seed culture of *B. brevis* Nagano. Figure 4a shows the effect of added GS on total GS content after growth, and the net concentration after subtraction of the added amount of GS. It can be seen that GS production was markedly inhibited by as little as 50 μg ml^{-1} of exogenous GS. Growth was unaffected by this level of GS addition; only a slight inhibition in final OD was observed with GS levels of 100 μg ml^{-1} and higher. On the other hand, sporulation was markedly inhibited at concentrations of higher than 50 μg/ml^{-1} (Figure 4b).

Effect of metal ions on GS production

Since $CaCl_2$ had such a marked reversal effect of GS inhibition of germination, we were interested in whether it had any effect on excretion of normally cell-bound GS during fermentation. We examined the effect of $CaCl_2$ and the other salts (NaCl, $MgSO_4$, $FeSO_4$, $CuSO_4$) on GS production, assaying the culture supernatant instead of the usual method of extracting whole broth. The expected

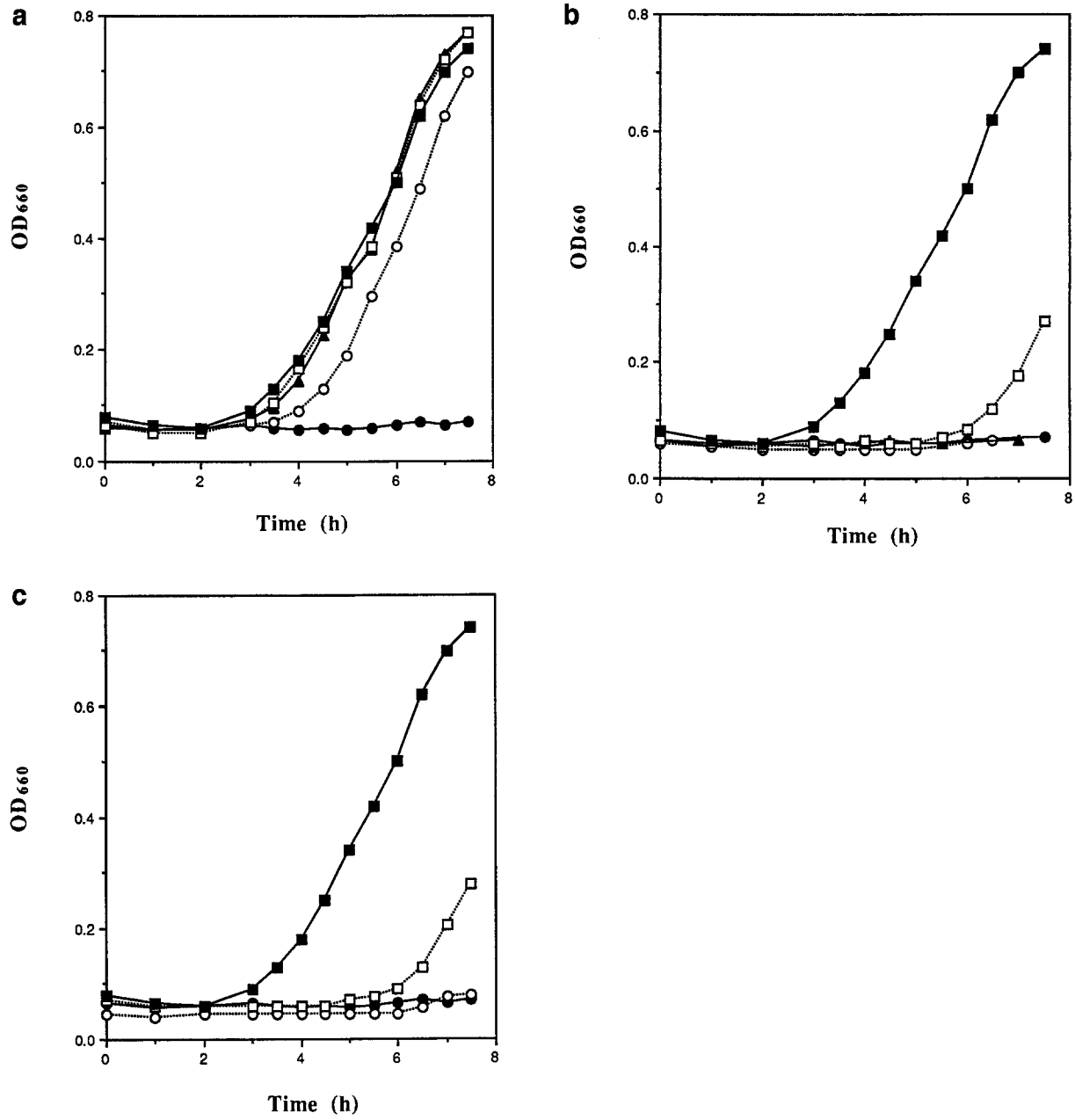

Figure 2 Effects of pretreatments on germination. (a) ●, GS-rich spores, no pretreatment; ○, GS-rich spores, 1% SDS, 0.5 h; ▲, GS-rich spores, 10% SDS, 0.5 h; □, GS-rich spores, 10% SDS, 1 h; ■, GS-poor spores, no pretreatment. (b) ●, GS-rich spores, no pretreatment; ○, GS-rich spores, 5% ethanol, 0.5 h; ▲, GS-rich spores, 10% ethanol, 0.5 h; □, GS-rich spores, 10% ethanol, 1 h; ■, GS-poor spores, no pretreatment. (c) ●, GS-rich spores, no pretreatment; ○, GS-rich spores, pH 4, 1 H; □, GS-rich spores, pH 10, 1 h; ■, GS-poor spores, no pretreatment.

absence of measurable GS in the culture supernatant was seen in the current experiment except in the case of $CaCl_2$ addition. About 80 μg ml^{-1} was found in the extracellular fluid when 1% $CaCl_2 \cdot 2H_2O$ was present; 0.1% $CaCl_2 \cdot 2H_2O$ was ineffective. A further experiment (Figure 5) showed that GS in the supernatant medium increased and cellular GS decreased as the concentration of calcium chloride was increased.

Discussion

It is clear that both endogenous GS and exogenous GS bind to *B. brevis* cells and spores. We have found that approximately 1.5×10^8 molecules of GS are required to saturate one spore. However, the site of GS binding has been unclear. Egorov *et al* [7,8] reported that spores contain 25–50% of the amount of GS synthesized by *B. brevis* cells and that none appears in the culture filtrate. Lobareva *et al* [11] found that 92% of endogenous GS was associated with the biomass and concluded from experiments using washed cells and spores that GS is excreted and attaches to the cell or spore surface by a labile bond. Not only does endogenous GS bind to cells and spores, but when GS was added to a non-producing *B. brevis* mutant, it was completely bound [11].

GS is membranotropic, binding to lipoprotein and phos-

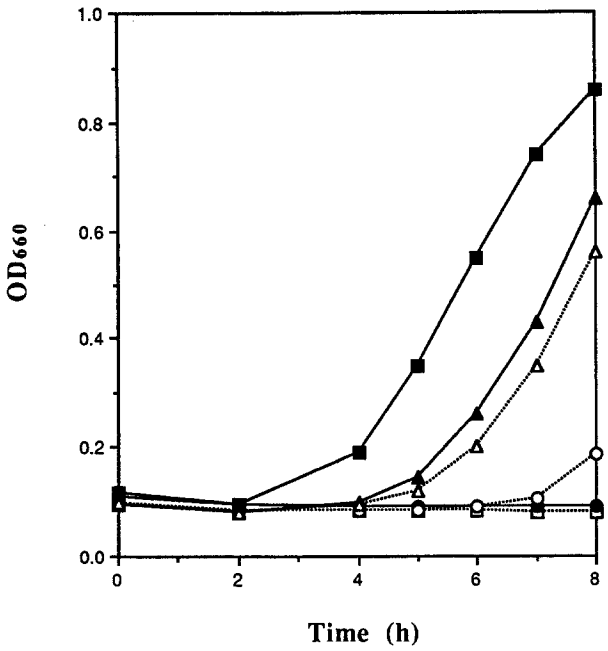

Figure 3 Effect of concentration of $CaCl_2$ during 1 h pretreatment of GS-rich spores on germination. □, 0%; ●, 0.1%; ○, 0.5%; ▲, 1%; △, 2%; ■, GS-poor spores, 0%.

pholipid membranes of many types of cells, eg bacteria, erythrocytes, mitochondria and artificial liposomes. The hydrophobic segments of GS become trapped in the interior lipid phase of cellular membranes due to hydrophobic interactions, while the positively charged hydrophilic ornithine groups on the other side of the GS molecule interact electrostatically with the negatively-charged phosphate moiety of the membrane's polar head groups [9,10]. In many cases, phospholipid is released from the membrane, increasing permeability and killing the target cells.

The mechanism by which *B. brevis* protects itself from the killing effects of endogenous GS is not known, but perhaps the delay in GS production until exponential growth ceases is the main reason. Furthermore, whatever is produced appears to be excreted and then bound to the exterior of the cells and/or spores [11]. It is not known to which part of the spore GS binds but the membranotropic character of GS suggests that lipids are involved. It is known that lipids can account for up to 13% of the dry weight of *Bacillus* spores [14], including 4–7% free lipids, 4–6% bound lipids, 2–3 mg non-esterified fatty acids and 13–17 mg phosphatides per 100 mg total lipids.

The outer layers of the bacterial endospore include the exosporium, the spore coat and the cortex. The exosporium is the outermost layer; it is chemically complex but nothing is known about its structure in *B. brevis*. However, in *Bacillus cereus*, the exosporium contains mainly phospholipoprotein (composed of 52% protein, 13% neutral lipid and 6% phospholipid [cardiolipin]) plus 20% polysaccharide and 4% inorganic ions [13,21]. Of the fatty acids, normal C_{16} and C_{18} predominate. Of the inorganic elements, calcium and phosphorus are the major constituents. The exosporium also contains enzymes apparently important in germination, ie alanine racemase and adenosine deaminase [4]. Exosporia are rather loose structures and may contain holes

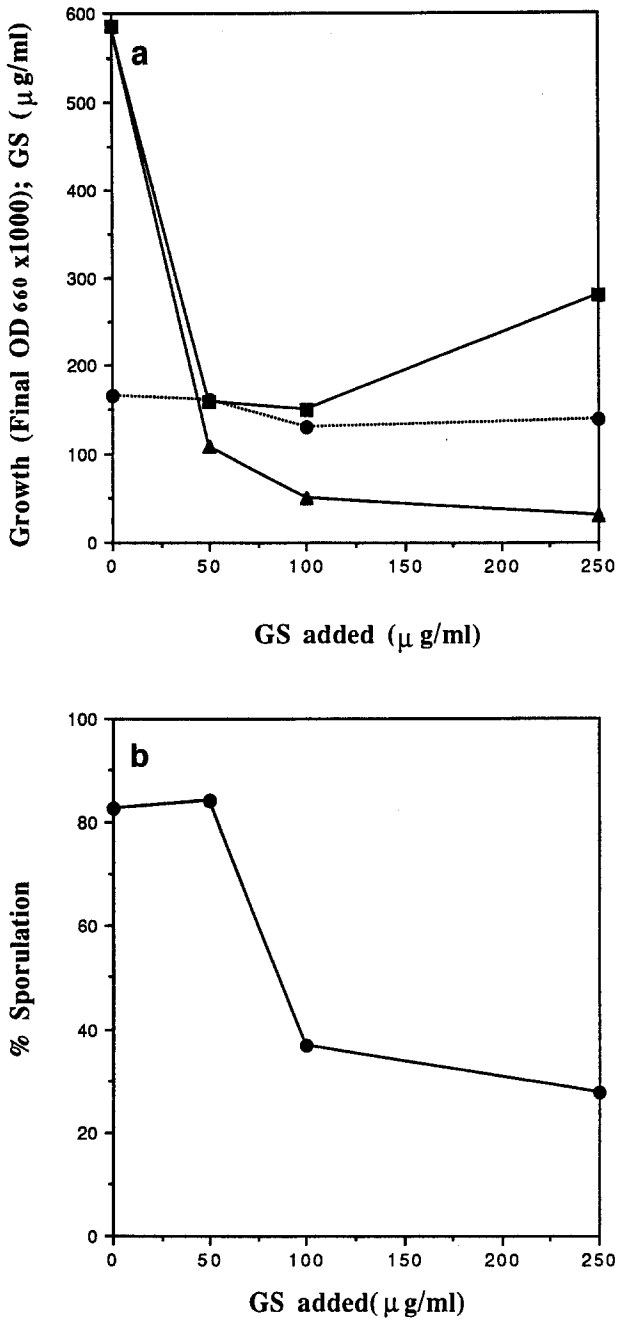

Figure 4 Effect of exogenous GS on (a) growth and GS production and (b) sporulation in medium LBA. (a) ●, growth (final $OD_{660} \times 1000$); ■, final GS concentration; ▲, net GS production. (b) % sporulation based on % of heat-resistant CFU compared to total CFU.

allowing GS to penetrate to the spore coat, the layer beneath the exosporium.

The spore coats of *Bacillus* generally contain about 80% of the spore protein and 30–60% of the spore dry weight [14,21]. Germination enzymes are present in the spore coats [1]. In *Bacillus megaterium*, the spore coat is the outermost layer [16] and contains exposed reactive groups such as sulfhydryl, hydroxyl, carboxylic, phosphoric and amino groups; the net surface charge is negative due to the high level of carboxylic and phosphate groups. The content of lipid (1–3%), phosphate (up to 3%) and the high protein content suggest the existence of phospholipoprotein [14].

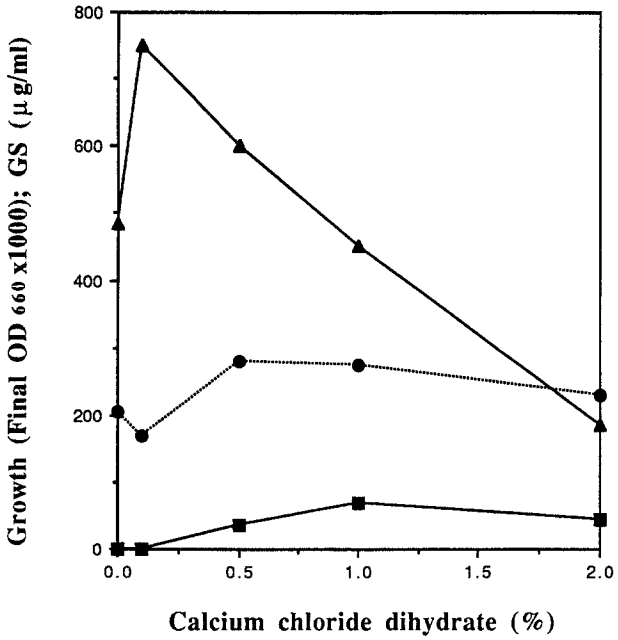

Figure 5 Effect of concentration of $CaCl_2 \cdot 2H_2O$ on growth and endogenous GS location. ●, growth (final $OD_{660} \times 1000$); ■, GS in supernatant medium; ▲, GS associated with cells and spores.

Underneath the spore coats of bacilli is the cortex, composed mainly of the electronegative peptidoglycan, dipicolinic acid and Ca^{2+}. Below the cortex are the cell wall, cell membrane and nuclear region. It appears likely that the major site of GS after synthesis and excretion by *B. brevis* would be the exosporium and/or the spore coat.

The complete reversal of GS inhibition of germination by SDS found in this work suggests that binding of GS to the spore is mainly due to hydrophobic and electrostatic interactions since SDS disrupts these with much lesser effect on hydrogen bonds and van der Waal's forces. SDS is an alkyl sulfate detergent which avidly binds to membranes. The action of ethanol in partially reversing the inhibition is apparently due to its ability to weaken hydrophobic interactions. Ethanol should have only a minor effect on electrostatic bonds and thus its lesser reversal activity compared to SDS suggests that electrostatic interactions are also involved in GS binding to the spore. This concept is supported by the data showing that pH 10 treatment also partially reverses GS inhibition. It would be expected that the ornithine side-chain amino groups of GS would become at least partly dissociated at pH 10, lose their positive charge, and weaken the electrostatic attraction to phosphate groups of spore surface phospholipids. It would be expected that pH 4 treatment would have no effect since the side-chain amino groups of the ornithine residues would be fully protonated at this pH.

Our finding that 1% $CaCl_2$ almost completely reversed GS inhibition of *B. brevis* germination can be explained by the ability of Ca^{2+} to form an electrostatic bond with the negatively charged groups of phospholipids [9]. Ca^{2+} thus might be expected to outcompete the ornithine residues of GS for the phospholipid polar groups; indeed, Ca^{2+} probably replaces GS in the electrostatic interactions. Ca^{2+} generally binds to cellular membranes, neutralizing their negative charge and contributing to maintenance of permeability and membrane stability, which is the opposite of GS action. The metal ion is essential to biological systems and usually is found at the exterior of cells. Cell surfaces are normally anionic and hydrophilic at physiological pH values due to the ionization of carboxylic and phosphate groups. These negatively charged surfaces are in equilibrium with environmental metal ions and bind them strongly. Bacterial spores are also highly interactive with certain metal ions during their formation and germination [5]. Spores have a higher affinity for divalent than monovalent cations [12], and divalent metals interact with the exosporium, spore coat and cortex during formation of bacterial endospores. However, the site of Ca^{2+} action when the metal is added to fully developed spores is probably restricted to the exosporium and the spore coat since the coat layers are relatively impermeable to multivalent ions. Our findings that Ca^{2+} addition to the growth medium leads to partial excretion of subsequently biosynthesized GS into the extracellular fluid lends support to the hypothesis that Ca^{2+} replaces GS in its interaction with exosporium/spore coat phospholipids.

All present and earlier findings discussed above point to: (i) a hydrophobic interaction between the valine and leucine moieties on one side of GS and the lipid phase of the exosporium/spore coat; and (ii) an electrostatic interaction between the ornithine residues on the other side of GS and the phosphate groups of the exosporium/spore coat phospholipids as being important in the action of GS as an inhibitor of germination outgrowth in *B. brevis*.

The finding that exogenous GS inhibits its own biosynthesis at concentrations as low as 50 $\mu g\ ml^{-1}$ in the absence of a marked effect on growth shows that a feedback effect is operative as a control mechanism in GS production. In this regard, the inhibition and destabilization of GS synthetase by GS [20] is probably the mechanism underlying this phenomenon. Our observation that exogenous GS interferes with sporulation is consistent with the earlier conclusion [6,18] that GS is not needed for sporulation in *B. brevis*.

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