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The influence of gramicidin S on hydrophobicity of germinating *Bacillus brevis* spores

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Abstract. Gramicidin S is known to prolong the outgrowth stage of spore germination in the producing culture. *Bacillus brevis* strain Nagano and its gramicidin S-negative mutant, BI-7, were compared with respect to cell-surface hydrophobicity and germination of their spores. Parental spores were hydrophobic as determined by adhesion to hexadecane, whereas mutant spores showed no affinity to hexadecane. Addition of gramicidin S to mutant spores resulted in a high cell surface hydrophobicity and a delay in germination outgrowth. The hydrophobicity of parental spores was retained throughout most of the germination period. Hydrophobicity was lost as outgrowing spores entered into the stage of vegetative growth. The data indicate that gramicidin S is responsible for the hydrophobicity of *B. brevis* spores. It is suggested that in making spores hydrophobic, the antibiotic plays a role in concentrating the spores at interfaces where there is a higher probability of finding nutrients for germination and growth.

Key words: Gramicidin S – Antibiotic function – Hydrophobicity – Germination – Spores – *Bacillus brevis*

The synthesis of one or more peptide antibiotics during the early stages of sporulation is common to most, if not all, members of the genus *Bacillus* (Katz and Demain 1977). *Bacillus brevis* strain Nagano produces the antibiotic gramicidin S [(GS); cyclo-(Val^1-Orn^2-Leu^3-D-Phe^4-Pro^5)$_2$] during late logarithmic growth and early stationary phase. Essentially all of the synthesized antibiotic is bound to the spores when they are released from the lysed sporangium (Matteo et al. 1975; Piret and Demain 1983).

Does GS play a role in the developmental cycle of *B. brevis*? One way to answer this question is to examine mutants blocked in GS synthesis. *B. brevis* BI-7 (Iwaki et al. 1972), a GS-negative mutant of the Nagano strain, lacks the light GS synthetase activity required for D-phenylalanine activation (Piret and Demain 1983; Lipmann 1973). Comparative studies of the parent and GS-negative strains showed no differences in the extent of sporulation or resistance of the mature spores to UV irradiation, organic solvents and heat (Piret and Demain 1983; Demain and Piret 1979; Lazaridis et al. 1980). On the other hand, the ability to produce GS had a marked effect on the outgrowth (but not the initiation) stage of spore germination. The presence of endogenously synthesized (or exogenously added) GS markedly lengthened the duration of outgrowth prior to entry into the stage of vegetative growth (Egorov et al. 1975; Nandi and Seddon 1978; Marahiel et al. 1979; Demain 1980; Piret and Demain 1982). Outgrowth is the stage of germination in which RNA, protein and DNA synthesis occurs, but the mechanism by which GS inhibits the onset of macromolecular synthesis for several hours after germination initiation is not known.

Although one of the fundamental changes that takes place during spore germination is the conversion of a hydrophobic spore into a hydrophilic vegetative cell (Gaudin et al. 1960; Sacks and Alderton 1961), the timing of this event has not been reported previously. Using a simple method for measuring cell-surface hydrophobicity based on adhesion of cells to hydrocarbons (Rosenberg et al. 1980; Rosenberg and Rosenberg 1981), data are presented in this paper indicating that the loss in cell-surface hydrophobicity of *B. brevis* spores during germination is a late event. Furthermore, we show that spores of the GS-negative mutant BI-7 are hydrophilic but convertible to the hydrophobic state by addition of GS.

Materials and methods

Strains

Cultures used in this study were *B. brevis* Nagano and its GS-negative mutant, strain BI-7 (Iwaki et al. 1972).

Media

All components are expressed in grams per liter. NBS medium contains: nutrient broth (Difco Laboratories, Detroit, MI, USA) 8; in salts solution I and II. LBS medium is composed of tryptone (Difco) 10; yeast extract (Difco) 5; in salts solution I and II, adjusted to pH 7.0. Tryptic soy agar (Gibco Laboratories, Madison, WI, USA) was used for determination of viable cell counts. Salts solution I was $CaCl_2 \cdot 2\ H_2O$, 0.1; $MnCl_2 \cdot 4\ H_2O$, 0.01 and $MgCl_2 \cdot 6\ H_2O$, 0.2. Salts solution II contained $FeCl_3$, 0.00027 in 0.0001 N HCl.

Spore production and storage

Fernbach flasks (2800 ml) containing 300 ml of NBS or LBS media were inoculated with 5 ml of overnight cultures of *B. brevis* Nagano and strain BI-7. The cultures were shaken at

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Abbreviation: GS, Gramicidin S

250 rpm at 37° C for 48 h in a New Brunswick model 26 gyrotary shaker. Spores were harvested by centrifugation at $5000 \times g$ for 30 min, washed four times in distilled water, suspended in distilled water to about 4×10^9 colony-forming units per ml, distributed (3 ml) into sterile vials, and stored frozen at −20° C. The colony-forming units per ml of all spore stocks were the same before and after heating at 80° C for 1 h.

Adhesion of spores to hexadecane

Cell-surface hydrophobicity of spores was determined by adhesion to hexadecane (Rosenberg et al. 1980; Rosenberg and Rosenberg 1981; Shoham et al. 1983). The frozen spore stocks were thawed and diluted into distilled water so that the absorbance at 400 nm of the spore suspension was 0.5 to 1.0 (Gilford Spectrophotometer, model 2600, 1-cm light path). Test tubes containing 2.0 ml of suspension and different volumes of hexadecane were vortexed vigorously for 2 min. After allowing the phases to separate for 15 min the absorbance of the lower aqueous phase was measured at 400 nm and compared with that of the unheated spore suspension.

Germination

Sterile NBS medium (20 ml) was added to 250-ml sidearm Erlenmeyer flasks. For experiments in the presence of GS, 0.2 ml of a GS solution in 50% ethanol was added to the experimental flasks and 0.2 ml of 50% ethanol was added to the control flasks. The flasks were inoculated with heat-activated spores (80° C, 15 min) and shaken at 250 rpm at 37° C.

Turbidity of the cultures was followed in a Klett-Summerson colorimeter using a red (no. 66) filter. Germination was also monitored by phase microscopy. Cell surface hydrophobicity of the germinating spores was determined by diluting samples 100-fold into distilled water and then extracting 2-ml portions with 0.2 ml hexadecane as described above. After phase separation, the number of cells remaining in the bottom aqueous phase was determined by spreading an appropriate dilution on TS agar. Diluted samples treated in the same manner served as controls. Plates were scored after 48 h incubation at 37° C.

Results

Adhesion of parental and BI-7 spores to hexadecane

When turbid suspensions of the parental *B. brevis* spores prepared in NBS medium were mixed with hexadecane in distilled water, the spores adhered avidly to the hydrocarbon droplets, giving rise to a turbid emulsified upper layer and a relatively clear aqueous bottom layer (Fig. 1 a). Microscopic examination of the upper layer showed refractile spores adhering to the hexadecane/water interface. The bottom layer showed some cell debris but very few spores.

In contrast, spores of the GS-negative mutant BI-7 showed no affinity to hexadecane even at relatively high hydrocarbon to water ratios (Fig. 1 a). The hexadecane droplets coalesced rapidly to form a clear upper layer containing no spores. The refractile BI-7 spores remained dispersed in the bottom aqueous phase. Over 95% of the

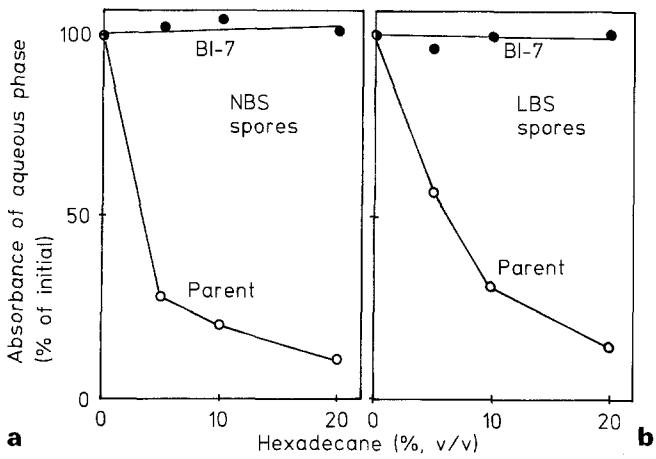

Fig. 1 a, b. Adhesion to hexadecane of *Bacillus brevis* spores prepared in NBS medium (**a**) and LBS medium (**b**). Spore suspensions (2 ml) in distilled water were mixed with varying volumes of hexadecane as described in the text. Results are expressed as percentage of the initial absorbance (A_{400}) of the aqueous phase as a function of volume percent hexadecane

hexadecane-extracted BI-7 spores were still heat-resistant (80° C, 1 h).

Examination of spores prepared in LBS medium (a medium supporting higher GS production than NBS medium) also indicated that the GS-negative mutant was significantly less hydrophobic than the parental strain (Fig. 1 b).

Exponentially growing vegetative cells of *B. brevis* Nagano and mutant BI-7 in NBS and LBS media showed no affinity for hexadecane (data not shown).

Effect of added GS on cell-surface hydrophobicity and germination of BI-7 spores

The non-adhesive spores of the GS negative mutant BI-7 spores became adhesive to hexadecane upon addition of the antibiotic (Fig. 2). With 1, 2 and 3 μg GS per ml, approximately 10, 40 and 80%, respectively, of the spores adhered to the hydrocarbon. The increased hydrophobicity of the spores was due to the binding of GS since incubation of spores with 5 μg GS per ml followed by centrifugation resulted in a spore preparation that adhered (over 90%) to hexadecane.

The concentration of GS required to delay the outgrowth stage of spore germination depends on the source of the spores and their population density (Piret and Demain 1982). With BI-7 spores prepared in NBS medium at a cell density of 3.9×10^7/ml ($A_{400} = 0.420$), outgrowth was delayed 0.5 h by 0.5 μg GS per ml and 8 h by 1 μg GS per ml (Fig. 3).

To ensure that the effect of added GS on cell-surface hydrophobicity and germination was not restricted to BI-7 spores prepared in NBS medium, the experiments were repeated with BI-7 spores prepared in LBS medium. Similar data were obtained.

Changes in cell-surface hydrophobicity of parental spores during germination

During the initial 30 min of incubation of heat-activated parental spores prepared in NBS medium, the cells lost their refractility (phase microscopy) and the turbidity of

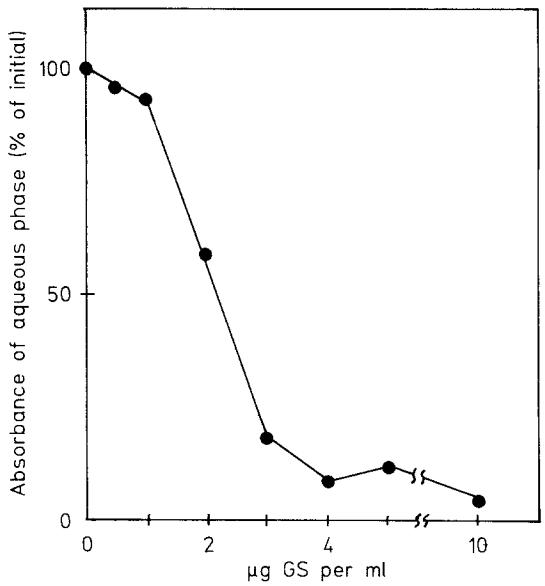

Fig. 2. Effect of added gramicidin S on cell-surface hydrophobicity of *Bacillus brevis* mutant BI-7 spores prepared in NBS medium. Spore suspensions in distilled water (2 ml, $A_{400} = 0.420$) containing varying concentrations of gramicidin S (the final concentrations of ethanol was 0.5%) were mixed with 0.2 ml hexadecane, and the absorbance of the aqueous phase determined after phase separation as described in the text

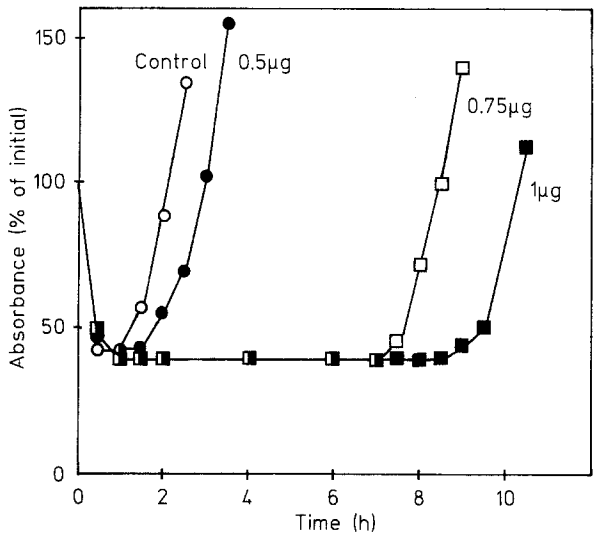

Fig. 3. Effect of added gramicidin S on germination outgrowth of *Bacillus brevis* mutant BI-7 spores prepared in NBS medium. Figures are μg per ml added gramicidin S

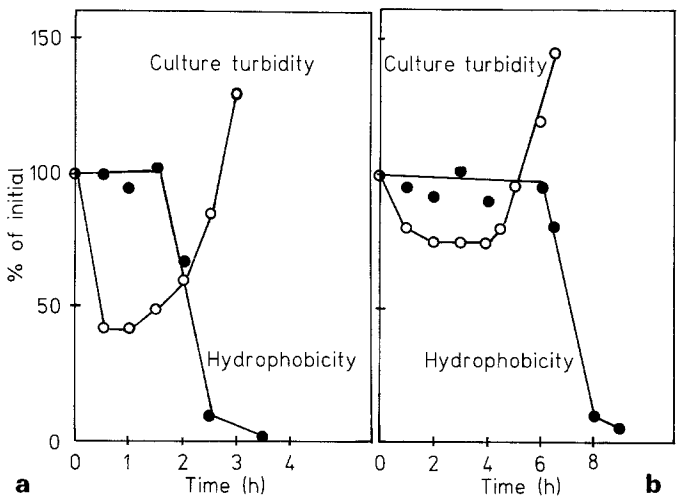

Fig. 4. a Changes in cell-surface hydrophobicity during germination of parental spores prepared in NBS medium. Spores were heat activated (80°C, 15 min), diluted into prewarmed NBS medium ($\sim 2 + 10^7$ spores per ml) and incubated at 37°C with shaking. At timed intervals, the optical absorbance of the cell suspension was measured and samples were removed for determination of viable cell number before and after extraction with 10% (vol/vol) hexadecane as described in the text. **b** Changes in cell-surface hydrophobicity during germination of parental spores prepared in LBS medium

the culture decreased by approximately 50% (Fig. 3). This degradative process, referred to as germination initiation, did not result in a measurable loss in cell-surface hydrophobicity (Fig. 4a). From 0.5–1.5 h (germination outgrowth stage), the turbidity of the culture remained relatively constant and the high cell-surface hydrophobicity was maintained. Decrease in hydrophobicity of the germinating cells occurred at approximately 2 h, when outgrowth was complete and vegetative growth began. Thus, loss of hydrophobicity is a relatively late event in *B. brevis* spore germination.

Loss of cell-surface hydrophobicity of parental spores prepared in LBS medium was also a late event (Fig. 4b). As expected (Piret and Demain 1982), the germination outgrowth period of *B. brevis* spores prepared in LBS medium was much longer than with NBS spores due to the higher level of GS present in LBS spores. Furthermore, the decrease in turbidity was less precipitous than for spores prepared in NBS medium because of a higher level of cell debris in LBS spore suspensions. Decrease in hydrophobicity of the germinating LBS spores occurred at approximately 6.5 h, when outgrowth was complete and vegetative growth began.

It is known that germination outgrowth can be delayed by addition of GS to parental spores prepared in NBS medium (Piret and Demain 1982). It was therefore of interest to determine if loss of cell-surface hydrophobicity would be similarly delayed. In the presence of 10 μg GS per ml, *B. brevis* parental spores completed their outgrowth period and lost their adhesiveness to hexadecane at approximately 7 h (Fig. 5) compared to 2 h in the absence of added antibiotic (Fig. 4a).

Discussion

Several lines of investigation indicate that GS is bound strongly to the outer surface of *B. brevis* spores (Piret and Demain 1982): (i) essentially all of the native or added antibiotic is associated with the spore pellet following centrifugation; (ii) the enzyme Nagarse can hydrolyze GS while the antibiotic is spore-bound; and (iii) the occurrence of the delay in germination outgrowth by GS is directly dependent on the size of the spore inoculum; if GS were present inside the cell, then one would not expect such a cell density-dependent phenomenon.

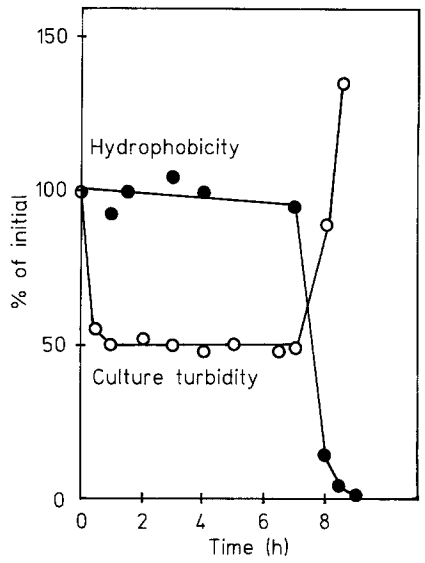

Fig. 5. Effect of added gramicidin S on germination outgrowth and loss of cell-surface hydrophobicity. The experiment was performed as described in Fig. 4 except that the NBS germination medium contained 10 μg gramicidin S per ml

Recently it has been shown that GS binds nucleotides (Krauss and Chan 1983) and nucleic acids (Krauss and Chan 1984) to form complexes which partition into organic solvents. This phase-transfer activity of GS is due to the rigidity of the peptide backbone which constrains the two positively charged ornithine side chains to one side of the ring, an average of 8 Å apart. The side chains of the remaining hydrophobic residues are oriented toward the opposite side of the ring. We suggest that a similar type of complex, formed between GS and the negatively charged surface of the spore, is responsible for the high cell surface hydrophobicity of GS-containing *B. brevis* spores.

The high cell-surface hydrophobicity of GS-containing *B. brevis* spores was retained for most of the germination period. Binding to hexadecane decreased only after the culture turbidity began to increase and cells initiated cell divisions. It is not known if the loss of cell-surface hydrophobicity is associated with hydrolysis of GS (Kurotsu et al. 1982) or the synthesis of a fresh vegetative cell envelope.

Cell-surface hydrophobicity is considered an important factor in the adhesion of microorganisms to solid surfaces (Marshall 1976) and water/air interfaces (Dahlback et al. 1981), and for the growth of certain bacteria on water-insoluble substrates (Rosenberg et al. 1980). The data reported in this study indicate that the antibiotic GS is responsible for the high cell-surface hydrophobicity of *B. brevis* spores. Thus, we suggest that at least one of the natural roles of the antibiotic GS is to assist the spore in adhering to interfaces where there is a higher probability of finding nutrients required for germination and growth. Another role is to lengthen the stage of germination outgrowth during which *B. brevis* Nagano, unlike other bacilli, is resistant to heat and other stress factors (Daher et al. 1985).

To our knowledge, the data presented in Fig. 2 represent the first case in which a specific polypeptide added to cells makes them hydrophobic. It will be interesting to examine the specificity of this interaction with regard to the antibiotic, the species and the stage of the growth cycle.

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References

Daher E, Demain AL (1985) Germination-initiated spores of *Bacillus brevis* Nagano retain their resistance properties. J Bacteriol 161:47–50

Dahlback B, Hermannson M, Kjelleberg S, Norkrans B (1981) The hydrophobicity of bacteria – an important factor in their initial adhesion at air-water interface. Arch Microbiol 128:267–270

Demain AL (1980) Do antibiotics function in nature? Search II:148–151

Demain AL, Piret JM (1979) Relationship between antibiotic biosynthesis and sporulation. In: Luckner M, Schreiber K (eds) Regulation of secondary product and plant hormone metabolism. Pergamon Press, New York, pp 183–188

Egorov NS, Vyipiyach AN, Zharikova GG, Maksimov VN (1975) Effect of various factors on spore germination in S and P-variants of *Bacillus brevis*. Mikrobiologiya 44:237–240

Gaudin AM, Mular AL, O'Connor RF (1960) Separation of microorganisms by flotation. Appl Microbiol 8:91–97

Iwaki M, Shimura K, Kanda M, Kaji E, Saito Y (1972). Some mutants of *Bacillus brevis* deficient in gramicidin S formation. Biochem Biophys Res Commun 48:113–118

Katz E, Demain AL (1977) The peptide antibiotics of *Bacillus*: chemistry, biogenesis and possible functions. Bacteriol Rev 41:449–474

Krauss EM, Chan IS (1983) Complexation and phase transfer of nucleotides by gramicidin S. Biochemistry 22:4280–4291

Krauss EM, Chan IS (1984) Complexation and phase transfer of nucleic acids by gramicidin S. Biochemistry 23:73–77

Kurotsu T, Marahiel MA, Muller KD, Kleinkauf H (1982) Characterization of an intracellular serine protease from sporulating cells of *Bacillus brevis*. J Bacteriol 151:1466–1472

Lazaridis I, Frangou-Lazaridis M, MacCuish FC, Nandi S, Seddon B (1980) Gramicidin S content and germination and outgrowth of *Bacillus brevis* spores. FEMS Microbiology Lett 7:229–232

Lipmann F (1973) Nonribosomal polypeptide synthesis on polyenzyme templates. Accounts Chem Res 6:361–367

Marahiel M, Danders W, Krause M, Kleinkauf H (1979) Biological role of gramicidin S in spore functions. Studies on gramicidin S-negative mutants of *Bacillus brevis* ATCC 9999. Eur J Biochem 99:49–55

Marshall KC (1976) Interfaces in microbial ecology. Harvard University Press, Cambridge, MA

Matteo CC, Glade M, Tanaka A, Piret JM, Demain AL (1975) Microbiological studies on the formation of gramicidin S synthetases. Biotechnol Bioeng 17:129–142

Nandi S, Seddon B (1978) Evidence of gramicidin S functioning as a bacterial hormone specifically regulating spore outgrowth in *Bacillus brevis* strain Nagano. Biochem Soc Trans 6:409–411

Piret JM, Demain AL (1982) Germination initiation and outgrowth of spores of *Bacillus brevis* strain Nagano and its gramicidin S-negative mutant. Arch Microbiol 133:38–43 (1982)

Piret JM, Demain AL (1983) Sporulation and spore properties of *Bacillus brevis* and its gramicidin S-negative mutant. J Gen Microbiol 129:1309–1319

Rosenberg M, Rosenberg E (1981) Role of adherence in growth of *Acinetobacter calcoaceticus* on hexadecane. J Bacteriol 148: 51–57

Rosenberg M, Rosenberg E, Gutnick D (1980) Bacterial adherence to hydrocarbons. In: Berkeley RCW, Lynch JM, Melling J, Rutter PR, Vincent B (eds) Microbial adhesion to surfaces. Society of Chemical Industry, London, pp 541–542

Sacks LE, Alderton G (1961) Behavior of bacterial spores in aqueous polymer two-phase systems. J Bacteriol 82:331–341

Shoham Y, Rosenberg M, Rosenberg E (1983) Bacterial degradation of emulsan. Appl Environ Microbiol 46:573–579

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