Biochemical Aspects of Germination and Outgrowth of Bacillus brevis Nagano and Control by Gramicidin S

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It has been demonstrated that gramicidin S inhibits the outgrowth of spores of the producer-organism Bacillus brevis Nagano (Nandi & Seddon, 1978). Once again we have used the antibiotic-negative mutants E-I and BI-9 (supplied by Professor Y. Saito) in an attempt to relate control by gramicidin S over outgrowth to respiration, RNA synthesis and protein synthesis of the spores.

Spores were prepared and germinated in nutrient broth at 37°C with aeration as described previously (Nandi & Seddon, 1978), except that for the present studies a concentration of $2 \times 10^8$ spores/ml was used. The gramicidin S-negative mutants were compared with wild-type spores with respect to biochemical parameters of outgrowth.

Outgrowth of the antibiotic-negative mutants after 80 min of incubation was accompanied by a simultaneous, rapid increase in respiration that continued to rise until at least 200 min (monitored in a Clark oxygen electrode). This was followed by the rapid development of RNA synthesis at 100 min, which reached a peak at 180–190 min. Protein synthesis developed rapidly at 180 min and continued to rise up to 250 min. RNA- and protein-synthesizing activities were monitored by pulse-labelling culture samples with $^{14}$C-labelled precursors (see legend to Table 1). The wild-type spores did not outgrow for at least 250 min and respiration, RNA synthesis and protein synthesis remained insignificant during this time. This was also true of mutant spores that germinated in the presence of 100 pg of gramicidin S/ml.

It was also discovered that if gramicidin S was removed from the wild-type spores, they would behave as mutant spores. This was effected by immersing an ethanol suspension of spores in a boiling-water bath for 2 min and then removing the spores by centrifugation. For a concentration of $10^{10}$ spores in 2 ml of ethanol, three such extractions were very effective at removing gramicidin S while leaving the viability of the spores relatively unimpaired. The washed, extracted spores would then outgrow after 80 min in nutrient broth, and respiration, RNA synthesis and protein synthesis would develop in a similar time-course to the mutant spores. Addition of appropriate concentrations of gramicidin S or wild-type extracts once again restored inhibition of outgrowth and associated activities.

Respiration and $[^{14}C]$uracil uptake are normally well established after 180 min and $[^{14}C]$lysine uptake is at 210 min. However, if gramicidin S is added, at physiological concentrations (10 μg/2 × 10^8 spores), at or any time before 80 min these activities fail to develop. As soon as outgrowth begins the culture becomes increasingly insensitive to gramicidin S. After 140 min there is active cell division and the early events of outgrowth, respiration and RNA synthesis are now relatively unaffected by addition of gramicidin S, even at high concentrations (Table 1).

In agreement with these observations, we find that gramicidin S does not inhibit $[^{14}C]$uracil uptake by vegetative cultures of B. brevis wild-type and E-I. Our studies were based on experiments of Sarkar & Paulus (1972) who found that tyrocidine inhibits RNA synthesis in B. brevis. Thus we have only evidence of a gramicidin S-effect on spores and no evidence for an effect on the vegetative cell.

Although respiration is the earliest outgrowth-associated phenomenon that we have observed, it appears to become significant only shortly before the onset of outgrowth in mutant spores germinated in nutrient broth. However, B. brevis Nagano spores suspended in 10 mM-potassium phosphate buffer, pH 7.4, are stimulated to germinate by 10 mM-L-alanine. Spore germination follows a similar course to that in nutrient broth, but in this simple system it is easier to follow the development of respiration in the very early stages. Respiration can be detected within 10 min of initiation with L-alanine and rises steadily to a concentration of 6–8 nmol of O_2/min per 10^8 spores after.
Table 1. Effect of gramicidin S on the development of respiration and [14C]uracil and [14C]-lysine uptake during spore outgrowth in B. brevis Nagano

Duplicate volumes (10 ml) of spore culture in nutrient broth were treated with gramicidin S, or an appropriate ethanol solution as control, after 80 min (onset of outgrowth) or 140 min (active cell division) of incubation. Gramicidin S concentrations were 10 µg/ml, if added after 80 min, or 50 µg/ml, if added after 140 min, to allow for increased cell numbers. Consumption of O₂ by the spores was measured in a Clark oxygen electrode. Radioactive-precursor uptake was determined by incubating duplicate samples (0.5 ml), for appropriate times with 0.05 µCi of [14C]uracil (sp. radioactivity 1 mCi/mmol) or 0.05 µCi of [14C]lysine (sp. radioactivity 33 mCi/mmol) for periods of 2 min with shaking at 37°C. The reaction was stopped by the addition of 0.5 ml of 10% (v/v) trichloroacetic acid on ice and samples were stored at 4°C until filtration on Whatman GF/C filters. The filters were washed with 5% trichloroacetic acid and ethanol and dried overnight at 60°C. The radioactivity on the filters was counted in a liquid-scintillation counter (Intertechnique SL30). Respiration and precursor-uptake values in the presence of gramicidin S are percentages of control values. The values shown are the averages of two experiments.

<table>
<thead>
<tr>
<th>Spore type</th>
<th>Time of gramicidin S addition (min)</th>
<th>Respiration after 180 min (% of control)</th>
<th>[14C]uracil uptake after 180 min (% of control)</th>
<th>[14C]lysine uptake after 210 min (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>140</td>
<td>80</td>
</tr>
<tr>
<td>E-I</td>
<td></td>
<td>0</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>BI-9</td>
<td></td>
<td>0</td>
<td>91</td>
<td>88</td>
</tr>
<tr>
<td>Wild-type extracted</td>
<td></td>
<td>0</td>
<td>86</td>
<td>—</td>
</tr>
</tbody>
</table>

240 min. [The electrode was calibrated by the method of Robinson & Cooper (1970).] At this stage, early signs of emergence of vegetative cells are visible in 1 or 2% of the spore population. Addition of 10 µg of gramicidin S/2 × 10⁸ spores at any time before 180 min inhibits respiration by 60% within the first minute and this rises to at least 90% thereafter. The O₂ consumption of wild-type spores corresponds only to this inhibited value.

Such respiration studies will undoubtedly help in the further characterization of gramicidin S inhibition of spore outgrowth.

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