A Bacitracin-negative Mutant of *Bacillus licheniformis* Which is Able to Sporulate

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The formation of peptide antibiotics is frequently associated with the sporulation process of the producer organisms (Bernlohr & Novelli, 1960; Winnick, Lis & Winnick, 1961; Paulus, 1967; Hurst, 1969). Generally, peptide antibiotics are produced after rapid growth and before sporulation (Bodanszky & Perlman, 1969; Weinberg, 1970).

The peptide antibiotic bacitracin is synthesized by *Bacillus licheniformis* only under cultural conditions that support spore formation and specific sporulation inhibitors inhibit bacitracin formation (Bernlohr & Novelli, 1960, 1963).

Investigation of mutants indicates the close connexion between antibiotic synthesis and sporulation (Balassa, 1969; Schaeffer, 1969). Mutants have been isolated which show no antibiotic activity which also produce no spores (Schaeffer, 1967; Schmitt & Freese, 1968). Mutants unable to sporulate normally can be blocked at different stages in the sporulation process (Young & Fitz-James, 1959). Mutants which are both antibiotic and sporulation negative are blocked at an early stage, whereas mutants blocked at later stages are found to be non-sporulating antibiotic producers and restoration of antibiotic production by reversion, transduction or transformation restores the ability to sporulate (Hoch & Spizizen, 1969; Schaeffer, 1969). Schmitt & Freese (1968) and Schaeffer (1967) conclude that the production of peptide antibiotics is essential for sporulation to reach stage one.

Schaeffer (1969) isolated five partially antibiotic deficient mutants of *Bacillus subtilis* which are capable of forming spores and suggests that one non-essential antibiotic out of several produced has been lost in these mutants.

Hodgson (1970) postulates that all mutants of Bacillus species which fail to produce antibiotics, also fail to sporulate and he concludes that these peptides are required for a normal sporulation process.

Ray & Bose (1971), however, have reported mycobacillin-negative spore-forming mutants of *Bacillus subtilis*.

We have isolated several bacitracin-negative mutants during selection for higher yielding bacitracin mutants of *Bacillus licheniformis*. This report describes one of these mutants, SB 319, which is bacitracin-negative and still able to sporulate.

**METHODS**

*Organisms and mutagenic treatment.* The bacitracin-producing strains *Bacillus licheniformis* AL and *B. licheniformis* ATCC 10716 were used as reference strains. Mutants were made from strain AL by u.v. treatment of spores (Philips UV-lamp DD 115). The dose most frequently used, was 520 erg/mm² at a rate of 65 erg/mm² s.

*Media and growth conditions.* The medium used for growth and bacitracin production had
the following composition (g/1000 ml distilled water): Bacto-soytone, 20; Bactopeptone, 10; Bacto-glucose, 10; K2SO4, 1·0; MnSO4·H2O, 0·01. The pH was adjusted to 7·0 before autoclaving at 121 °C for 20 min. In this medium strain AL normally yields about 80 i.u. bacitracin/ml in 28 h. Growth occurred in 500 ml Erlenmeyer flasks at 37 °C at 400 rev./min on a New Brunswick rotatory shaker (Model G-53). The sporulation medium used was Bacto-soytone (10 g/l).

Microbiological assay of bacitracin. Bacitracin was determined by an agar-diffusion method according to Dahl et al. (1972). 10 ml of seed agar inoculated with a predetermined concentration of Micrococcus flavus was added directly to the 9 cm Petri dishes. Six stainless steel cylinders were placed on the surface of the seeded plates by a mechanical dropping device. Three of the cylinders were filled with our working standard of concentration 1 i.u. bacitracin/ml. The other three cylinders were filled with the concentration to be determined. The diameters of the zones of inhibition were measured with a Fisher-Lilly zone reader.

Thin-layer chromatography (t.l.c.). Bacitracin was deleted by t.l.c., slightly modified from the method given by Styczyliska, Niemczyk & Mazoń (1971). The centrifuged supernatants of fermentation broths (2 to 5 μl) were separated on thin-layer plates (Eastman, Silica Gel 6060) for 3 h. The solvent used was a mixture of n-butanol:acetic acid:water (4:1:2). The antibiotic appeared on the chromatograms as a distinct yellow-brown spot after development with ninhydrin. Microbiologically, the antibiotic was identified on chromatograms by overlaying with Micrococcus flavus. By using low concentrations of M. flavus in the agar overlay, it was possible to detect as little as 0·1 i.u. purified bacitracin/ml. In this system the bacitracin inhibition zone had a Rf value of approximately 0·5.

Spore count. Spores were counted after heat treatment at 80 °C for 30 min.

Biochemical tests. Motility was examined in Bacto-Penassay Broth (Difco, Michigan, Detroit, U.S.A.) with 0·4 % agar. Urease production, in Urea Agar Base (Difco) with 2 % urea added. H2S production, in TSI-Agar (Difco) stab cultures. Indole production in casein-peptone (0·5 %) and Na2HPO4·2H2O (0·2 %). O-F tests were carried out in Hugh and Leifson’s medium. Methyl red reaction, in Clark and Lub’s medium. Acid production from carbohydrates was examined in Beef extract-Peptone Broth with 1 % carbohydrate and 0·002 % bromthymol blue. The acetyl-methylcarbinol test was carried out in Proteose-peptone (0·7 %), glucose (0·5 %) and NaCl (0·5 %). Nitrate and nitrite reduction, arginine dehydro-lase and gelatinase tests were all carried out in standard preparations.

Assay of intracellular bacitracin. For the assay of intracellular bacitracin, cultures were harvested after about 26 h of incubation, washed twice in cold saline, and resuspended in a small volume of distilled water. The cells were opened with 1 % lysozyme and the lysate was evaporated to dryness under reduced pressure in a rotavapor. The dried mass was dissolved in 1 ml of ethanol and examined for bacitracin by t.l.c. and subsequent overlaying with Micrococcus flavus.

RESULTS AND DISCUSSION

Bacitracin production and the degree of sporulation of Bacillus licheniformis AL and the mutant SB319, are listed in Table 1. The two strains show approximately the same degree of sporulation, whereas bacitracin production could not be demonstrated in SB319.

No significant differences in sporulation of the two strains could be demonstrated. They started to sporulate at the same time and at the same rate, beginning normally after about 15 h of incubation, when sporangia could be seen.

Schmitt & Freese (1968) described a mutant of Bacillus subtilis which shows 20 times less
Table 1. Sporulation and bacitracin production in Bacillus licheniformis strain AL and strain SB319

<table>
<thead>
<tr>
<th>Strain</th>
<th>Degree of sporulation</th>
<th>Maximum bacitracin titre (i.u./ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>After 16 h (%)</td>
<td>After 24 h (%)</td>
</tr>
<tr>
<td>AL</td>
<td>0.1</td>
<td>3.5</td>
</tr>
<tr>
<td>SB 319</td>
<td>0.1</td>
<td>2.5</td>
</tr>
</tbody>
</table>

n.d., not detected.

antibiotic activity than the reference strain B. subtilis 60015 but still shows normal sporulation. Attempts were therefore made to ascertain if the bacitracin-negative strain, SB319, could produce small amounts of bacitracin which may be sufficient for sporulation to occur. The bacitracin produced by interesting mutants during selection was usually identified by t.l.c. and subsequent overlaying with Micrococcus flavus. However, by using this technique it was not possible to detect bacitracin production by SB319, either by ninhydrin development of the chromatograms, or by overlaying with low concentrations of M. flavus.

The data show that, if strain SB319 produces any bacitracin, production must be less than 0.1 i.u./ml (the lowest concentration of bacitracin detectable by this analytical method). This amount would, however, be negligible when compared to both the 80 i.u. bacitracin/ml produced by the reference strain AL and the 50 i.u. bacitracin/ml usually produced by strain ATCC 10716.

It was possible that SB319 produced bacitracin but did not excrete it. The cells were therefore disrupted with lysozyme, and the cell content was examined for bacitracin as described under Methods. No bacitracin was detected in SB319 by this technique. When cells of strain AL were treated in the same manner, bacitracin was detected. This indicates that the lack of bacitracin in cultures of SB319 is not due to a defect in excretion.

Strain AL and SB319 showed the same growth rate and identical morphology during all stages of growth in the production medium. Both showed growth at 56 °C, in 7 % NaCl and anaerobic growth. In an O-F test both organisms fermented glucose. The two strains also showed identical reactions in several other biochemical tests. The following tests were positive: Gram reaction, growth in Cosers citrate medium, nitrate reduction, arginine dehydrolase, acetylmethylcarbinol production, acid production after 24 h from: starch, glucose, sucrose, arabinose, mannitol, sorbitol.

The following tests were negative: H₂S production, nitrite reduction, urease, gelatinase 22 °C, indole production, methyl red reaction, acid production after 24 h from: lactose, xylose, raffinose. Both strains showed motility.

The data show that SB319 is a mutant of Bacillus licheniformis strain AL, and that this mutant is able to sporulate, although it is bacitracin-negative. The existence of such mutants, rules out a direct role for the peptide antibiotic bacitracin in the formation of spores in this species.

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REFERENCES


