Antibiotic Production and Sporulation in *Bacillus subtilis*

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Experiments of several different kinds have led to the suggestion that the ability of certain Gram-positive bacilli to sporulate is connected with antibiotic production by these organisms. Schmitt & Freese (1968) reported that an antistaphylococcal substance produced by a strain of *Bacillus subtilis* (60 015) in a late phase of growth caused the lysis of cells of this strain that were in the phase of exponential growth. They suggested that the antibiotic was involved in the commitment of the cells to sporulation. Studies with a series of mutants of *B. subtilis* Marburg showed that those able to sporulate (Sp⁺) produced an antibiotic active against staphylococci (Ab⁺). Although some antibiotic-producing strains did not yield spores (Sp⁻Ab⁺), no Sp⁺Ab⁻ strain was encountered (Balassa, Ionesco & Schaeffer, 1963; Schaeffer, 1969). It was therefore concluded that there was a 'physiological connexion' between sporulation and antibiotic formation.

In the studies with mutants of *B. subtilis* Marburg little information has been obtained about the nature of the antibiotic detected (Schaeffer, 1967). Work with a different strain of *B. subtilis* (A14, Sp⁺) showed that the latter produced an extracellular dipeptide, bacilysin, that lysed growing staphylococci (Rogers, Lomakina & Abraham, 1965). This antibiotic has now been assigned the structure (I) (Walker & Abraham, 1970a). It was therefore decided to study the relationship between the antibiotics produced by the A14 strain and the Marburg strain of *B. subtilis*.

B. subtilis Marburg was grown at 33-34°C in 5-litre whirling flasks under the conditions used for the production of bacilysin by strain A14 (Walker & Abraham, 1970a). Estimations of bacterial growth, antibacterial assays and bioautography

after paper electrophoresis and chromatography were carried out as described by Roscoe & Abraham (1966).

The growth of *B. subtilis* Marburg and the appearance of antibiotic in the culture fluid under the conditions used are shown in Fig. 1. After a lag of nearly 9h the culture entered exponential growth. This was followed by a phase of slower growth in which most of the antibiotic was produced.

When assayed against *Staphylococcus aureus* N.C.T.C. 6571 the culture supernatants produced zones of inhibition containing resistant colonies in a manner characteristic of bacilysin (Abraham, Callow & Gilliver, 1946). On plates seeded with this resistant strain of *S. aureus* bacilysin (30 units/ml) produced no zone of inhibition.

Concentrates of the antibiotic in the culture supernatant that were suitable for analysis on paper were prepared by the method of Roscoe & Abraham (1966). After electrophoresis at pH 1.8 followed by chromatography in a second dimension in butan-1-ol-acetic acid-water (4:1:4, by vol.) the antibiotic from *B. subtilis* Marburg was found by bioautography in a position characteristic of bacilysin (Roscoe & Abraham, 1966). When a mixture of the two antibiotics was analysed no resolution was observed. It thus appears that the antibiotic produced under these conditions by *B. subtilis* Marburg is bacilysin. As with strain A14, antibiotic production occurs after the first phase of rapid growth has come to an end (Roscoe & Abraham, 1966).

In the medium used in these experiments (Walker & Abraham, 1970a) virtually no spores were formed. However, under similar conditions in a sporulation medium (Schaeffer, Ionesco, Ryter & Balassa, 1965) bacilysin was produced by the Marburg strain and by the A14 strain (11 and 14 units/ml respectively after 19h) and massive spore formation was subsequently observed after 48h.

When either the Marburg strain or the A14 strain of *B. subtilis* was grown in a streak on Oxoid nutrient agar plates for 24h at 37°C, cross-streaks of *S. aureus* N.C.T.C. 6571 were made up to the central streak and the plates were then incubated for a further 16h, complete inhibition of growth of the staphylococcus within about 10mm of the

Fig. 1. Production of an extracellular antibiotic active against *S. aureus* by *B. subtilis* Marburg. ●, Bacterial growth; ○, pH; △, antibacterial activity.

central streak indicated antibiotic formation by the organisms in the latter. By this time extensive sporulation had occurred. In comparable experiments cross-streaks of a bacilysin-resistant strain of *S. aureus* were inhibited to a similar extent. Hence the antibiotic produced under these conditions was not bacilysin.

Further studies showed that one or more antibiotics active against bacilysin-resistant *S. aureus* were produced when *B. subtilis* Marburg was grown on Lemco agar (Schaeffer *et al.* 1965) or on a cellophan sheet covering an agar based on the chemically defined medium used for bacilysin production in liquid cultures (Walker & Abraham, 1970a). In the latter case the active material was extracted by freezing at -20°C and removal of the extruded liquid. After precipitation of impurities with 70% (v/v) ethanol and concentration of the supernatant *in vacuo* a solution was obtained (containing 1.5 units/ml) that was subjected to electrophoresis and chromatography on paper. Bioautography revealed the presence of one active substance with $R_{\text{bacilysin}} 1.5$ in butan-1-ol-acetic acid-water (4:1:4, by vol.) and showing no net charge at pH 4.5. This substance was not extracted from water by ether or butan-1-ol at pH 2, 7 or 10.

In contrast with the antibiotic described by Schmitt & Freese (1968), no lysis of either strain

A14 or strain Marburg occurred when bacilysin (final concentration 30 units/ml) was added during exponential growth.

Thus it is clear from the results described here that both *B. subtilis* Marburg and *B. subtilis* A14 can produce at least two antibiotics, one of which is bacilysin (I), and that the nature of the antibiotic formed depends on the conditions of culture. In its failure to cause the lysis of exponentially growing cultures of strains of *B. subtilis* by which it is produced, bacilysin lacks the property that led Schmitt & Freese (1968) to suggest that an antibiotic was concerned in the commitment of a different strain of *B. subtilis* to sporulation. The antibiotic with $R_{\text{bacilysin}} 1.5$ in butan-1-ol-acetic acid-water (4:1:4, by vol.) differs in its solubility properties from the substance described by Schmitt & Freese (1968), but it is not known whether the former substance has any action against the strain of *B. subtilis* that produces it.

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