Relationship Between Butirosin Biosynthesis and Sporulation in 

Bacillus circulans

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The relationship between butirosin biosynthesis and certain biochemical characteristics related to sporulation in a strain of Bacillus circulans NRRL B-3313 was examined. The cellular content of dipicolinic acid increased while the amount of poly-β-hydroxybutyrate decreased with changes in antibiotic productivity. Oligosporogenous mutants failed to synthesize the antibiotic and to degrade poly-β-hydroxybutyrate. These observations suggest that sporulation may be related to antibiotic production in this strain of B. circulans.

A close relationship between sporulation and the production of secondary metabolites in microorganisms has been demonstrated by biochemical and genetic analysis of some organisms. In bacilli, the polypeptide antibiotics produced have been found to affect the spore formation directly or indirectly (2, 6, 9, 10, 16, 18-20, 23). It has also been observed that antibiotic production is closely related to the production of serine proteases during sporulation (14, 23) and that some polypeptide antibiotics produced are specific inhibitors of RNA synthesis (10, 18, 19). The relationship between antibiotic production and morphological differentiation in Cephalosporium acremonium, a cephalosporin C producer (11, 13, 17), in Streptomyces lactamdurans, a cephamycin C producer (3), and in Penicillium urticae, a patulin and griseofulvin producer (21), has also been reported.

The production of butirosin and another related compound, Bu-1975 (4'-deoxybutirosin), by a strain of Bacillus circulans (Fig. 1) (4, 7) represents the only reported example of production of an organism other than the filamentous Actinomycetes. In this unique aminoglycoside-producing strain of bacillus, the relationship between the production of butirosin and several biochemical characteristics related to sporulation was studied.

MATERIALS AND METHODS

Microorganisms and cultivation. A strain of B. circulans NRRL B-3313 was used in this study. Four strains, B-3313-SM1 to B-3313-SM4, were independently isolated as spontaneous oligosporogenous mutants from B. circulans NRRL B-3313.

The inoculum was prepared in 100 ml of medium (pH 7.5) containing 3.0% soybean meal, 0.4% ammonium chloride, and 0.5% calcium carbonate in a 500-ml Erlenmeyer flask and incubated at 30°C for 48 h on a rotary shaker at 300 rpm. The same medium supplemented with 4.0% glycerol was seeded with 5% 48-h inoculum and fermented for 5 days under the same conditions used for the inoculum (4).

Analytical procedures. To investigate the relationship between antibiotic production and sporulation, the production of butirosin and several other biochemical products related to the spore formation was measured after centrifugation of 120-h culture broth. The antibacterial activity of butirosin in the supernatant was estimated by an agar diffusion method as reported earlier (12). Dipicolinic acid found in the spor coat was assayed by the method of Janssen et al. (5), and poly-β-hydroxybutyrate in the cell mass was determined by the procedure described by Law and Slepecky (8). The number of refractile sporulated bacilli was counted with a hemacytometer and a phase-contrast microscope. The cell mass was measured after treatment with 0.1 N hydrochloric acid to remove residual calcium carbonate.

RESULTS

In the fermentative production of butirosin with the B. circulans strain, antibiotic productivity was closely associated with the state of the microorganism, including its sporulation potential. A higher productivity of butirosin was achieved by the more highly sporulated strains.

In the sporulation process for bacilli, dipicolinic acid is known to be one of the major endospore components (14), and poly-β-hydroxybutyrate is considered to be a lipid inclusion as an energy source during sporulation (22). To investigate the relationship between antibiotic production and the sporulation process, the amounts of dipicolinic acid and poly-β-hydroxybutyrate in B. circulans were determined during the fermentation. As seen in Fig. 2, butirosin biosynthesis followed soon after the production of dipicolinic acid and consumption of poly-β-hydroxybutyrate during the fermentation. These observations suggest that the antibiotic biosynthesis was related to the sporulation process.


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During propagation of *B. circulans* on agar plates, some spontaneous mutants with different sporulation potentials appeared and were isolated. With these oligosporogenous mutants, the butirosin fermentation was performed, and the sporulation potential was examined (Table 1). Compared with the original strain, the oligosporogenous mutant strains SM-3 and SM-4 contained negligible amounts of dipicolinic acid and significantly larger amounts of poly-δ-hydroxybutyrate. Concomitantly, these produced only small quantities of butirosin. Partially sporulating strains (SM-1, SM-2, and SM-3) also showed similar biochemical properties which also correspond to sporulation potential and antibiotic production. To show the relationship between antibiotic biosynthesis and sporulation potential, the data obtained from the original parental strain and resulting mutants were compared by graphical analysis on a semi-logarithmic plot. As shown in Fig. 3 and 4, the amount of antibiotic produced can be correlated with sporulation, which is expressed in terms of the amount of dipicolinic acid produced and the amount of poly-δ-hydroxybutyrate consumed. A good correlation between antibiotic production and the sporulation potential in *B. circulans* was found. The oligosporogenous mutants could not form mature spores due to a blockage in a sequence of morphological changes associated with spore formation (15, 20). Although the immature spores could be counted by the microscopic examination, they could not be correlated directly with the antibiotic productivity. For this reason, it was difficult to ascertain the direct correlation between the oligosporogenous mutant formation and antibiotic productivity.

The effect of carbon sources on butirosin production and sporulation of *B. circulans* was also evaluated (Table 2). Glycerol was found to be the most suitable for antibiotic production among the carbon sources tested. Xylose and lactose were found to be poor carbon sources for cell growth and butirosin production. The highest level of sporulation in *B. circulans* was observed in a glycerol medium. However, cultivation on glucose medium resulted in a relatively low level of sporulation and butirosin production. Propagation on starch and maltose showed a somewhat different pattern (Table 2), in that a high level of butirosin was synthesized despite a relatively low level of dipicolinic acid production.

### Table 1. Comparison of antibiotic productivity and sporulation characteristics of oligosporogenous mutants and the original strain

<table>
<thead>
<tr>
<th><em>B. circulans</em> strain</th>
<th>Final pH</th>
<th>DCW (mg/ml)</th>
<th>Dipicolinic acid (μg/mg of DCW)</th>
<th>Poly-δ-hydroxybutyrate (μg/mg of DCW)</th>
<th>Butirosin productivity (μg/mg of DCW)</th>
<th>Spore content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL B-3313</td>
<td>6.87</td>
<td>4.20</td>
<td>68</td>
<td>2.9</td>
<td>186</td>
<td>85</td>
</tr>
<tr>
<td>SM-1</td>
<td>6.95</td>
<td>4.55</td>
<td>54</td>
<td>4.1</td>
<td>107</td>
<td>85</td>
</tr>
<tr>
<td>SM-2</td>
<td>6.96</td>
<td>4.42</td>
<td>53</td>
<td>5.8</td>
<td>83</td>
<td>88</td>
</tr>
<tr>
<td>SM-3</td>
<td>7.51</td>
<td>3.33</td>
<td>22</td>
<td>18</td>
<td>13</td>
<td>72</td>
</tr>
<tr>
<td>SM-4</td>
<td>7.23</td>
<td>4.44</td>
<td>6.5</td>
<td>15</td>
<td>6</td>
<td>28</td>
</tr>
</tbody>
</table>

*Samples taken after 120 h of cultivation were assayed for butirosin, dipicolinic acid, and poly-δ-hydroxybutyrate with the final pH and dry cell weight (DCW) as indicated.*
This was especially evident when *B. circulans* was propagated in malose.

**DISCUSSION**

The relationship between antibiotic synthesis and sporulation of *B. circulans* was investigated by studying biochemical characteristics associated with sporulation, i.e., dipicolinic acid and poly-β-hydroxybutyrate. During the fermentation of butirosin, it was observed that the synthesis of antibiotic initiated with the concomitant appearance of dipicolinic acid and disappearance of poly-β-hydroxybutyrate. In another strain of bacilli producing peptide antibiotics, antibiotic production was shown to be regulated in coordination with physiological changes occurring during sporulation (2, 9, 18, 23).

The relationship between antibiotic biosynthesis and sporulation in *B. circulans* was evaluated quantitatively. Oligosporogenous mutants appear to produce little butirosin, but the parental strain, which possessed a greater sporulation capacity, produced correspondingly larger amounts of butirosin. When the relationship among the amounts of dipicolinic acid, poly-β-hydroxybutyrate, and antibiotic productivity was examined, an exponential relationship was found. The results obtained were somewhat different from those of the other published reports (3, 11, 13, 23) in that the other results showed a linear relationship between morphological differentiation and the synthesis of secondary metabolites. The exponential relationship found here empirically is considered to be consistent with the theoretical expectation. If it were to have a linear relationship, the productivity of genetically mutated microorganisms with a limited degree of sporulation could not be accounted for. It is speculated that once cells go into the antibiotic-producing phase (or idiophase), an autocatalytic mechanism may function inside individual cells where antibiotic-synthesizing enzymes are produced at an exponential rate. This observation is analogous to the runaway type of plasmid replication in certain recombinant organisms which harbor genes responsible for the antibiotic-synthesizing enzymes.

The propagation of *B. circulans* on a glycerol medium gave maximal sporulation and antibiotic production. A catabolite repression of antibiotic biosynthesis in this organism grown on glucose medium was observed previously (1). This resulted in a relatively low level of sporulation and antibiotic production. The finding that growth on starch and malose showed somewhat different relationships between the butirosin biosynthesis and sporulation may be attributed to the observation that the rate of assimilation of carbon source varies and that *B. circulans* cells are committed to sporulation only after starvation of the carbon source (20).

Further quantitative studies of this kind should provide a better understanding of the biochemical basis of morphological differentiation of microorganisms and its relationship to the biosynthesis of antibiotics.

**LITERATURE CITED**


