Fractionation of the mycobacillin-synthesizing enzyme system

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The mycobacillin-synthesizing enzyme system was highly purified by fractionation at 30-55% (NH₄)₂SO₄ saturation. The enzyme concentrate on Sephadex G-200 gel chromatography was resolved into three distinct fragments. Each of the fragments on further purification by DEAE-cellulose ion-exchange chromatography behaved as a single-component system, as clearly indicated by the sharpness of the peaks in the elution diagram. None of the fragments alone nor any two of them in all possible combinations possessed mycobacillin-synthesizing activity, which was restored only when the three fragments were used together in the test system.

Materials and methods

Chemicals and radiochemicals

Lysozyme from egg white and ATP were obtained from Sigma Chemical Co (St Louis, MO, USA). U-¹⁴C-labelled Chlorella protein hydrolysate (sp. radioactivity 42 mCi/mg atom of C) and Na₂H₂¹⁵PO₄ (sp. radioactivity 10 mCi/mmol) were purchased from Bhabha Atomic Research Centre ( Trombay, India). Other chemicals used in the experiments were obtained from commercial sources.

Organism

Bacillus subtilis B₂ (Majumder & Bose, 1958) was used to study the biosynthesis of mycobacillin.

Preparation of 20000 g supernatant

The method used to prepare 20000 g supernatant was based on that described by Sengupta & Bose (1971). The producer organism B. subtilis B₂ was grown in a fresh nutrient broth (4.5 litres) supplemented with 1% glucose for 15-16h at 30±1°C. Then the broth was centrifuged and washed once with 0.9% NaCl and then with 5mM-MgCl₂. The cells (20g) were resuspended in 80ml of 50mM-Tris/HCl buffer, pH 7.8, containing 2mM-EDTA, 10mM-MgCl₂ and 1mM-dithiothreitol (buffer A), to which lysozyme (800µg/ml) had been added. After incubation for 20 min at room temperature the
cell-free system was quickly chilled at 0°C for 20 min and centrifuged at 20000 g for 30 min

Ultra-centrifugation and streptomycin sulphate/\(\text{\(\text{NH}_4\)}\text{\(_2\)}\text{\(\text{SO}_4\)}\) fractionation

The cell-free extract was centrifuged at 20000 g A portion of the supernatant was further centrifuged at 105000 g (Beckman ultracentrifuge) for 90 min, and the supernatant from this was returned for biosynthesis studies. To another portion of the 20000 g supernatant 10% (w/v) streptomycin sulphate solution was added dropwise to make the final concentration 1%. The precipitate collected by centrifugation at 20000 g for 30 min was discarded. The supernatant was then precipitated with \(\text{\(\text{NH}_4\)}\text{\(_2\)}\text{\(\text{SO}_4\)}\) (30–55% saturation), and the precipitate was collected by centrifugation at 20000 g for 30 min. The precipitate was dissolved in buffer B (50 mM-Tris/HCl buffer, pH 7.8, containing 0.25 mM-EDTA, 10 mM-MgCl\(_2\) and 1 mM-dithiothreitol) and dialysed for 10 h against 2 litres of the same buffer (buffer B)

Sephadex G-200 gel filtration

The dialysed 30–55% satn \(\text{\(\text{NH}_4\)}\text{\(_2\)}\text{\(\text{SO}_4\)}\) fraction (8 ml) was applied to a Sephadex G-200 column (2.5 cm x 70 cm) equilibrated with buffer B and eluted with the same buffer in 5 ml fractions (flow rate 15 ml/h). The protein content and ATP-\([\text{\(\text{P}\)}\text{\(_3\)}\text{\(\text{P}\)}]\) exchange capacity dependent on mycobacillin constituent amino acids of each of the fractions were measured. The peak fractions as indicated by the exchange activity were separately pooled together and precipitated with \(\text{\(\text{NH}_4\)}\text{\(_2\)}\text{\(\text{SO}_4\)}\), and the precipitate was dissolved in a small volume of buffer B and dialysed for 5 h against 2 litres of buffer B

DEAE-cellulose column chromatography

The three dialyzed peak fractions from the Sephadex G-200 gel column were applied separately to a DEAE-cellulose column (1.5 cm x 16 cm) equilibrated with buffer B and eluted stepwise with the same buffer containing increasing concentrations of KCl in 4 ml fractions (flow rate 24 ml/h), and the activity of each fraction was determined on the basis of ATP-\([\text{\(\text{P}\)}\text{\(_3\)}\text{\(\text{P}\)}]\) exchange capacity. The peak fractions as indicated by the exchange activity were pooled together and further concentrated separately by freeze-drying

Determination of mycobacillin biosynthesis

The incubation mixture contained the following, in a total volume of 2 ml: Tris/HCl buffer, pH 7.8, 100 μM, MgCl\(_2\), 10 μM, ATP, 5 μM, phosphoenolpyruvate, 5 μM, pyruvate kinase, 20 μg, dithiothreitol, 10 μM, U-\[^{14}\text{C}\] labelled Chlorella-protein hydrolysate (sp radioactivity 42 mCi/mg atom of C), 2.5 μCl, l-leucine, l-alanine, l-proline, l-serine, l-aspartic acid, 1 μM each, d-aspartic acid, 4 μM, d-glutamic acid, 2 μM, l-tyrosine, 2 μM, enzyme to be assayed, 5 mg. The reaction was terminated by adding 4 vol of butan-1-ol, and 2 mg of unlabelled mycobacillin was added as carrier. Mycobacillin was isolated from the butanol extract as described by Majumder & Bose (1960) and finally dissolved in 2 ml of ethanol, and the ethanol solution was used directly for counting of radioactivity

Measurement of ATP-\[^{32}\text{P}\]\ exchange dependent on mixture of constituent amino acids of mycobacillin

The exchange was studied as described by Stulberg & Novelli (1960). The reaction mixture contained, in a final volume of 300 μl, 0.1 M-Tris/HCl buffer, pH 8.0, 3 mM-ATP, 5 mM-MgCl\(_2\), 10 mM-KF, 2 mM-dithiothreitol, a mixture of constituent amino acids of mycobacillin (each 5 mM), 2 mM-Na\(_2\)H\(_3\)P\(_4\) (10 μCi, sp radioactivity 10 mCi/mmole) and 200 μl of Sephadex G-200 eluate as a source of the enzyme. The mixture was incubated for 30 min at 30°C, and the reaction was terminated by mixing with 0.5 ml of a solution of 2.5% of charcoal (acid-washed Norit A) in 0.2 M-Na\(_2\)HPO\(_4\), and 10% (w/v) trichloroacetic acid. After 30 min in the cold the suspension was filtered on a Whatman GF/C disc (2.5 cm diam.), washed with 5 x 5 ml of water, and the filter was dried and radioactivity of the adsorbed materials measured in a liquid-scintillation counter

Determination of protein concentration

The protein concentration was measured by the method of Lowry et al. (1951), with bovine serum albumin as the standard

Results

Purification of mycobacillin-synthesizing enzyme by ultracentrifugation

The attempt to purify the mycobacillin-synthesizing enzyme system by centrifugation shows that the protein content was 8.0 mg/ml for the 20000 g supernatant, as against 6.2 mg/ml for the 105000 g supernatant, and that the incorporating ability of the mycobacillin-synthesizing enzyme per mg of protein was also slightly greater for the 20000 g supernatant than for the 105000 g supernatant, which retained approx 80% of the activity

Purification by streptomycin sulphate/\(\text{\(\text{NH}_4\)}\text{\(_2\)}\text{\(\text{SO}_4\)}\) fractionation

Table 1 indicates that streptomycin sulphate did not increase the purification (fold) of the enzymes. However, the step was retained, since it removed much of the non-protein macromolecular constituents. Attempts at further purification with

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The 30–55% satn (NH₄)₂SO₄ fraction obtained from 20 g wet wt of cells, (4.5 litres of culture) was loaded on a Sephadex G-200 column (2.5 cm x 70 cm) equilibrated with buffer B at 5°C. The column was then eluted with buffer B (15 ml/h). The ATP-[γ-32P]P₆ exchange was measured in each fraction (5 ml). ATP-[γ-32P]P₆ exchange activity dependent on a mixture of mycobacillin constituent amino acids was measured in a 200 μl portion of each fraction. The total amount of radioactivity in each incubation mixture was 10 μCi. The radioactivity measured in control tubes (without amino acid mixture) was subtracted.

### Table 1: Streptomycin/(NH₄)₂SO₄ fractionation of the mycobacillin-synthesizing system

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total incorporated radioactivity (c.p.m.)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>688</td>
<td>1513</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>668</td>
<td>1560</td>
</tr>
<tr>
<td>30–55% satn (NH₄)₂SO₄</td>
<td>144</td>
<td>5368</td>
</tr>
</tbody>
</table>

(NH₄)₂SO₄ shows that most of the active enzyme protein was precipitated at 30–55% (NH₄)₂SO₄ saturation, which resulted in 3.5-fold purification with respect to the crude-cell-free extract.

### Purification of mycobacillin-synthesizing enzyme on the Sephadex G-200 gel column

The protein content and ATP-[γ-32P]P₆ exchange-activity profiles of the different fractions from a Sephadex G-200 column are shown in Fig. 1, which clearly indicates that the mycobacillin-synthesizing enzyme system was resolved into three peak fractions, on the basis of both protein content and exchange activity in the presence of mycobacillin constituent amino acids. It is noteworthy that the three peaks of protein content almost coincided with those of exchange activity.

### Mycobacillin-synthesizing activity of three peak fractions either alone or in different combinations

The fractions constituting the three different peaks obtained from Sephadex G-200 gel filtration on the basis of ATP-[γ-32P]P₆ exchange activity were separately pooled together, and mycobacillin-synthesizing activity was examined in each fraction alone, or in all possible combinations of two fractions, and also in a combination of the three. The results are shown in Table 2, which indicates that the mycobacillin-synthesizing enzyme system could be separated into three fractions and that neither any one nor any two of them in any combination was active, whereas all the three peak fractions taken together could bring about the synthesis.

### DEAE-cellulose ion-exchange-chromatographic behaviour of peak fractions obtained from Sephadex G-200 gel column

All the three peak fractions from the Sephadex column on the basis of ATP-[γ-32P]P₆ exchange activity were separately pooled together and passed separately through a DEAE-cellulose column, the results are shown in Figs 2(a), 2(b) and 2(c). It appears that the peaks in the elution diagram on the basis of either protein concentration or exchange activity were much sharper and showed greater coincidence.
Fractionation of mycobacillin-synthesizing activity

The three peak fractions from the DEAE-cellulose column were tested again for mycobacillin-synthesizing activity either alone, or in groups of two in all possible combinations, or finally in a combination of all three peak fractions Table 3 shows that mycobacillin synthesis occurred only when all the three peak fractions were taken together.

Discussion

Although exhaustive studies on the biosynthesis of oligopeptides indicate the presence of two enzyme components in gramicidin-S synthetase (Gevers et al., 1968) and of three in tyrocidine synthetase (Roskoski et al., 1970), the present work was undertaken to purify and if possible to fractionate the mycobacillin-synthesizing enzyme to test whether the synthesizing system is a multi-site single enzyme or a multiple-component enzyme complex. Different techniques of protein purification were tried: Ultracentrifugation at 105,000 g did not purify the enzyme, although it directly proved that the enzyme was present in a soluble form in the cytosol (NH₄)₂SO₄ fractionation considerably purified the enzyme, a 3.5-fold purification was achieved by fractionation at 30-55% saturation. The active enzyme concentrate so obtained on further purification on Sephadex G-200 was resolved into three components on the basis of both their protein content and also their ATP-[³²P] exchange activity. The three components on the basis of their exchange activity, when tested for mycobacillin-synthesizing activity either alone or in all possible combinations, gave results clearly indicating that the mycobacillin-synthesizing system consists of three enzyme components. DEAE-cellulose column chromatography further purified each of the components, and also confirmed the three-component nature of the mycobacillin-synthesizing enzyme complex, which resembles tyrocidine and bacitracin (cyclic oligopeptide with a side chain) synthetase (Frølyshov, 1974) but not gramicidin-S synthetase.

The fold purification, which was 3.5 after streptomycin/(NH₄)₂SO₄ fractionation, could not be calculated for the components obtained after gel or ion-exchange chromatography. However, on the basis of protein requirement for equal synthesis in the presence of equal but not equivalent amounts of these components, the values were 22-fold and
36-fold for gel and ion-exchange chromatography respectively.

Thus the purification of mycobacillin-synthesizing enzyme complex and its fractionation into three components have been achieved.

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Standardization of Parameters for the Mycobacillin Synthetase Activity

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ABSTRACT An effective method of preparation involving sonication was developed for cell-free mycobacillin synthetase from Bacillus subtilis. The enzyme showed optimum activity at a buffer concentration of 0.5 M Tris-HCl and pH 7.5. ATP and Mg²⁺ were essential for synthase activity, while ADP and AMP were not effective. The synthetase was inhibited by MgCl₂ but not by ADP and ATP. The optimum ratio of MgCl₂ to ATP was 1:1. Homogenous addition of pantothenic acid had no effect.

Spore-forming bacilli produce a variety of bioactive peptides. The cell-free synthesis of peptide antibiotics like gramicidin S and tyrocidine, produced by different strains of Bacillus brevis, have been described by several groups (Berg et al., 1965; Yukioka et al., 1966; Fujikawa et al., 1966; Roskoski et al., 1970). Mycobacillin (Majumder and Bose, 1968), an antifungal cyclic tripeptide antibiotic, isolated and characterized in this laboratory (Majumder and Bose, 1969; Banerjee and Bose, 1963, Banerjee and Bose, 1964a, b), was synthesized by the enzymes present in the 10 000 g supernatant fraction prepared by protoplast lysis of the producer organism, Bacillus subtilis. A method of enzyme preparation involving sonication and the essential parameters for optimum enzymic synthesis of mycobacillin are described here.

MATERIALS AND METHODS

**Streptococcus subtilis** B₃, originally isolated in this laboratory, is capable of producing mycobacillin.

*Media and growth conditions.* Nutrient broth (pH 7.2) containing glucose 1%, peptone 0.5%, and beef extract 0.3%, was used. Cells were harvested from a rotary-shaker culture after the required incubation at 30 ± 1 °C by centrifugation at 0—4 °C and at 3000 g for 15 min and washed twice with cold 0.9% saline and once with cold distilled water.

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Table 1 The effect of the mode of enzyme preparation on the yield of protein as well as synthetic activity:

<table>
<thead>
<tr>
<th>Type of treatment</th>
<th>Amount of protein per mL supernatant</th>
<th>Average enzyme activitya</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysosomeb</td>
<td>0.5 mg</td>
<td>561</td>
<td>445</td>
</tr>
<tr>
<td></td>
<td>0.9 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sonication</td>
<td>9.8 mg</td>
<td>5085</td>
<td>636</td>
</tr>
<tr>
<td></td>
<td>10 mg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Age of culture 16–17 h, wet mass of cells 2.5 g, incubation period 2 h
b Per 8 mg of enzyme protein
c 20 000 g supernatant was used

Preparation of buffer A A 50 mM Tris-HCl buffer (pH 7.4) was prepared which contained 10 mM MgCl₂, 10 mM 1,4-dithiothreitol, 2 mM EDTA and 1 mM phenylmethanesulfonyl fluoride.

Preparation of cell-free extract by lysosome-EDTA treatment 2.5 g wet mass cells collected from 600 mL of broth were suspended in 4 volumes of buffer A and subjected to lysozyme (EC 3.2.1.17) digestion by the method of Sengupta and Bose (1971). The lyzate was centrifuged (20 000 g, 30 min, 0–2 °C) in a Sorval-RC-5B refrigerated superspeed centrifuge.

Preparation of cell-free extract by sonication A homogenized washed cell suspension of the organism prepared as above in the same buffer was taken in a specially made glass tube. The tube was immersed in an ice-salt bath and the suspension sonicated for two 30-s periods at 100 W and 20 kHz in MSE ultrasonicator. The suspension was cooled for 3 mm between bursts to maintain the temperature below 5 °C. The lyzate was centrifuged at 20 000 g for 30 mm at 0–2 °C.

![Graph](image)

Fig. 1 Effect of pH and buffer concentration (mm) on mycobacterial synthesis enzyme activity (A, ppm > 10⁻²). Measurements were carried out with 20 000 g supernatant as the enzyme source in 50 mm Tris-HCl buffer. The pH values were determined with a glass electrode (model L-110) at the incubation temperature.
Preparation of purified enzyme. The crude enzyme from the lyzate obtained by sonication was purified following (NH₄)₂SO₄ precipitation, Sephadex G-200 gel filtration and DEAE ion exchange chromatography (Ghosh et al. 1983)

Estimation of protein. Protein was estimated by the Lowry method taking bovine serum albumin (BSA) as standard.

![Graph](attachment:image.jpg)

Fig. 2. Dependence of ¹³C incorporation (activity) into mycobactin upon ATP and Mg²⁺ concentration (mm) 20 000 g supernatant was used as enzyme source. Incubation period was 1 h.

Assay of mycobactin synthetase activity. Enzyme activity was assayed according to Sengupta and Bose (1971). The incubation mixture (2 mL) contained 185 kBq chlorella protein hydrolyzate, 5 μM ATP, 5 mM phosphoenolpyruvate, 20 μg pyruvate kinase (EC 2.7.1.40), 10 μM MgCl₂, 100 μM chloramphenicol, 4 μM d-Asp, 2 μM each of L-Glu and L-Tyr and 1 μM each of L-Ileu, L-Ala, L-Pro and L-Ser. Incubation was carried out at 30 °C.

RESULTS

It appears from comparative studies on the mode of enzyme preparation by lysozyme treatment and sonication (Table I) that the latter method was more effective in the sense that not only the yield of protein per wet cells but also the specific enzyme activity per mg protein was increased by 42.8 and 42.5 %, respectively.

Experiments relating the effect of pH on enzyme activity showed that the mycobactin-synthesizing enzyme showed maximum activity at pH 7.5 at a buffer concentration of 50 mM. The buffer concentration was then varied at the optimum pH which shows that the optimum concentration was also 50 mM at the optimum pH of 7.5 (Fig. 1).

An ATP concentration of 5 mM was optimal for synthesis and the ATP concentration above 5 mM was inhibitory (Fig. 2). The optimum Mg²⁺ con-
centration required at the optimum ATP concentration was about 5 mM corresponding to an ATP to Mg2+ ratio of nearly 1:1.

Mg2+ was essential for mycobacillm-synthesizing activity (Table II). Omission of Mg2+ resulted in a marked decrease in 14C-amino acid incorporation into mycobacillin. Among the other divalent cations, such as Co2+, Fe2+, Mn2+, Ni2+, and Zn2+, only Co2+ and Mn2+ could substitute for Mg2+

some extent. Addition of monovalent ions (K+ or Na+) in combination with Mg2+ appeared to have neither stimulatory nor inhibitory effect on the incorporating ability.

The incorporation of the 14C-amino acids into mycobacillin by the cell-free extract was energy-dependent (Table III). The omission of both ATP and the energy-generating system completely inhibited the incorporation although ATP* or phosphoenolpyruvate and pyruvate kinase could to some extent substitute for each other. No incorporation into mycobacillin took place in the presence of GTP, UTP, or CTP.

### Table III: Effect of energy sources on enzyme activity

<table>
<thead>
<tr>
<th>Changes in incubation mixture</th>
<th>14C Amino acid incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.80</td>
</tr>
<tr>
<td>--ATP</td>
<td>0.50</td>
</tr>
<tr>
<td>--phosphoenolpyruvate, pyruvate kinase</td>
<td>2.15</td>
</tr>
<tr>
<td>--ATP, phosphoenolpyruvate, pyruvate kinase</td>
<td>0.00</td>
</tr>
<tr>
<td>TP, GTP, CTP</td>
<td>1.20</td>
</tr>
</tbody>
</table>

* Incubation period 1 h, temperature of incubation 30°C; 20,000 g supernatant was used as a source of enzyme.

### Table II: Effect of metal ions on mycobacillin synthetase activity

<table>
<thead>
<tr>
<th>Salt</th>
<th>14C Amino acid incorporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.50</td>
</tr>
<tr>
<td>- MgCl2</td>
<td>8.25</td>
</tr>
<tr>
<td>+ MnCl2</td>
<td>15.45</td>
</tr>
<tr>
<td>+ CoCl2</td>
<td>12.00</td>
</tr>
<tr>
<td>+ FeCl2</td>
<td>9.25</td>
</tr>
<tr>
<td>+ NiCl2</td>
<td>9.00</td>
</tr>
<tr>
<td>+ ZnCl2</td>
<td>7.00</td>
</tr>
<tr>
<td>+ KCl</td>
<td>40.00</td>
</tr>
<tr>
<td>+ NaCl</td>
<td>21.00</td>
</tr>
</tbody>
</table>

* Enzyme activity was measured by the standard assay condition as described in the text. Divalent cations instead of Mg2+ were added to the incubation system as desired. K+ and Na+ ions were added in combination with Mg2+. The purified enzyme was used at a concentration of 100 μg per mL of the incubation mixture.
ADP markedly inhibited antibiotic formation but AMP was practically without any effect (Fig 3).

Mycobacillin-synthesizing enzyme activity was markedly inhibited by mercapto reagents (Table IV). The inhibition of enzyme activity by 1 mM mercuric chloride or 4-chloromercuribenzoate was higher than that by N-ethylmaleimide and 5,5'-dithio-bis-2-nitrobenzoate at the same concentration.

Free pathothentic acid if added exogenously to the incubation system did not enhance the activity of mycobacillin synthetase (Table V).

DISCUSSION

The work was done to standardize some parameters including enzymic preparation history to unreported to secure maximum cell-free enzyme synthesis of mycobacillin. Previously, the cell-free enzyme was prepared by lysozyme treatment. This method was found to be ineffective in the case of spores. An alternative method involving sonication applicable to both vegetative cells and spores was therefore developed for comparison and subsequent use for studies on the localization of the enzyme system. The yields of protein including its specific enzyme activity were high if prepared by the sonication method.

The optimum pH 7.5 for mycobacillin synthesis was not very different from that for other cyclic peptide antibiotics, such as gramicidin S or tyrocidine, the pH optimum range being 7.2–8. ATP and Mg$^{2+}$ were necessary for mycobacillin synthetase activity. An ATP to Mg$^{2+}$ ratio of 1.1 which was found to be optimum for enzyme biosynthesis of mycobacillin appeared to be remarkably similar for optimum synthesis in most other cases including gramicidin S, tyrocidine, etc. Among the divalent metal ions only Mn$^{2+}$ and Co$^{2+}$ could substitute for Mg$^{2+}$ to some extent. The ATP generating system phosphoenolpyruvate and pyruvate kinase could substitute for ATP to some extent but GTP, UTP or CTP had no effect.

Mycobacillin synthesis was inhibited by ADP, stimulated by diphosphate and unaffected by AMP, as otherwise observed for gramicidin S, tyrocidine.
bacitracin, etc., in which case the synthesis was inhibited both by AMP and diphosphate and unaffected by ADP. This might follow directly from the earlier observation that amino acid activation with mycobacillin occurred through ATP—monophosphate and not through ATP—diphosphate exchange as in the case of other peptide antibiotics.

The mercapto group seemed to be essential for mycobacillin because the synthesizing activity was markedly inhibited by mercapto reagents, HgCl₂, 4-chloroacetanilide, 5,5'-dithio-bis-2-nitrobenzoate, and N-ethylmaleimide.

The summation method of enzyme preparation and the parameters for optimum enzymic synthesis of mycobacillin are thus indicated.

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Accumulation of Peptides by Mycobacillin-negative Mutants of Bacillus subtilis B3

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Thirteen mycobacillin-negative (Mv-) mutants of Bacillus subtilis B3 were isolated from an auxotrophically tagged mycobacillin producer organism. The wild-type produces three feeble producers and three strictly My- mutants did not accumulate any ninhydrin-positive peptide in the culture medium while the remaining seven My- mutants did accumulate ten such peptides whose amino acid composition indicated that there might be only three different peptides. The N-terminal and C-terminal amino acid residues implicated one of these peptides as a pentapeptide intermediate in mycobacillin synthesis. This was further confirmed by its molecular weight and sequence. Studies on cell-free synthesis showed that only the enzyme system from the wild-type strain synthesized mycobacillin while the defective ones from all the My- mutants synthesized one and the same pentapeptide as found in the culture broth of some of the mutants. Further studies in which the enzymes responsible for mycobacillin synthesis by cell-free extracts were separated into three fractions, A, B and C, showed that seven of the mutants were defective in fraction B whereas the other three mutants had defects in both fractions B and C. Thus, the pentapeptide Pro-Asp-Glu-Tyr-Asp appears to be implicated in mycobacillin biosynthesis.

INTRODUCTION

The use of 'blocked' mutants, as developed by Beadle & Tatum (1941) has proved very effective in the study of intermediary metabolism. The technique has also been extended to the biosynthesis of antibiotics, e.g. penicillin (Edwards & Holt, 1974), cephalosporin C (Queen & et al. 1974) and tetracycline (McCormick et al. 1968). Loder & Abraham (1971a, b) isolated and identified the tripeptide L-a-aminoadipyl-L-cysteinyl-D-valine (ACV) from cell-free extracts of Cephalosporium acremonium. Lemke & Nash (1972) isolated from blocked mutants of C. acremonium ACV-like compounds implicated as intermediates in the elaboration of penicillin N and cephalosporin C. Fujisawa et al. (1973) isolated a mutant of C. acremonium blocked in the conversion of deacetylcephalosporin C (DCPC) to cephalosporin C. McCormick et al. (1968) isolated mutants of a chlorotetraacycline producer organism that were blocked in the reduction of dehydrochlorotetraacycline to chlorotetraacycline, and therefore accumulated dehydrochlorotetraacycline. The biosynthesis of mycobacillin, a cyclic antifungal antibiotic produced by Bacillus subtilis B3 (Majumder & Bose, 1958), does not involve an RNA template activation of the constituent amino acids occurs as a result of AIP-P exchange, proline being the initiating amino acid in the synthesis (Sengupta & Bose, 1974). An amino acid deprivation technique was developed for the isolation of possible intermediates in mycobacillin synthesis (Sengupta & Bose, 1974). However, these intermediates, in the presence of complementary amino acids, were not incorporated into mycobacillin (Sengupta & Bose, 1982).
The present communication describes the isolation of some mycobactin-negative mutants from an auxotrophically tagged mutant of the producer *B. subtilis* B, and the use of these blocked mutants in whole-cell fermentation and cell-free *in vitro* synthesis, both to isolate and characterize some peptides that might be involved in mycobactin biosynthesis, and to locate the enzyme defect in the three fractions (Ghosh et al., 1983) of the mycobactin synthetase complex

**METHODS**

Organisms, cultural conditions and media

*B. subtilis* strain BM, originally isolated in this laboratory (Majumder & Bose, 1960a) produces 0.37 mg mycobactin per ml of nutrient broth, recovery by the standard process is 65%, (Majumder & Bose, 1960a) This organism was used to produce auxotrophic mutants which were subsequently used to isolate mycobactin-negative (My-) mutants

For the isolation of auxotrophs as well as My- mutants, we used a complete medium containing (per litre) glucose, 10 g; peptone, 5 g and beef extract, 3 g (pH 7.0), and a minimal medium containing (per litre) (NH₄)₂SO₄, 20 g; K₂HPO₄, 14 g; KH₂PO₄, 6 g; sodium citrate, 1 g; MgSO₄ · 7H₂O, 2 g and glucose, 5 g (pH 7.0). For isolation of peptide intermediates by whole-cell fermentation, a synthetic medium was used containing (per litre) glucose, 10 g; glutamic acid, 5 g; K₂HPO₄, 1 g; MgSO₄ · 7H₂O, 1 g; MnSO₄ · 4H₂O, 0.01 g; K₂HPO₄, 0.01 g (pH 7.2)

Isolation of auxotrophic mutants

The isolation was based on the method of Adelberg et al. (1965) NG (N-acetyl-4-N-turo-N nitrosoquinoline) (100 μg ml⁻¹) was added to a shaken culture of the organism in mid-exponential phase. The treated culture was incubated for 50 min in Tris/maleic buffer (0.05 M) at pH 6.0 and then transferred to complete medium for 24 h at 32 °C. Potential auxotrophs were detected by replica plating on minimal medium and characterized by the methods of Ledelger (1950) and Holliday (1956)

Isolation of mycobactin-negative (My-) mutants

Two methods were used

(1) One mutant arbitrarily selected from among the auxotrophs and designated A-1 was treated with NG as described above and then plated on complete medium. The plates containing the established colonies were then flooded with agar seeded with the mycobactin-sensitive fungus *Aspergillus oryzae* to detect non-producer strains according to the method of Banerjee & Bose (1964). Two thousand colonies were screened and My- mutants thus identified were then transferred to nutrient broth containing 250 μg mycobactin ml⁻¹ and incubated for 24 h at 30 °C the blocked mutants, now free from *A. oryzae* Br, were maintained on nutrient agar slopes

(2) This method was based on one developed by Raetz (1975) for isolating mutants blocked in membrane lipid synthesis, and further modified by Mukhopadhyay & Paulus (1977) for the isolation of gramicidin-negative mutants. Fluorescein auxotrophic mutant A-1 was treated with NG as before and then plated on complete medium. After incubation for 24 h, plates containing 50-80 colonies were illuminated under sterile disks of Whatman No. 42 filter paper using a double Neubauer chamber under a phase-contrast microscope at 400× magnification

Confirmation of the My- character of the isolated mutants

This was done by a cross-streak method against the sensitive organism *A. oryzae* CB, and also by silica gel thin-layer chromatography. For thin-layer chromatography analysis, mutants were inoculated into 100 ml minimal synthetic medium and incubated for 6 d at 27 °C without shaking for optimum synthesis (Majumder & Bose, 1960b). The culture fluid was extracted three times with chloroform/methanol (1:4, v/v) and the extract was evaporated under reduced pressure and the residue dissolved in a minimal volume of ethanol and then proceed further by the method of Majumder & Bose (1960a). The residue was finally characterized by silica gel thin-layer chromatography using 1-propanol/ammonia (2:1, v/v) at the developing solvent (Ladawan & Bose, 1982)

The My- character was finally confirmed by screening for cell-free synthesis by the method of Sethupathy et al. (1984). Cells (2 g wet wt) of wild-type and My- strains, collected from 1 litre of nutrient broth after 15-16 h incubation at 30 °C, were suspended in 4.6 ml 50 mM Tri-HCl buffer at pH 7.4 containing 2 mM EDTA, 10 mM DTT, 10 mM MgCl₂ and 1 mM PMSF (phenylmethylsulphonyl fluoride) in a glass tube. The tube was immersed in an ice salt bath and the suspension sonicated for two 30 s periods at 100 W and 20 kHz in an MSE ultrasonicator. The suspension was cooled for 3 min between bursts to maintain a
Peptide accumulation by mycobacteria-negative mutants

temperature below 5°C. The sonicate was centrifuged at 20,000 g for 30 min at 0°C and the supernatant used as enzyme source. The protein concentration of the supernatant was measured by the Lowry method with bovine serum albumin as the standard. The incubation mixture contained the following: a total volume of 2 ml 5 mM KCl, 2.5 mM -labeled 1-butyrolactone, 10 mM MgCl₂, 0.5% soybean phosphatase, 0.5 mM pyridoxal 5′-phosphate, 0.5 mM pyruvate kinase, 100 mM Tris/HCl buffer (pH 7.4), 100 mM sodium pyruvate, 2000 units of pyruvate kinase/ml, 100 mM sodium pyruvate, 100 mM Tris/HCl buffer (pH 7.4), 100 mg chloramphenicol, 8 mg enzyme protein, 4 mM-d-glutamic acid, 1 mM-L-glutamic acid, 2 mM-glycine, 1 mM-glutamine, 1 mM-L-lysine, 1 mM-arginine, 1 mM-tryptophan and 1 mM-threonine. The mixture was incubated at 30°C for 20 min and the reaction terminated by the addition of 1 vol. of butanol. Unlabeled mycobacteria (2 mg) was added as carrier and mycobacteria was isolated from the butanol extract as described by Marmur and Yob (1966a). The radioactivity was measured at the final time of incubation, after its recovery from the butanol extract without allowing it to crystallize.

Isolation of peptides from My mutants. The My mutants were incubated without shaking for 6 days at 27°C. The culture fluid was extracted three times with chloroform/1-butanol (1:4, v/v), the solvent layer dried under reduced pressure and the residue dissolved in a minimal volume of ethanol containing 10% (v/v) 10 M-HCl. After centrifugation, the centrifuged residue was then chromatographed in two dimensions in solvent systems (i) phenol/water (1:1, v/v) and (ii) 1-butanol/acetic acid/water (4:1:4, by vol) and sprayed with a solution of 0.2% ninhydrin in 70% (v/v) ethanol to test their homogeneity. Each of the spots obtained in the chromatogram was then eluted with 70% (v/v) ethanol.

Identification and determination of the molar proportion of the constituent amino acids. The ninhydrin-positive spots other than those representing residual amino acids added initially to the fermentation medium were eluted from an unsprayed chromatogram and rechromatographed in the same two solvent systems and also in 70% (v/v) ethanol, to test their homogeneity. Each of the spots obtained in the chromatogram was then eluted with 70% (v/v) ethanol.

Determination of the C- and N-terminal amino acid of the peptides. The C-terminal amino acid of each peptide isolated from the chromatogram was determined by the method of Akabori et al. (1956). Each peptide was incubated at 60°C for 2 h with two drops of 6N hydrochloric acid in a sealed tube. The tubes were opened and excess hydrochloric acid was removed in a vacuum desiccator over H₂SO₄. The dried mass was then dissolved in water and shaken overnight with the carboxylic resin Amberlite IRA 68 (H⁺ form). After removal of the supernatant (2 ml) was shaken with benzyl alcohol (0.2 ml) and pyridine (0.1 ml) overnight in a stoppered cylinder. The aqueous layer was then dried and chromatographed in two dimensions in solvent systems (i) and (ii) as above. The amino acid appearing in the chromatogram was identified and then molar proportions were determined by a ninhydrin method (Giris et al., 1952).

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results

Effect of auxotrophic mutations on mycobacilln production. Five auxotrophic mutant derivatives of wild-type Bacillus subtilis B1 were obtained. Mutants A-1 and A-3 required aspartic acid, A-4 and A-5 proline and A-1 arginine. All these mutants retained their My+ (myleobacilln-producing) and Spo+ (sporogenous) character (Table 1).

Characterization of My+ strains. Two thousand colonies were screened by method (1) (see Methods) and 12 My+ mutants were isolated (strains N-201 to N-10 listed in Table 1). Only one My+ mutant (N-1111) was isolated after screening 1000 colonies by method (2). Of the 13 My+ mutants, three were feeble mycobacilln producers and the remaining ten were non-producers as tested by thin-layer chromatography. The My+ character of these ten mutants was confirmed by testing their inability to produce 14C-labelled mycobacilln in the cell-free system. There was negligible incorporation of radioactivity into mycobacilln by these ten mutants as compared with that by the wild-type (Table 1).

All the 13 My+ mutants were Spo+. Four of them (N-20, N-14, N-4 and N-6) were originally Spo+ but they spontaneously reverted to Spo+ while still retaining their My+ character. The degree of sporulation of these My+ mutants was similar to that of the wild-type Bacillus subtilis B1 (Table 1).

Amino acid composition, C- and N-terminal amino acid residue and molecular weight of peptides accumulating in the blocked mutants. Culture supernatants from the wild-type mycobacilln producer strain Bacillus subtilis B1, three feeble antibiotic producer mutants (N-201, N-20 and N-101) and also three strictly My+ mutants (N-54, N-20 and N-6) contained no ninhydrin-positive material other than aspartic acid and glutamic acid as used initially in the fermentation broth. Culture supernatants from the remaining seven strictly My+ mutants (N-10, N-9, N-14, N-7, N-1111, N-6 and N-4) contained additional ninhydrin-positive materials. Four of them, N-10, N-4, N-14 and N-6, produced only a single peptide, designated M1, MII, MIII and MIV, respectively, whereas the remaining three produced two peptides each. M and MV by N-7, MVII and MVIII by N-1111, and MIX and MX by N-9 (Table 2). Peptides M1, MIV, MVIII and MX appeared to be identical as indicated by their Rf values and amino acid composition. In the same way peptides MII, MIII and MV appeared to be identical as did peptides MVI, MVII and MIX. Hence, the mutants produced only three chemically different peptides, two containing proline whereas the other one (MVI/MVII/MIX) did not.

Of the two proline-containing peptides, the first one (M1/MIV/MVIII/MX) contained four mycobacilln amino acids whose molar proportions were the same as that of the equivalent segment of the mycobacilln molecule (see Fig 1). The second peptide (MII/MIII/MV) contained eight amino acids of which five were...
Peptide accumulation by mycobactin negative mutants

Table 1 Characterization of auxotrophic and My~ mutants of B. subtilis B1

<table>
<thead>
<tr>
<th>Mycobactin activity</th>
<th>Radioactivity incorporated during cell-free synthesis (c.p.m.)</th>
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<tbody>
<tr>
<td>Stain</td>
<td>Radioactivity in copluted during Sporulation frequency</td>
</tr>
<tr>
<td></td>
<td>[μg (ml broth)]</td>
</tr>
<tr>
<td>B1 (wild-type)</td>
<td>152</td>
</tr>
<tr>
<td>A-1</td>
<td>150</td>
</tr>
<tr>
<td>A-3</td>
<td>152</td>
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<tr>
<td>A-5</td>
<td>150</td>
</tr>
<tr>
<td>N-201</td>
<td>46</td>
</tr>
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<td>N-101</td>
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</tr>
<tr>
<td>N-16</td>
<td>ND</td>
</tr>
<tr>
<td>N-7</td>
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<tr>
<td>N-10</td>
<td>ND</td>
</tr>
<tr>
<td>N-1111</td>
<td>ND</td>
</tr>
</tbody>
</table>

* + Antibiotic producer, -- antibiotic non-producer
† Antibiotic was assayed by the cup-plate method. ND Not detectable (detection limit 40 μg ml⁻¹)
‡ Spore counts (determined at 48 h) are expressed as a percentage of the viable count at the end of exponential growth.

mycobactin amino acids and three (glycine, valine and methionine) were not. The third non-proline peptide (MVI/MVII/MIX) contained five amino acids of which four were present in mycobactin and one (cysteine) was not.

The two proline-containing peptides contained proline as the N-terminal and aspartic acid as the C-terminal residue and the third peptide contained aspartic acid as the N-terminal and cysteine as the C-terminal residue (Table 2).

The molecular weights of peptides M1, MIV, MVIII and MX, elaborated by mutants N-10, N-16, N-1111, N-9, respectively, were found to be 640 by gel-filtration on Sephadex G-25 on the basis of migration rates relative to cytochrome c of a series of calibration peptides (see Methods).

Amino acid sequence of the mycobactin amino acid-containing peptide accumulating in the blocked mutants: The C-terminal amino acid of peptide M1/MIV/MVIII/MX was aspartic acid (Table 2). The three consecutive amino acids from the N-terminus were Pro→Asp→Glu. From the molar proportions of the constituent amino acids and the molecular weight of the peptide, the fourth amino acid from the N-terminus was tyrosine. Hence the sequence of amino acids in peptide M1/MIV/MVIII/MX was Pro→Asp→Glu→Tyr→Asp.

Isolation of peptides from the cell-free synthesizing system obtained from wild-type and blocked mutants: The cell-free synthesis of mycobactin and related incomplete peptides was studied by using enzyme systems from the wild-type and blocked mutant strains. The full complement of amino acids present in mycobactin and also in the three other peptides isolated from My~ mutants was present in the reaction mixture. The seven strictly My~ mutants (N-14, N-4, N-9, N-16, N-7, N-10 and N-1111) synthesized only one peptide, which was the same as M1/MIV/MVIII/MX (Table 2). The three other My~ mutants (N-201, N-301 and N-105) and wild-type Bacillus subtilis B1 produced mycobactin only. We also studied the other three strict My~ mutants (N-20, N-6 and N-54). Although they did not accumulate any peptide in the
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<tr>
<td>N-10</td>
<td>MI</td>
<td>0.61</td>
<td>0.69</td>
<td>Pro, Asp, Glu, Tyr</td>
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<td>Pro</td>
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<tr>
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<td>0.72</td>
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<td>0.62</td>
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<td>Cys</td>
<td>Asp</td>
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<tr>
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<td>0.62</td>
<td>Asp, Glu, Tyr, Leu, Cys</td>
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<td>Asp</td>
</tr>
<tr>
<td></td>
<td>MX</td>
<td>0.61</td>
<td>0.69</td>
<td>Pro, Asp, Glu, Tyr</td>
<td>Asp</td>
<td>Pro</td>
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Peptide accumulation by mycobactin-negative mutants

Table 3  Cell-free synthesis of mycobactin by different combinations of the fractionated enzyme complex of wild-type and My* strains

<table>
<thead>
<tr>
<th>Mutant</th>
<th>AM 1 BM</th>
<th>AW 1 BM</th>
<th>AM 1 BW</th>
<th>AW 1 BW</th>
<th>AM 1 BM</th>
<th>AW 1 BM</th>
<th>AM 1 BW</th>
<th>AW 1 BW</th>
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<td>275</td>
<td>322</td>
<td>321</td>
<td>120</td>
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<td>115</td>
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<tr>
<td>N 16</td>
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<td>107</td>
<td>1162</td>
<td>1412</td>
<td>299</td>
<td>311</td>
<td>1182</td>
<td></td>
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<tr>
<td>N-1111</td>
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<td>268</td>
<td>1704</td>
<td>1448</td>
<td>100</td>
<td>287</td>
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<tr>
<td>N 9</td>
<td>172</td>
<td>172</td>
<td>1980</td>
<td>1175</td>
<td>302</td>
<td>318</td>
<td>2995</td>
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<tr>
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<td>270</td>
<td>255</td>
<td>1312</td>
<td>1403</td>
<td>289</td>
<td>287</td>
<td>3112</td>
<td></td>
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<tr>
<td>N 6</td>
<td>101</td>
<td>282</td>
<td>1422</td>
<td>1382</td>
<td>295</td>
<td>299</td>
<td>401</td>
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<tr>
<td>N-54</td>
<td>252</td>
<td>263</td>
<td>1904</td>
<td>1471</td>
<td>282</td>
<td>332</td>
<td>1117</td>
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<tr>
<td>N 4</td>
<td>127</td>
<td>109</td>
<td>1662</td>
<td>1175</td>
<td>299</td>
<td>309</td>
<td>287</td>
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<tr>
<td>N-14</td>
<td>302</td>
<td>126</td>
<td>1111</td>
<td>247</td>
<td>301</td>
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<td>307</td>
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<tr>
<td>N 7</td>
<td>245</td>
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<td>1301</td>
<td>288</td>
<td>272</td>
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</table>

In vitro synthesis of mycobactin by reconstituted cell extracts. In a series of reconstitution experiments, cell-free extracts from wild-type and mutant bacteria were separated into fractions A, B and C as described by Ghosh et al. (1983). When these fractions from wild-type bacteria are combined, mycobactin is synthesized from its constituent amino acids (Ghosh et al., 1981). The results of these experiments in which mutant and wild-type fractions were mixed in various combinations (Table 3) indicated that the seven mutants N-10, N-16, N-1111 and N-9 have a defect in enzyme fraction B and the other three N-4, N-14 and N-7 have a defect in both fractions B and C.

DISCUSSION

In the study of the biosynthesis of mycobactin we have isolated thirteen My* mutants from an auxotrophically tagged mycobactin producer strain of *B. subtilis* B. These mutants were all Sp* with the exception of four isolates which were originally Sp* but subsequently reverted spontaneously to Sp*. The degree of sporulation of all of these strains was similar to that of the wild-type. In a study on biosynthesis using genetically blocked strains it is essential to use structural gene mutants rather than pleiotropic regulatory ones. Studies of peptide accumulation were therefore undertaken with these mutants, these led to the isolation of three chemically different peptides whose amino acid composition and N- and C-terminal residues were determined. Since our earlier studies had indicated that proline was the initiating amino acid for mycobactin biosynthesis (Sengupta & Bose, 1974), peptides lacking N-terminal proline and also those containing non-mycobactin amino acids were considered unlikely to be intermediates in mycobactin biosynthesis. There was only one pentapeptide (as determined by molecular weight) which contained not only four mycobactin amino acids but also proline at the N-terminal and aspartic acid as the C-terminal amino acid. The formation of such a peptide could be visualized if mycobactin synthesis started with proline and was blocked between aspartate and tyrosine (see Fig. 1), in which case the product should be a pentapeptide with a sequence equivalent to that of the corresponding region of the mycobactin molecule. The amino acid sequence of this peptide entirely agreed with the prediction.
Sengupta & Bose (1972) reported that the enzyme system obtained from a wild-type producer strain gave rise to a hexapeptide when incubated in a cell-free system in the presence of the four amino acids that constitute the present pentapeptide. The limitation imposed in the earlier work was not in the enzyme system but in the availability of amino acids, whereas in the present case the limitation was in the enzyme system and not in the availability of amino acids, which resulted in the formation of a pentapeptide due to a genetic lesion as indicated in Fig 1.

That the pentapeptide is an intermediate in the biosynthesis of mycobacilhn was further confirmed by studies on cell-free synthesis using the blocked mutants. Extracts from the blocked mutants produced only one peptide in the cell-free system instead of the three peptides of different chain lengths that were observed in the case of whole-cell fermentation by the blocked mutants. The amino acid composition, molecular weight and sequence of this peptide showed it to be identical to the pentapeptide referred to above. The lack of peptide products other than the pentapeptide might be due to the use of a somewhat purified enzyme system from the blocked mutants. Thus the pentapeptide Pro-Asp-Glu-Tyr-Asp which accumulated both in the whole-cell culture supernatant and during in vitro synthesis with the blocked mutants might be considered an intermediate in the biosynthesis of mycobacilhn.

Ghosh et al. (1983) successfully fractionated cell extracts to produce three fractions (A, B and C), all of which were necessary for the in vitro synthesis of mycobacilhn from its constituent amino acids. To determine which of these fractions is defective in the My" mutants, fractions from wild-type and mutant strains were mixed in various combinations. Mutants N-10 and N-16, which produced only the above pentapeptide both in culture broth and in the cell-free system, have a defect in the enzyme fraction B. Mutants N-111 and N-9, which produced the same pentapeptide and another peptide in the culture broth but only the pentapeptide in the cell-free system, also have a defect in the enzyme fraction B. Mutants N-20, N-6 and N-54, which did not produce any peptide in the culture broth but did produce the same pentapeptide as mentioned above in the cell-free system, also have a defect in the enzyme fraction B. Mutants N-4 and N-14 produced one peptide in the culture broth but produced the same pentapeptide in the cell-free system, and have defects in both enzyme fractions B and C. The remaining mutant, N-7, which produced two peptides in the culture supernatant but produced the same pentapeptide in the cell-free system, also has a defect in both enzyme fractions B and C. All these mycobacilhn-negative strains are therefore structural gene mutants.

The importance of the pentapeptide Pro-Asp-Glu-Tyr-Asp in the biosynthesis of mycobacilhn is thus indicated.
Peptide accumulation by mycobactin-negative mutants

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Translocation of mycobacillin synthetase in *Bacillus subtilis*

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(Received 16 July 1984/Accepted 12 October 1984)

The extracellular release of mycobacillin from *Bacillus subtilis* first occurred in the medium at the onset of stationary phase and continued at a high rate even after 6 days. Mycobacillin synthetase activity appeared earlier than late-exponential phase in the cytosol of producer cells and was not sedimentable even at 105000 g. The activity then quickly reached the maximum late in the stationary phase. With further increase in the age of the culture, the activity gradually disappeared from the cytosol, to reappear concomitantly in the membrane in an insoluble particulate form, even in absence of protein synthesis. The membrane-bound synthetase activity was sedimentable at 10000 g and was fairly active even after 5 days.

The biosynthesis of the antibiotic peptides gramicidin S and tyrocidine produced by different strains of *Bacillus brevis* has been elucidated by several groups (Roskoski *et al.*, 1970, Lipmann, 1980, Kurahashi, 1981, Kleinkauf & Von Dohren, 1987). It was observed that the synthesizing-enzyme system, which happened to be present in the soluble supernatant in the early stage of growth, changed from a soluble to a membrane-bound form with the age of the culture in the case of tyrocidine (Lee, 1974), gramicidin S (Vandamme & Demain, 1976, Vandamme, 1981, Nimu *et al.*, 1982) and bacitracin (Trayshov, 1977), whereas in case of polymyxin (Balakrishnan *et al.*, 1980, Vasantha *et al.*, 1980) the reverse phenomenon occurred. However, in the case of edemacin, the synthesizing-polyenzyme system has been shown to be associated with a membrane DNA complex (Kurylo-Borowska, 1975) in the post-exponential-phase cells of *Bacillus brevis Vin 4*.

Mycobacillin (Mapumoder & Bose, 1958) is an antifungal cyclic tripeptide antibiotic whose synthesizing-enzyme system has been shown to be present in the soluble supernatant of the producer *Bacillus subtilis* B3 during the early phase of growth (Sengupta & Bose, 1971). The synthesizing enzyme, mycobacillin synthetase, has been purified and appears to be a polyenzyme system that resolves itself into three fractions, which cannot carry out the synthesis unless added together (Ghosh *et al.*, 1983). We therefore decided to study the localization of the three-fraction enzyme in relation to the age of the culture.

**Materials and methods**

Chemicals and radiochemicals

Lysozyme from egg white, ATP and sucrose were obtained from Sigma Chemical Co (St Louis, MO, USA). U-14C-labelled *Chlorella* protein hydrolysate (sp radioactivity 42 mCi/mg atom of C) was purchased from Bhabha Atomic Research Centre (Bombay, India). Other chemicals used were from commercial sources.

**Strain, media and growth conditions**

*Bacillus subtilis* B3 producing mycobacillin was grown in a shaking incubator at pH 7.2 and at 30 ± 1 °C in nutrient broth as reported previously (Ghosh *et al.*, 1983).

**Measurement of growth**

The growth under agitation was monitored by measurement of A660 in a photoelectric colorimeter.

**Microbial assay**

The extracellular and intracellular mycobacillin production under agitation was assayed, by a cup-plate method, by its activity against *Aspergillus niger* G,4B (Banerjee & Bose, 1969, Ray & Bose, 1971). The concentration of the antibiotic was...
determined from the standard curve of mycobacillin

Preparation and fractionation of sonic extract

A homogenized washed cell suspension of B. subtilis in 50mM-Tris/HC1 buffer was sonicated (Mukhopadhyay et al., 1984). Portions of the sonicated extract were then separately centrifuged at 10000g for 30 min in a Sorvall RC-5B refrigerated superspeed centrifuge and also at 105000g for 90min in the ultracentrifuge below 5°C. The pellet and supernatant at each step were assayed for enzyme activity. In characterization, the 10000g pellet as obtained from 80h-old cells was suspended in buffer and centrifuged first at 10000g and then at 10000g to give a heavy and a light pellet respectively. The light pellet was further fractionated by discontinuous 20-60% (w/v)-sucrose density-gradient centrifugation and its activity assayed at each step.

Preparation of protoplasts

Protoplasts were prepared by the method of Sengupta & Bose (1971).

Assay of mycobacillin synthetase activity

The incubation mixture and the assay procedure was the same as described by Ghosh et al. (1983). The concentration of the enzyme and the period of incubation in the assay procedure were so adjusted as to maintain a linear relationship during the process. In practice, the specific activity of the soluble enzyme preparations was 400c p m /h per mg of protein, and the enzyme synthesized mycobacillin at enzyme-protein concentrations of 3mg/ml for at least 120h, whereas at higher concentrations (4 and 5mg/ml) a slight decline was observed after 90h, the pellet preparations had a specific activity of 320c p m /h per mg of protein and produced mycobacillin for at least 120h.

Protein determination

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

Results

Kinetics of cellular growth, antibiotic production and synthetase activity in subcellular fractions

The extracellular mycobacillin production started at the onset of stationary phase (Fig 1) and continued for 6 days or more, the peak value being attained at about 136h (stationary phase). The intracellular accumulation of mycobacillin occurred first at the lower limit of the assay, 40pg/ml, at late stationary phase, and continued during the observed period. Fig 1 also shows that mycobacillin synthetase activity first appeared in the 105000g supernatant at the late-exponential phase.

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![Graph](https://via.placeholder.com/150)

**Fig 1** Correlation between cell growth, antibiotic production and mycobacillin synthetase activity

The enzyme activity in the supernatant as well as in the pellet fraction was determined as described in the Materials and methods section. The age of the culture was varied as required. Incubation temperature and period were respectively 30°C and 90min. Key to symbols: A, Cell growth; Q, extracellular and Δ, intracellular, mycobacillin activity; B, 105000g-supernatant and L, 10000g-pellet mycobacillin synthetase activity; , change in pH of fermentation medium.

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Translocation of mycobacillin synthetase in *Bacillus subtilis*

of growth, increased abruptly and reached the maximum at the onset of stationary phase (16h). The activity then declined slowly to become almost zero at about 72h. The synthetase activity in the 10000g pellet appeared first at about 20h, reached the maximum at about 80h and then slowly declined to attain, at 100h, 50% of its peak activity, which thereafter remained constant all through the observed period.

**Distribution of synthetase activity under conditions of inhibition of protein synthesis**

Studies on the pattern of distribution of mycobacillin synthetase activity between the 10000g supernatant and the pellet over a short period, namely 40-46h, when the activity was present in both the pellet and the supernatant (Table 1) and under conditions where protein synthesis was completely blocked by chloramphenicol, indicate that the total synthetase activity showed a slight downward trend over this period. However, whereas the activity in the pellet fraction increased continuously, that in the supernatant decreased during this period, even in the absence of any protein synthesis.

**Localization of the 10000g particulate synthetase activity of stationary-phase cells**

Studies on the localization of the particulate enzyme obtained in the 10000g pellet of sonicated stationary-phase cells indicated that the activity was absent from the heavy pellet (30000g) but present in the light one (10000g). On further fractionation by sucrose-density-gradient centrifugation, the activity in the light pellet resolved itself into three fractions. The major synthesizing activity was found to be associated with fractions sedimented at 40% (w/v) sucrose (Fig. 2), whereas a minor one was sedimented at 20% (w/v) sucrose. The heavy precipitate sedimented at the bottom of the 60% (w/v) sucrose was devoid of any synthesizing activity.

**Characterization of particulate mycobacillin synthetase system**

Mycobacillin synthetase activity of the unfractonated 10000g pellet was decreased by 15% after lysozyme treatment (Table 2), whereas the fractions obtained from the sucrose density gradient possessed lysozyme-insensitive enzyme activity.

Table 2 further indicates that succinate dehydrogenase activity was present in the 10000g pellet and in the fractions sedimented at 40% (w/v) sucrose, but was completely absent from the fractions sedimented at 20% (w/v) sucrose.

The particulate enzyme activity of the 10000g light pellet and that of the fractions sedimented at 40% (w/v) sucrose was destroyed almost totally (75%) by treatment with sodium deoxycholate and sodium dodecyl sulphate (Table 2), and this loss of activity was linearly related to detergent concentration. The enzyme activity in the 105000g supernatant of exponential-phase cells was unaffected by the treatment (result not shown).

**Site of membrane binding of particulate mycobacillin synthetase activity**

The mycobacillin synthetase activity was determined not only in the supernatant from lysozyme-treated cells but also in the heavy membrane. The activity was absent from the heavy membrane (30000g) but present in the light membrane (10000g). On further fractionation by sucrose-density-gradient centrifugation, the activity in the light membrane resolved itself into three fractions. The major synthesizing activity was found to be associated with fractions sedimented at 40% (w/v) sucrose (Fig. 2), whereas a minor one was sedimented at 20% (w/v) sucrose. The heavy membrane-bound enzyme activity was determined separately for the 10000g pellet and supernatant as described in the text.

**Table 1** Effect of chloramphenicol on mycobacillin synthetase activity

Chloramphenicol (30μg/ml) was added to the growing culture (40h) at 30°C, which was further incubated with aeration. The cells of different age groups (200ml) were harvested and the protein concentration as well as the synthesizing activity were determined separately for the 10000g pellet and supernatant as described in the text.

<table>
<thead>
<tr>
<th>Age of the culture (h)</th>
<th>Incorporation of 14C-labelled amino acid into mycobacillin (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
</tr>
<tr>
<td>40</td>
<td>2890</td>
</tr>
<tr>
<td>42</td>
<td>2700</td>
</tr>
<tr>
<td>44</td>
<td>2545</td>
</tr>
<tr>
<td>46</td>
<td>2304</td>
</tr>
</tbody>
</table>

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Table 2  Nature of the 10000g particulate enzyme synthesizing mycobactin

<table>
<thead>
<tr>
<th>System</th>
<th>Lysozyme-sensitivity</th>
<th>Succinate dehydrogenase activity</th>
<th>Effect of 1% detergent (c.p.m.)</th>
<th>Incorporation ability (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10000g pellet</td>
<td>+</td>
<td>+</td>
<td>505</td>
<td>1657</td>
</tr>
<tr>
<td>Enzyme system at. 20% (w/v) Sucrose</td>
<td>-</td>
<td>-</td>
<td>1088</td>
<td>1068</td>
</tr>
<tr>
<td>40% (w/v) Sucrose</td>
<td>-</td>
<td>+</td>
<td>450</td>
<td>2496</td>
</tr>
</tbody>
</table>

Different fractions were treated with lysozyme (1 mg/ml) for 10 min at 30°C and centrifuged at 10000g in the cold. The supernatant from each fraction was taken for sugar detection by the anthrone reaction (Gilbert, 1957). The enzyme activity of each fraction before and after lysozyme treatment, expressed as c.p.m., was determined as described in the text. The presence of the enzyme succinate dehydrogenase in different fractions was assayed by the method of Slater & Bonnes (1952). The different fractions were treated with different concentrations of the detergents sodium deoxycholate (DOC) and sodium dodecyl sulphate (SDS) for 10 min in the cold, washed twice in buffer and enzyme activity assayed. (For brevity, only the results for 1% detergent are shown.) The control was run with denatured protein.

Discussion

In contrast with tyrothricin, which in the course of its production accumulated within cells of *Bacillus brevis* ATCC 8185, mycobactin was rapidly released to the growth medium and only a negligible concentration was found within the cells of the producer at the late stage of growth. This situation was more or less similar to that of edetane in *Bacillus brevis* Vm4.

The pattern of distribution of mycobactin synthetase activity in different subcellular fractions indicates that all the three fractions of the three-fraction mycobactin synthetase are present, firstly in a soluble form in the cytosol not sedimentable at 105000g and secondly in a particulate form sedimentable at 10000g, depending on the stages of growth of the producer cell. Interestingly, the increase in synthesizing activity in the particulate form is accompanied by a concomitant decrease in the activity of the soluble form of the enzyme complex with the age of the culture in the absence of protein (and so of mycobactin synthetase) synthesis. This might be considered as a clear case for the translocation of all the three fractions of the enzyme complex from the cytosol to the particulate fraction with the age of the culture.

Characterization of the particulate enzyme seems to indicate its presence in the membrane, it being associated with the membrane marker enzyme succinate dehydrogenase and completely free from cell-wall components (as indicated by the absence of sugar after lysozyme treatment) This membrane-bound activity persisted up to the observed period of 120 h, although at a gradually decreasing rate, in contrast with tyrothricin, whose membrane-bound synthetase enzyme activity disappeared soon after attachment.

There remains a question regarding the site of membrane binding of the particulate enzyme. To this end we sought the activity in the still-lysozyme-sensitive cells when the enzyme was present in both the soluble and the particulate form. The presence of the enzyme activity in the protoplast lysate and its absence from the intact protoplast or in the supernatant from lysozyme-treated cell suspension might be taken to mean that the mycobactin synthetase was not a periplasmic enzyme or attached to the exterior surface of the protoplast, but localized within the membrane itself. That the enzyme activity was intimately associated with the membrane was further supported by a 75% decrease in its activity after detergent treatment, which has no effect on the soluble form of the enzyme activity. However, the enzyme, like other peripheral membrane proteins, may not be bound very strongly to the membrane, being partly released during sucrose-density-gradient purification in the 20% (w/v)-sucrose fraction, which did not contain any membrane marker enzyme.

Thus mycobactin synthetase activity, which appears to be present in the cytosol of vegetative cells, becomes associated with the membrane of the producer cells in the stationary phase.
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