Influence of Metal Ions on the Formation of Mycobactin and Salicylic Acid in *Mycobacterium smegmatis* Grown in Static Culture

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Received for publication 13 May 1971

*Mycobacterium smegmatis* was grown on trace-metal-free medium in static culture. Throughout the growth phase, the concentration of mycobactin increased continuously, reaching a maximum of about 30 to 40 μg of mycobactin/mg of cell dry weight after 6 days; the concentration of salicylic acid remained approximately constant at 1 to 2 μg of salicylic acid/μg of cell dry weight. Fe²⁺ (or Fe³⁺), Zn²⁺, Mn²⁺, and Mg²⁺ were all essential to a maximum formation of mycobactin. Optimum concentrations required were: Fe²⁺, about 1.8 μM; Mn²⁺ and Zn²⁺, about 0.5 μM; and Mg²⁺, at least 0.17 mM. Higher levels of Fe²⁺ (9 to 90 μM) and Zn²⁺ (2 to 7 μM) repressed mycobactin to about half the maximum value. No other cation or anion apparently is required for mycobactin biosynthesis. Salicylic acid concentration increased about fourfold when iron was omitted from the medium, but this is not as great as the increase reported previously for this strain of *M. smegmatis*. Mycobactin formation in another strain of *M. smegmatis*, NCIB 8548, showed similar dependencies on Fe²⁺, Zn²⁺, and Mn²⁺. Maximum accumulation of mycobactin with this strain was 85 μg of mycobactin/mg of dry cell weight, under iron-deficient (1.8 μM Fe²⁺) conditions.

Mycobactins have been isolated in several modified forms from different species of *Mycobacterium* (14), and it is likely that mycobactins are produced only by mycobacteria (9, 14). The structure of mycobactin S, isolated from *M. smegmatis*, is shown in Fig. 1. The most outstanding feature of the mycobactins is their ability to form strong chelates with trivalent metal ions, particularly the ferric ion (12, 13).

Salicylic acid, which is formed from shikimic acid (5, 8) through chorismic acid (6), is found only extracellularly (11) but is subsequently taken up by the bacteria to form part of the mycobactin S molecule (10). The role of mycobactins in mycobacteria is unknown, although they do serve as specific growth factors for *M. paratuberculosis* (4). As concentrations of specific mycobactins are increased in mycobacteria grown under conditions of iron limitation (1, 17), Snow has suggested that mycobactin could be a means of gathering iron for the mycobacterial cell (14). Because there is only scant information on the factors influencing mycobactin biosynthesis (7), we examined the role of metal ions, particularly the ferrous-ferric ion, in the formation of mycobactin S.

**MATERIALS AND METHODS**

**Organisms.** The strain of *M. smegmatis* used in the main part of this work is of unknown origin and is the same strain used in all previous studies (18). *M. smegmatis* NCIB 8548 was obtained from G. A. Snow, Imperial Chemical Industries, Ltd., Alderley Park, Cheshire, England.

**Medium and removal of trace elements.** The medium contained: glycerol, 20 ml; L-asparagine, 5 g; KH₂PO₄, 5 g; and distilled water to make 1 liter, with pH adjusted to 7.5 with NaOH. The medium was autoclaved with 0.5% (w/v) alumina ("for chromatography" grade, May & Baker Ltd., Dagenham, Essex, England) at 121°C for 15 min, shaken vigorously while still hot, and allowed to cool slowly (3). The medium was filtered through Whatman no. 541 paper. After the first runs were rejected, the medium was adjusted to pH 6.8 with 2 M H₂SO₄ and dispensed in 100-ml portions into previously cleaned, but not sterile, 250-ml conical flasks. All glassware was cleaned as previously described (11). The medium finally was sterilized at 121°C for 15 min. Unless stated otherwise, MgSO₄·7H₂O, from a 10% (w/v) solution, was added to give a final concentration of 1.7 mM. Additions of other metal ions
were made from sterile solutions of the appropriate metal salts. Fe\(^{2+}\) (as FeSO\(_4\)·7H\(_2\)O), Zn\(^{2+}\) (as ZnSO\(_4\)·6H\(_2\)O), and Mn\(^{2+}\) (as MnSO\(_4\)·4H\(_2\)O) were used as “Specpure” chemicals obtained from Johnson Matthey Chemicals Ltd., London.

**Growth and harvesting of organism.** All cultures were inoculated with 0.2 ml of a suspension of 3- or 4-day-old bacteria grown on “normal” medium (2 µg of Fe\(^{2+}\)/ml, 36 µm, and 0.46 µg of Zn\(^{2+}\)/ml, 7 µm). This suspension was formed by shaking several loops of the pellicle of bacteria with glass beads (5 mm diameter) in sterile medium for about 2 min. All cultures were grown at 37°C without shaking.

**Determination of bacterial dry weight; extraction and assay of mycobactin.** Bacteria were harvested by filtration with suction through Whatman no. 1 paper. The cells and the filter paper were transferred into ethanol (usually 100 ml) for 24 hr at room temperature. The filter paper was washed with alcohol to remove bacteria and then was discarded. The bacteria were collected by filtration through a sintered-glass funnel (no. 2 porosity) and were dried to a constant weight under vacuum, over P\(_2\)O\(_5\), at 50°C. This procedure extracts all mycobactin from cells in which it exists principally in the desferri form; further extraction with ethanol (hot or cold) or chloroform-methanol (2:1, v/v) produces negligible mycobactin (unpublished data).

Mycobactin in the ethanol extract was converted to the ferric form and assayed by the method of White and Snow (16). The extinction in methanol at 450 nm was read and corrected for residual color after acidification with an equal volume of 6 M HCl. Mycobactin concentrations were calculated from \(E_{1\text{cm}}^1\text{cm}^1 = \text{42} \) (reference 18).

**Extraction and assay of salicylic acid.** A pH of 2 to 3 in the cell-free medium was attained with 2 M H\(_2\)SO\(_4\), a known amount of \(^{14}C\)-carboxyl salicylic acid (5 to 10 nCi, specific activity, about 200 nCi/µg) was added, and the medium was extracted with ether (3 × equal volume). The ether extract was dried over MgSO\(_4\), evaporated, and chromatographed on Whatman no. 1 or no. 3 MM paper using 2-propanol-water-aqueous NH\(_4\)OH, specific gravity, 0.880 (8:1:1, by volume). Salicylic acid, located by its fluorescence under ultraviolet light, was eluted from the chromatogram with ethanol. The amount of salicylic acid was estimated from its extinction at 298 nm (\(ε = 3,890\)), and the percentage of recovery of salicylic acid from the medium was found by measuring the radioactivity of the sample. This procedure afforded an accurate estimate of the level of salicylic acid in the medium. The spectrum of the salicylic acid so extracted was always determined from 160 to 200 nm; no signs of interfering materials were detected.

**Instruments.** Spectrophotometers models SP500, SP600, and SP800 (Unicam Instruments Ltd., Cambridge, England) were used. Radioactivity was measured in a liquid scintillation counter (model LS233, Beckman Instruments Ltd., Glenrothes, Fife, Scotland), with the scintillation fluid of Bray (2).

**RESULTS**

The production of mycobactin and salicylic acid during growth of *M. smegmatis* under static conditions on iron-deficient (3.6 µm Fe\(^{2+}\)) and iron-sufficient (36 µm) medium was recorded from the 3rd to 7th day after inoculation (Fig. 2 and 3). Based on these results, subsequent cultures were grown on iron-deficient medium supplemented with nonlimiting levels of Zn\(^{2+}\) (7 µm) and Mg\(^{2+}\) (1.7 mM) and were incubated for at least 6 days before mycobactin levels were deter-
an outstanding increase in the mycobactin levels. Without Mn\(^{2+}\), only 2 \mu g of mycobactin/mg of dry cell weight was formed; with Mn\(^{2+}\) added at 50, 5, or 0.5 \mu M, the concentration increased to more than 32 \mu g of mycobactin/mg of dry cell weight. Concomitantly, the cell yield after 6 days of growth was only slightly improved (400 mg of dry cell weight/100 ml without Mn\(^{2+}\) and 550 mg of dry cell weight/100 ml when Mn\(^{2+}\) was added). Mn\(^{2+}\) at 50 nm did not produce the same marked improvement in mycobactin formation as the higher Mn\(^{2+}\) concentrations. Addition of other elements mentioned above, alone or in combination, along with Mn\(^{2+}\) did not increase the effect of the addition of Mn\(^{2+}\). Salicylic acid accumulation was not significantly affected by the presence or absence of 5 \mu M Mn\(^{2+}\) at low (3.6 \mu M) or high (36 \mu M) levels of Fe\(^{2+}\).

As cultures now were obtainable which consistently produced high concentrations of mycobactin, the influence of various concentrations of Fe\(^{2+}\), Zn\(^{2+}\), and Mg\(^{2+}\) on mycobactin and salicylic acid formation were examined (Fig. 4 and 5; Table 1). No change in concentration of mycobactin or salicylic acid occurred when Fe\(^{2+}\) (as FeCl\(_2\cdot6H_2O\)) at 36 \mu M was substituted for Fe\(^{2+}\). Fe\(^{2+}\) at 90 \mu M produced no change in the values for Fe\(^{2+}\) at 36 \mu M (Fig. 4).

Although mycobactin formation was studied previously (1, 17), the dependence of its biosynthesis on trace amounts of Zn\(^{2+}\) and Mn\(^{2+}\) in the medium has not been mentioned. However, the amounts of mycobactin produced in cultures grown in earlier investigations were about the same as those produced when our organism was grown on medium supplemented with Zn\(^{2+}\) and Mn\(^{2+}\). It is probable, therefore, that there was no actual deficiency of Zn\(^{2+}\) or Mn\(^{2+}\) in the medium used previously. But to determine whether our results were attributable to some characteristic unique to the strain of *M. smegmatis* being used, we obtained a new, fresh subculture of it from F. G. Winder, Trinity College, Dublin, Ireland. *M. smegmatis* NCIB 8548, as used by White and Snow (17), was also obtained, and both of these were compared to the organism used by us in the first part of our investigation. The effects of Fe\(^{2+}\), Zn\(^{2+}\), and Mn\(^{2+}\) on growth and mycobactin formation (Table 2) showed a marked difference in mycobactin levels between *M. smegmatis* NCIB 8548 and the untyped *M. smegmatis* strain (both subcultures), but there was little difference between the two subcultures of the untyped *M. smegmatis* strain. All three strains, however, showed both the same dependence upon the presence of Zn\(^{2+}\) and Mn\(^{2+}\) and the need for iron deficiency to achieve maximum formation of mycobactin.
DISCUSSION

The accumulation of salicylic acid by *M. smegmatis* does not show the same dependence upon the concentration of iron in the growth medium as reported originally (11). Previously the accumulation of salicylic acid was increased by about 50-fold when an iron-deficient instead of an iron-sufficient medium was used, and its production progressed uniformly from the 2nd day of growth until the 4th day. These events were not observed here. In a single experiment (Fig. 4), the maximum increase in salicylic acid concentration between iron-deficient and iron-sufficient medium was about fourfold. The explanation for these different results is not clear, although the concentrations of salicylic acid in the present investigation generally were as high as those obtained previously with iron-deficient medium.

From this investigation, we concluded that mycobactin formation is dependent upon small but significant amounts of iron, zinc, and manganese. When any one of these three elements is

![Graph](http://jb.asm.org/)  

**FIG. 4.** Effect of various concentrations of Fe**2⁺** on mycobactin and salicylic acid formation after growth of *Mycobacterium smegmatis* for 6 days. Results were derived from two separate experiments, but the medium used was prepared as a single batch and was supplemented with 7 µM Zn**2⁺** and 5 µM Mn**2⁺**. Symbols: bacterial dry weight (experiment 1, ○; experiment 2, □); mycobactin (experiment 1, △; experiment 2, Δ); and salicylic acid (experiment 1 only, ■).

![Graph](http://jb.asm.org/)  

**FIG. 5.** Effect of various concentrations of Zn**2⁺** on mycobactin and salicylic acid formation after iron-deficient growth of *Mycobacterium smegmatis* for 6 days. Metal ions were removed from the medium by alumina treatment. The medium was supplemented with 0.9 µM Fe**2⁺** and 5 µM Mn**2⁺**. Symbols: bacterial yield (○), mycobactin (△), and salicylic acid (■).

**TABLE 1.** Effect of Mg**2⁺** on bacterial yield, mycobactin, and salicylic acid formation

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Supplement to medium</th>
<th>Bacterial dry wt (mg/100 ml of medium)</th>
<th>Mycobactin extracted (µg/mg of cell dry wt)</th>
<th>Salicylic acid (µg/mg of cell dry wt)</th>
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<tbody>
<tr>
<td>Fe<strong>2⁺</strong> (µM)</td>
<td>Mg<strong>2⁺</strong> (mM)</td>
<td></td>
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<td></td>
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<tr>
<td>1</td>
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<tr>
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<td>0.9</td>
<td>0.0017</td>
<td>29</td>
<td>2.0</td>
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</tbody>
</table>

* Medium was autoclaved with alumina (0.5%, w/v) and supplemented with 7 µM Zn**2⁺** and 5 µM Mn**2⁺**. Cultures were harvested after 6 days of growth.

* Amount insufficient to assay with accuracy.
on magnesium is also evident from this investigation. When the concentration of magnesium was decreased, mycobactin concentration declined to a minimal concentration of about 1.5 μg/mg of cell dry weight. However, magnesium is a major microbial nutrient, and gram-positive bacilli depend upon an adequate extracellular concentration of it for their structural integrity (15). Therefore, one should not assume any direct connection between this metal ion and mycobactin formation until more evidence is available. Salicylic acid concentration, on the other hand, was stimulated by simultaneous magnesium and iron deprivation but was suppressed by magnesium deprivation when iron was in sufficient supply. Thus, under these rather special circumstances, salicylic acid concentration was considerably higher in iron-deficient medium than in iron-sufficient medium.

ACKNOWLEDGMENT
This investigation was supported by a grant from the Wellcome Trust.

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