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USE OF *ARTHROBACTER TERREGENS* FOR BIOASSAY OF MYCOBACTIN

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ABSTRACT

REICH, CLAUDE V. (Johns Hopkins-Leonard Wood Memorial Leprosy Research Laboratory, Johns Hopkins University, Baltimore, Md.), AND JOHN H. HANKS. Use of *Arthrobacter terregens* for bioassay of mycobactin. J. Bacteriol. **87**:1317-1320. 1964.—*Arthrobacter terregens* was used to assay mycobactin, a growth factor for *Mycobacterium paratuberculosis*. Within 7 days, *A. terregens* gave a linear photometric growth response to mycobactin in the range of 0.05 to 0.2 μg/ml. Preparations found to be active (or inactive) by this assay produced corresponding effects on the growth of *M. paratuberculosis* after 6 weeks to 4 months. Mycobactin was produced routinely from pellicles of *M. phlei* on a peptone-glycerol-beef heart infusion medium, and was extracted from both cells and medium by organic solvents. The mycobactin content per cell rose rapidly after the third day and attained a maximum at 4 to 6 days. The decline to less than one-half this value by the tenth day was associated with excretion into the medium. Production on synthetic media occurred after increasing the usual levels of asparagine. The demonstrated effects of crude mycobactin on the donor strain were (i) to catalyze the onset of growth and (ii) to reverse the effect of conditions which cause the formation of abnormal cells.

The first known demonstration of microbial requirements for special growth factors was the discovery by Twort and Ingram (1912) that *Mycobacterium paratuberculosis* can be induced to grow by the addition of killed cells, hot glycerol extracts, or solvent extracts of *M. phlei* and other growth-competent species of mycobacteria.

Purification and characterization of the active principle, mycobactin, by Francis et al. (1953) permitted Snow (1954) to suggest a chemical structure with a molecular weight of 870. This compound has two interesting properties: (i) solubility in lipid rather than aqueous solvents and (ii) marked chelation of heavy metals due to a phenolic hydroxyl and two secondary hydroxamic acid groups. Purified mycobactin can be assayed spectrophotometrically. However, it is not economically feasible or necessary to use the pure compound for routine cultivation of *M. paratuberculosis*.

Although the growth response of *M. paratuberculosis* has been used to monitor the production and purification of mycobactin (Francis et al., 1953), this method is not practical because of slow growth and cumbersome measurements. Since analogous requirements for microbially synthesized chelators of heavy metals exist in certain soil microbes (Nielands, 1957), it was of interest to see whether any of these could be employed for the assay of mycobactin. *Arthrobacter terregens* (Lochhead and Burton, 1953) was chosen for study because of its relatively smooth and rapid growth. A note on early phases of this study was presented previously (Reich and Hanks, 1961).

MATERIALS AND METHODS

Bioassay. *A. terregens*, obtained from A. G. Lochhead, was grown in Terregens Assay Medium (TAM) containing (per liter): Casamino Acids, Technical, 2.0 g; yeast extract (Difco), 1.0 g; glucose, 1.0 g; ammonium hydrogen citrate, 1.0 g; K_2HPO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and $FeCl_3 \cdot 6H_2O$, 10 mg. For maintenance on agar slants and crop production in liquid media, 0.1 μg/ml of Terregens Factor (TF; Burton, Sowden, and Lochhead, 1954) was added. The medium was dispensed as desired and autoclaved at 15 psi for 15 min.

For the bioassay, this medium minus TF was dispensed at 5 ml per 16-mm screw-cap tube, autoclaved, and then stored at 4 C until required. In initial titrations of mycobactin, 0.1 ml of each decimal dilution of mycobacterial extracts was

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added to six tubes and autoclaved. As much as 0.2 ml of 40% glycerol per 5 ml (1.6%) has no influence on assays or controls. After inoculation with 0.05 ml of *A. terregens*, the screw caps were loosened slightly, and the cultures were rotated 1 rev/min at 25 C. Inoculated controls consisted of two tubes containing 0.1 μg of TF per ml and two tubes without this factor. Two uninoculated tubes were incubated as controls for 100% light transmission.

To seed the bioassay, 5-day cultures of *A. terregens* were washed twice in 0.85% saline and resuspended to 50% light transmission at 500 mμ in a Coleman Junior spectrophotometer. As noted, 0.05 ml was used per 5 ml of assay system.

Photometric readings were made at 3 and 7 days, directly in the culture tubes, after 5 sec of agitation of each tube with a Clay-Adams Cyclo-Mixer. The 3-day readings were to detect contamination. At 7 days, the mean value from each set of six tubes was referred to the "standard curve" for mycobactin assay (Fig. 1). Only those values which fell within the range of 20 to 80% light transmission were used for final quantitation. If none of the values for the decimal dilutions satisfied this requirement, a finer series of dilutions was evaluated.

The standard curve was defined by use of purified mycobactin from G. A. Snow of Imperial Chemical Industries, England. The molar extinction coefficients at 250 mμ (ϵ = 14,000) and at 311 mμ (ϵ = 3,700) in methanol (Francis et al., 1953) were used to determine the concentration of purified mycobactin.

Preparation of crude mycobactin in 40% glycerol. M. phlei strain 72, obtained from H. W. Smith (1953), was grown at 37 C as pellicles in Roux bottles, on beef heart infusion medium supplemented with 2% Myosate (BBL), 2% Phytone (BBL), and 10% (w/v) glycerol. After chosen intervals, the cells were separated from the medium by filtration and washed on the filter with distilled water. The medium also was retained for extraction of mycobactin.

The cells were extracted at room temperature for 3 days in ether-ethanol (50:50) on a magnetic mixer, by use of 100 ml of solvent for each Roux bottle. The solvent and the resultant extracts were dried at 40 C in vacuo. These residues were refluxed over steam for 1 hr with 100 ml of methanol, cooled in an ice bath, and then filtered through Whatman no. 1 filter paper. This filtrate was dried in an evaporating dish over steam. Glycerol (12 ml) was added, the mixture was stirred for 20 min, and then 16 ml of distilled water were added. The mixture was then filtered hot.

The medium from each flask was placed in a 500-ml separatory funnel along with an equal volume of chloroform. Each was allowed to stand for 3 days with occasional shaking. After the chloroform extracts had been dried in vacuo, the residues were treated with methanol and suspended in glycerol in the manner described for cells.

Growth of M. paratuberculosis. The mycobactin-dependent *M. paratuberculosis* strain 68 was provided by H. W. Smith (1953). It was grown on a medium (no. 8a) containing (per liter): Trypticase, 3.0 g; L-asparagine, 1.0 g; yeast extract (Difco), 1.0 g; diethyl glutamic ester, 2.0 g; ammonium hydrogen citrate, 1.0 g; K_2HPO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeCl_3 \cdot 6H_2O$, 0.005 g; agar, 15 g; and glycerol, 25 ml. For agar slants, 10 ml of the medium were dispensed in 20-mm screw-cap tubes and autoclaved at 15 psi for 15 min. For routine cultures, this medium was supplemented with enough crude mycobactin extract to give a total of 1 μg of mycobactin per ml. The 40% glycerol contained in this extract was deducted from the normal glycerol addition.

Test inocula were prepared by rubbing and suspending the cells from 6- to 12-week-old cultures in 0.05% Tween 80, and by adjusting to a density of 50% light transmission. Cultures were inoculated with 0.05 ml and incubated upright at 37 C. Results were considered to be negative if no growth occurred within 4 months.

Results

Assay of mycobactin by A. terregens. There was a direct relationship between concentrations of purified mycobactin in the range of 0.05 to 0.2 μg/ml and the growth of *A. terregens* (Fig. 1). In suitable dilutions, nearly all crude extracts from *M. phlei* and from the medium gave similar results.

Whereas purified mycobactin at 0.25 and 0.30 μg/ml caused modest, nonlinear increments of growth, the crude mycobactins sometimes caused inhibition at 0.3 μg/ml. On several occasions, crude mycobactins failed to stimulate growth during the first few days, but began to exceed the controls (−TF) at 7 days and caused normal rates and extent of growth by 12 or 14 days. Such

results were attributed to metabolizable impurities in crude preparations of mycobactin.

The validity of the bioassay rests upon two types of evidence.

First, *M. paratuberculosis* did not grow in the presence of mycobacterial extracts which yielded a negative assay with *A. terregens*. Such extracts were derived from cells of: (i) *M. phlei* grown on synthetic medium with a low asparagine content, (ii) *M. smegmatis* grown on beef heart infusion, (iii) three rapidly growing acid-fast isolates recovered in the course of these experiments as contaminants, or perhaps mutants, of our *M. paratuberculosis* cultures, (iv) the mycobactin-requiring *M. paratuberculosis* strain 68, or (v) two slow-growing "mycobactin-independent" strains (Teps and III–V) of *M. paratuberculosis*.

Second, extracts of the attenuated tubercle bacillus, strain R_1R_v, grown on Sauton medium and of *M. phlei* grown on synthetic media with a high asparagine content supplied the factor requirement of both test organisms. Furthermore, the growth response of *M. paratuberculosis* was roughly proportional to the mycobactin concentrations as determined by the *A. terregens* assay.

Production of mycobactin by M. phlei. Figure 2 shows the activities of crude mycobactin extractable from cells and from the medium during 14 days of growth. The mycobactin content of cells incubated for 15, 20, 25, and 30 days remained relatively constant.

The peak for cell-bound mycobactin at 4 and 6 days occurred much earlier than was expected

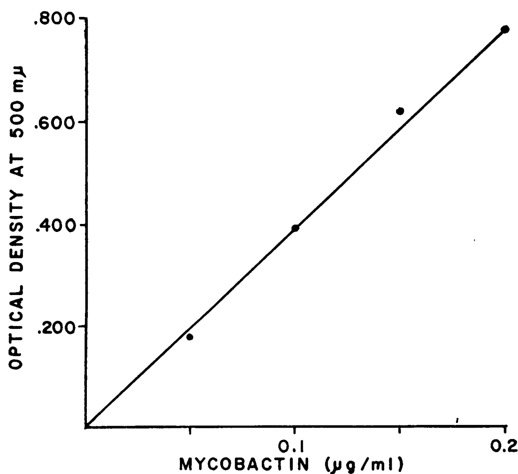

FIG. 1. *Standard curve. Growth response of Arthrobacter terregens. Purified mycobactin, 25 C, 7 days.*

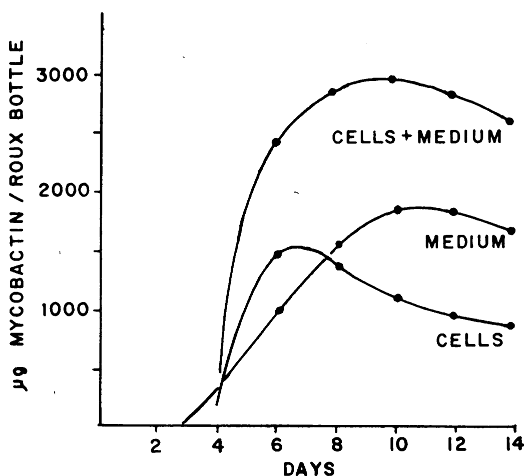

FIG. 2. *Rates of mycobactin production and liberation. Mycobacterium phlei strain 72, beef heart infusion medium, 37 C. Each point on the curves represents the mean of analyses of 2 cultures from a series of 24 identical cultures.*

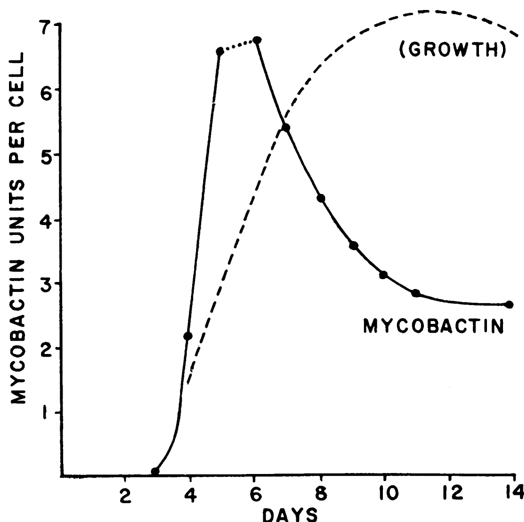

FIG. 3. *Relationship between mycobactin production and growth. Conditions as in Fig. 2.*

from the usual practice of harvesting cells after 10 to 14 days. Calculations of the mycobactin per cell emphasize this point (see Fig. 3). The growth curve in Fig. 3 shows the stages of cell growth at the time of mycobactin extractions.

Role of mycobactin in the growth of M. phlei. Since the onset of rapid growth coincided with the production of demonstrable mycobactin, the direct effect of mycobactin on the "donor" strain was studied. The addition to beef heart infusion and to synthetic media of mycobactin at 1.0

μg/ml hastened the onset of rapid growth by approximately 24 hr but did not influence subsequent rates of growth or total yield of cells to a significant degree.

The crude preparations of mycobactin induced reversals of the growth-inhibitory effects of 20% serum or serum plus glycine (0.1 M) at pH values above 7.3. Details of these experiments are being prepared for publication.

Discussion

When we described our plans to G. A. Snow in May, 1960, he informed us that T. Emory of J. B. Nieland's laboratory had observed that purified mycobactin meets the growth requirements of *A. terregens*. Burnham and Nielands (1961) reported that mycobactin supplies the factor requirements of *Arthrobacter* JG-9. *M. phlei* cells (strain not specified) contained bound hydroxylamine but did not produce a "ferrichrome type" stimulation of JG-9 in their method of screening.

The validity of the *A. terregens* assay can be judged only by making clear distinction between (i) the nonspecificity of the molecular requirements of *A. terregens* and (ii) the significance of the test as applied to mycobacterial derivatives.

A. terregens does not have a specific growth requirement for mycobactin, since it shows similar responses to TF, ferrichromes, coprogen, aspergillic acid, soil extracts, and hemin (Burton, 1957; Lochhead, 1958). We have also found a moderate response to some undefined factor in Trypticase (BBL). These compounds, however, do not meet the growth requirements of *M. paratuberculosis*. This means that *A. terregens* alone cannot be trusted in a search for mycobactin-type factors derived from miscellaneous microbes or other natural materials.

Significance, on the other hand, resides in the fact that the "donor" species of mycobacteria appear to make only mycobactin and not the more water-soluble chelators of heavy metals.

The relationship between the onset of rapid growth and of mycobactin production by *M. phlei* emphasizes that a major role of mycobactin in the economy of this organism is catalytic. Other experiments have shown that *M. phlei* can be converted to mycobactin-requiring states. It, therefore, seems evident that, with further investigation, the "donor" strain itself could be used as an alternative system for the assay of mycobactin.

From a practical point of view, discovery of the liberation of mycobactin from cells to medium indicates that, although 11- to 14-day cells have commonly been extracted (Francis et al., 1953), it is far better to recover mycobactin from both cells and medium as soon as maximal growth is attained.

Acknowledgments

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