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# Circumvention of the Mycobactin Requirement of *Mycobacterium paratuberculosis*

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ABSTRACT

MORRISON, NORMAN E. (Johns Hopkins University-Leonard Wood Memorial Leprosy Research Laboratory, Baltimore, Md.). Circumvention of the mycobactin requirement of *Mycobacterium paratuberculosis*. J. Bacteriol. **89**:762–767. 1965.—The mycobactin growth requirement of *Mycobacterium paratuberculosis* was circumvented on glucose-containing synthetic medium with an initial *p*H of 5.5. Mycobactin was required during the first transfer on the synthetic medium. Subsequent transfers have grown in the absence of mycobactin. The growth of mycobactin-"independent" strains of *M. paratuberculosis* on the synthetic medium was found to be stimulated by low concentrations of mycobactin. The circumvention of the mycobactin requirement appears to depend upon the properties of the medium and not upon having created conditions which promote endogenous mycobactin synthesis. Investigation of the glucose-containing synthetic medium showed that: (i) growth stimulatory compounds were formed during autoclaving, and (ii) compared with neutrality a *p*H of 5.5 gave markedly increased pellicle yields. It was suggested that the growth-stimulatory compounds formed during autoclaving may in part be responsible for the circumvention of the mycobactin requirement.

The natural survival of a number of soil microbes is dependent upon requirements for microbially synthesized metal-chelating growth factors termed sideramines (Zähner et al., 1962). The seemingly fastidious *Mycobacterium paratuberculosis*, which causes chronic enteritis in cattle and certain other ruminants (Hole, 1958), is a unique example of a pathogenic organism which resembles many soil microbes in respect to such requirements. A growth factor from growth-competent mycobacteria is required for its in vitro isolation and cultivation (Twort and Ingram, 1913). The growth factor, purified by Francis et al. (1953), was named mycobactin. Since mycobactins from *M. phlei* and *M. tuberculosis* have small chemical differences (Snow, 1954*a*, *b*, 1961), each mycobacterial species may produce a characteristic mycobactin type of growth factor. The *M. phlei* mycobactin contains two secondary (i.e., nitrogen-substituted) hydroxamic acid groups and forms chelates with heavy metals, such as iron or copper, which are soluble in lipid solvents. Because mycobactin occurs in the avirulent tubercle bacillus as an iron complex (Snow, 1961), the mycobactins can be tentatively placed among the iron-containing sideramines, which possess nitrogen-substituted hydroxamic acid groups as the iron-binding sites (Zähner et al., 1962). The sideramines of fungal origin generally contain three nitrogen-substituted hydroxamic acid groups per molecule (Prelog, 1963).

The mycobactin-requiring *M. paratuberculosis* differs from the heterotrophic sideramine-requiring organisms such as *Arthrobacter terregens* or *Microbacterium lacticum* (Zähner et al., 1962) in that the mycobactin requirement cannot be replaced by the following sideramines: terregens factor (Reich, *personal communication*), ferrichrome (Snow, *personal communication*), or coprogen. These observations suggest that *M. paratuberculosis* be designated a homotrophic sideramine requirer. Current studies with this fastidious pathogen seem to provide the first example of defined conditions under which a strict sideramine requirement had been circumvented.

The existence of slow-growing mycobactin-"independent" strains of *M. paratuberculosis* suggested that an investigation of the nutritional conditions whereby "independence" had been established might result in a better understanding of the conditions or factors which circumvent the mycobactin requirement. As is emphasized in the work of Wheeler and Hanks (1965), there is reason to believe that mycobactin circumvention occurs naturally during the existence of *M. para-*

*tuberculosis* within the infected cells of the animal host.

The present paper describes the circumvention of the mycobactin requirement for pellicle growth of *M. paratuberculosis* on synthetic medium at low *p*H, and demonstrates that "independent" growth has not resulted in any change in the growth response to mycobactin.

## MATERIALS AND METHODS

The designations and origins of the strains used in this series of studies were as follows. *M. paratuberculosis* strains used were the mycobactin-dependent strain, 68, from H. W. Smith, The Animal Health Trust, Stock, England, and two mycobactin-"independent" strains, Teps and III-V, from I. W. Lesslie, The Central Veterinary Laboratory, Weybridge, England. Strains of the Scandinavian wood pigeon bacillus were two mycobactin-dependent strains, WP-8 and WP-9, from H. E. Ottosen, State Serum Institute, Copenhagen, Denmark.

The five strains were isolated originally on media containing heat-killed mycobacteria as the source of mycobactin. All are stimulated by mycobactin under the conditions investigated in this laboratory. Stock cultures of strains 68, WP-8, and WP-9 were maintained at 37 C on slants of Trypticase-glycerol medium containing 1 μg/ml of mycobactin (Reich and Hanks, 1964). Stock ultures of strains Teps and III-V were maintained on modified Watson-Reid medium (Watson, 1935), solidified with 1.5% Agarose (Marine Colloids, Inc., Rockland, Me.).

Growth experiments were carried out by triplicate determinations of dry weights of pellicles during incubation at 37 C on 50 ml of modified Watson-Reid medium which contained (per liter): L-asparagine·$H_2O$, 5.0 g; D-glucose, 10.0 g; glycerol, 63.0 g; ammonium hydrogen citrate, 2.0 g; ferric ammonium citrate (green granular), 0.075 g; $KH_2PO_4$, 2.0 g; NaCl, 2.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $ZnSO_4 \cdot 7H_2O$, 0.01 g; $CaCl_2 \cdot 2H_2O$, 0.02 g; $CoCl_2 \cdot 6H_2O$, 0.002 g; the *p*H was adjusted to 5.5 with 1 N $NH_4OH$. This medium was autoclaved at 121 C for 15 min or sterilized by filtration through a 5-cm (diameter) Polypore membrane filter, type AM-6 (Gelman Instrument Co., Ann Arbor, Mich.).

Purified metal-free mycobactin was kindly provided by G. A. Snow, Imperial Chemical Industries Ltd., Macclesfield, England. Small quantities (0.5 to 2.0 mg/ml) were dissolved in ethyl alcohol prior to medium addition. Ethyl alcohol was also added to nonmycobactin controls. Mycobactin concentration was confirmed in a Beckman DU spectrophotometer at 311 mμ, $\epsilon$ = 3,700 (Francis et al., 1953).

## RESULTS

*Circumvention of mycobactin dependency.* Investigation of the nutritional conditions whereby mycobactin-dependent strains acquire "independence" demonstrated the ease with which a seemingly fastidious pathogen can adapt to grow on an appropriately prepared synthetic medium.

During the first transfer of *M. paratuberculosis* strain 68 from the mycobactin-containing Trypticase-glycerol medium (Reich and Hanks, 1964) onto Watson-Reid medium at *p*H 5.5, a mycobactin requirement was observed. However, 18 subsequent transfers have grown in the absence of added mycobactin. This result is analogous to that reported by Watson (1935), and indicates that mycobactin is necessary for *M. paratuberculosis* to adapt to the Watson-Reid medium.

*Effect of added mycobactin.* The evidence suggests that mycobactin "independence" results from a special property of the Watson-Reid medium rather than a loss of mycobactin dependence by *M. paratuberculosis*. The Watson-Reid medium at an initial *p*H of 5.5 supports the slow growth of the mycobactin-"independent" strains of *M. paratuberculosis* (within 6 to 12 weeks) and results in the formation of thick, crinkled pellicles.

Purified mycobactin, when added to the Watson-Reid medium at 1 μg/ml, stimulated the onset of growth and produced a 45 to 65% increase in pellicle yields from strain Teps or strain III-V after 6 weeks. Figure 1 shows that mycobactin (1 μg/ml) caused maximal stimulation of strain Teps during the 8th week of growth but that eventually the same approximate yield of pellicle occurred in the absence of mycobactin. If a plot is made of the logarithm of the weight increase due to added mycobactin versus time in weeks, a linear relationship is found. Thus, mycobactin produced an earlier onset of growth and accelerated growth during the first half of the relatively rapid phase of growth. An almost identical effect has been reported for ferrichrome

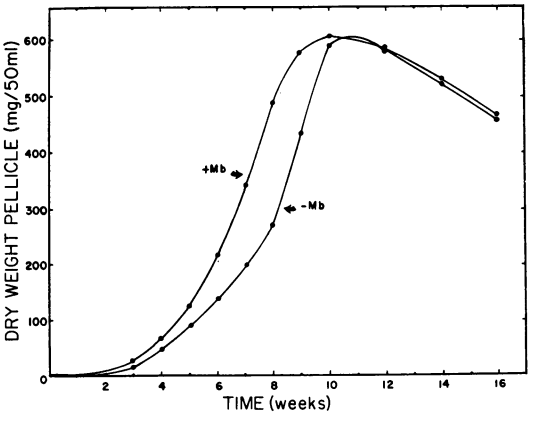


FIG. 1. *Stimulation of the growth of Mycobacterium paratuberculosis strain Teps by 1 μg/ml of mycobactin (Mb).*

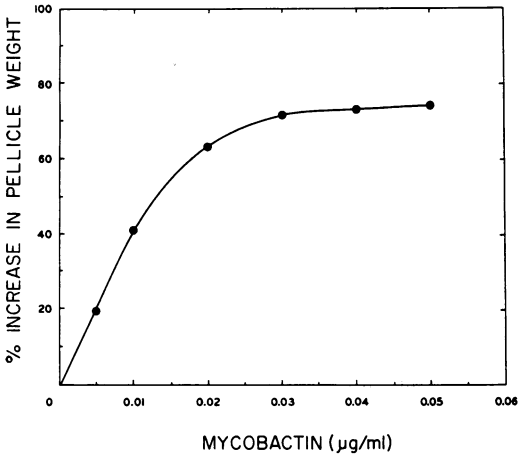


FIG. 2. *Effect of mycobactin concentration on the growth of Mycobacterium paratuberculosis strain Teps. Growth period: 8 weeks. Each point represents the per cent increase in the average of triplicate determinations.*

TABLE 1. *Growth of "independent" strains of Mycobacterium paratuberculosis on autoclaved or filtered medium*

| Treatment of Watson-Reid medium* | Dry wt of pellicles (6 weeks) | |
|---|---|---|
| | Strain Teps | Strain III-V |
| | *mg* | *mg* |
| Autoclaved | 342 | 340 |
| Autoclaved and filtered | 338 | 362 |
| Filtered | 247 | 221 |
| Filtered and autoclaved | 363 | 366 |

* Medium autoclaved at 121 C for 15 min.

TABLE 2. *Effect of time of autoclaving on growth of "independent" strains of Mycobacterium paratuberculosis*

| Autoclaving time* | Dry wt of pellicles (6 weeks) | |
|---|---|---|
| | Strain Teps | Strain III-V |
| *min* | *mg* | *mg* |
| 0 (filtered) | 267 | 248 |
| 5 | 305 | 296 |
| 10 | 348 | 396 |
| 15 | 354 | 400 |
| 20 | 346 | 402 |
| 30 | 316 | 395 |
| 40 | 302 | 396 |

* Watson-Reid medium autoclaved at 121 C.

in stimulating the growth of a species of *Microbacterium* (Demain and Hendlin, 1959).

The effect of adding mycobactin at concentrations from 0.005 to 0.05 μg/ml is shown in Fig. 2. After 8 weeks of growth, maximal stimulation of strain Teps occurred at 0.03 μg/ml. These results are consistent with those of Snow (1961), who reported that mycobactin at 0.03 μg/ml provided maximal growth rates for a mycobactin-dependent strain of *M. paratuberculosis*. The similarity of the mycobactin concentrations required for maximal effects on an "independent" and a dependent strain may be of particular significance in the question of mycobactin function and circumvention.

The possibility was considered that strain Teps and strain III-V endogenously synthesize a mycobactin-type of growth factor when growing "independently" on Watson-Reid medium. Solvent extracts from cells of both strains, prepared according to Reich and Hanks (1964), did not stimulate the growth of *A. terregens*, which is used for the bioassay of mycobactin (Antoine, Morrison, and Hanks, 1964). Further investigation has shown that, when strain Teps, III-V, or 68 is grown on Watson-Reid medium containing no added iron, it was not possible to detect chemically in cells significant levels of nitrogen-substituted hydroxamic acid groups (Csaky, 1948) which are characteristic for the mycobactin-type of growth factor. Under such conditions of low-iron growth, *M. phlei*, *M. smegmatis*, or *M. tuberculosis* strain R1Rv produce high levels of nitrogen-substituted hydroxamic acid groups.

*Effect of autoclaving the medium.* Factors which contribute to the special properties of the Watson-Reid medium have been demonstrated in the following experiments.

Autoclaved Watson-Reid medium was shown to produce more rapid growth than filtered medium. Comparisons demonstrated that autoclaving improved the yields of strain Teps by 39% and of strain III-V by 54% during the first 6 weeks of growth (Table 1). Control experiments involving autoclaving and filtering similar quantities of medium demonstrated that the presence of growth inhibitors originating from the membrane filter or adsorption of essential trace metals by the membrane filter cannot explain the results observed.

The effects of varying the time of autoclaving are shown in Table 2. Both strains gave maximal weight yields on media autoclaved for 15 min, but the growth of strain Teps (but not strain III-V) was decreased when the time of autoclaving was increased to 40 min.

The value of the simultaneous presence of glucose plus glycerol is shown in Table 3. Even at *p*H 5.5, satisfactory growth of the three strains occurred only when both carbon sources were present. D-Fructose or D-sorbitol cannot replace glucose in support of growth.

When Watson-Reid medium was autoclaved at 121 C for 15 min, a moderate yellow coloration indicated an alteration in some of the medium components. Spectrophotometrically, it was determined that autoclaving caused the formation of a compound(s) with an absorption maximum at 290 mμ and strong end absorption below 250 mμ. When glucose (10 g per liter) and potassium dihydrogen phosphate (2 g per liter) were autoclaved at *p*H 5.5, the compound(s) formed exhibited an absorption maximum at 290 mμ with a second stronger peak at 230 mμ. The absorption peak at 290 mμ decreased slowly during storage at room temperature.

The inclusion of glycerol (63 g per liter) with glucose and phosphate stabilized, after a period of 2 days, the compound(s) formed on autoclaving against loss of absorption at 290 mμ. It was also found that there is a slow formation of the 290 mμ-absorbing compound(s) during the incubation of filtered medium at 37 C.

Growth experiments have demonstrated that separate autoclaving of a mixture containing glucose, glycerol, and phosphate provides greater growth stimulation than is obtained with filtered media. However, the results were not equivalent to those obtained by simultaneously autoclaving all the components of the Watson-Reid medium. The evidence available at present shows that both glucose and glycerol autoclaved in the complete medium are required for satisfactory growth of *M. paratuberculosis*. The spectral changes induced by autoclaving require as a minimum the combination of glucose plus phosphate, and the compound(s) formed from these two components appears to be stabilized by the inclusion of glycerol.

*Growth in relation to pH of the medium.* A *p*H of 5.5 is unconventional for mycobacterial media. However, Watson (1935) reported successful growth of *M. paratuberculosis* at *p*H 5.6 to 5.8. Comparisons of growth at *p*H 5.5 and 7.0 (Table 4) showed that, although Watson-Reid medium may be autoclaved at *p*H 7.0, the *p*H must be adjusted to 5.5 prior to inoculation. The importance of low *p*H for the initiation of growth was confirmed by Wheeler and Hanks (1965), who, by use of declumped, diluted suspensions, showed that *p*H 4.5 is equivalent to *p*H 5.5 for the growth of strain 68. Hanks and Wheeler (*unpublished data*) further demonstrated with strains of *M. paratuberculosis* and the wood pigeon bacillus that *p*H 5.0 is equivalent to *p*H 5.5, which in turn is superior to *p*H 6.5 or 7.5 for growth.

TABLE 3. *Effect of glucose and glycerol on the growth of "independent" strains of Mycobacterium paratuberculosis*

| Addition to basal medium* | Dry wt of pellicles (8 weeks) | | |
|---|---|---|---|
| | Strain Teps | Strain III-V | Strain 68 |
| | *mg* | *mg* | *mg* |
| None | 4 | 5 | 4 |
| Glucose | 15 | 13 | 12 |
| Glycerol | 43 | 42 | 23 |
| Glucose, glycerol | 528 | 516 | 88 |

* The basal medium consisted of Watson-Reid medium lacking glucose and glycerol. Additions included: glucose, 2.0 g/50 ml; glycerol, 4.0 g/50 ml; and combined glucose and glycerol in amounts present in Watson-Reid medium. All carbon sources were autoclaved in medium.

TABLE 4. *Effects of pH on the autoclaving of medium and on the growth of "independent" strains of Mycobacterium paratuberculosis*

| Medium *p*H* | Dry wt of pellicles (6 weeks) | |
|---|---|---|
| | Strain Teps | Strain III-V |
| | *mg* | *mg* |
| Autoclaved at 5.5 | 309 | 386 |
| Autoclaved at 7.0 | 121 | 168 |
| Autoclaved at 7.0, then adjusted to 5.5 | 287 | 364 |
| Autoclaved at 5.5, then adjusted to 7.0 | 103 | 108 |

* Watson-Reid medium autoclaved at 121 C for 15 min.

## DISCUSSION

Special interest is attached to the findings that (i) mycobactin "independence" of *M. paratuberculosis* on autoclaved media has not eliminated a requirement for low *p*H or eliminated mycobactin stimulations even after propagation under these conditions for prolonged periods of time, and (ii) the circumvention of the mycobactin requirement is determined to an important degree by the properties of the medium. The second finding was further supported by the fact that no evidence was obtained for the endogenous synthesis of mycobactin by "independent" strains. Furthermore, Hanks and Wheeler (*unpublished data*) have made the unusual observation that a mycobactin dependence for growth by "independent" strains can be found on complex media at neutral *p*H. Thus, the evidence suggests that mycobactin requirements are determined largely through the nature of the nutritional background under which the organism is grown. Another noteworthy feature of mycobactin activity is that the growth

factor effect is not solely restricted to *M. paratuberculosis*. *M. tuberculosis* (Marks, 1954), *M. smegmatis* (Zygmunt, 1963), *M. phlei* (Reich and Hanks, 1964), and the heterotrophic sideramine requirer, *A. terregens*, all show growth stimulation with low concentrations of mycobactin.

The properties of the Watson-Reid medium, namely, the importance of low *p*H and the effects arising from autoclaving, require further comment, for it is possible that the compound(s) formed during autoclaving may in part be responsible for the circumvention of the mycobactin requirement. With regard to growth stimulation by breakdown products of glucose, the fastidious *M. paratuberculosis* is analogous to organisms belonging to species of lactobacilli (Snell, Kitay, and Hoff-Jorgensen, 1948; Rogers, King, and Cheldelin, 1953; Ramsey and Lankford, 1956), streptococci (Smiley, Niven, and Sherman, 1943; Rabinowitz and Snell, 1947), propionibacteria (Field and Lichstein, 1958), and the genus *Bacillus* (Sergeant, Lankford, and Traxler, 1957; Lankford, Kustoff, and Sergeant, 1957). As indicated, it is indeed possible that the very slow growth of *M. paratuberculosis* on filtered media at *p*H 5.5 can be accomplished only because the essential growth-stimulating factor(s) is formed very slowly during incubations at 37 C. Speculation regarding the function of the autoclave factor(s) for *M. paratuberculosis* must await chemical definition of the factor(s) itself. As shown by Demain and Hendlin (1959) with a species of *Microbacterium* which is less strict than *M. paratuberculosis* in its requirements for sideramines, the need for an "iron-transport factor" has been circumvented by glucosylglycine.

Implications arising from this work, particularly with respect to the importance of low *p*H and the properties of the Watson-Reid medium, have immediate application to the problem of cultivating mycobacterial pathogens such as *M. lepraemurium* and *M. leprae*. One such successful application by Chatterjee (1964) has resulted in a limited multiplication of L forms of *M. leprae* isolated from clinical biopsies.

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