
MYCOBACTERIOLOGY

Mycobacterium paratuberculosis Factors That Influence Mycobactin Dependence

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Mycobacterium paratuberculosis does not produce any detectable mycobactin, an iron-binding compound that is synthesized by most *Mycobacterium* spp. and necessary for the growth of all mycobacteria. This study examined the influence of various culture conditions on mycobactin dependence in *M. paratuberculosis*. Using a radiometric growth assay, we found the minimal concentration of mycobactin necessary for growth of *M. paratuberculosis* to be 0.006 μM , whereas 1.2 μM (1 $\mu\text{g/ml}$) was required for optimal growth. In media without mycobactin at iron concentrations $\leq 100 \mu\text{M}$, growth of *M. paratuberculosis* occurred at pH 5.0, but not pH 6.8. Iron concentrations $>100 \mu\text{M}$ did not significantly increase

growth at pH 5.0, but at pH 6.8 the growth rate increased with increasing amounts of iron reaching a rate equal to control cultures containing mycobactin. *Mycobacterium paratuberculosis* appeared to lose mycobactin dependence when subcultured; however, this was subsequently shown to be a result of mycobactin carried over from primary medium. Removal of this contaminating cell-wall-associated mycobactin reestablished mycobactin dependence. We conclude that mycobactin dependence must be carefully determined because it is a key test used in identification of *M. paratuberculosis* and may be easily influenced by media pH, iron concentration, and mycobactin carryover from primary media.

INTRODUCTION

Mycobacterium paratuberculosis is the causative agent of paratuberculosis (Johne's disease) and has been isolated from intestinal tissues of several patients with Crohn's disease for which the etiology is unknown (Chiodini et al., 1984; Twort and Ingram, 1912). Both Johne's and Crohn's disease are chronic inflammatory bowel conditions of ruminant animals and humans, respectively, for which there is no cure. *Mycobacterium paratuberculosis* often requires 6–10 weeks incubation before colonies appear on primary isolation media. Unlike most species of mycobacteria, *M. paratuberculosis* is a mycobactin auxotroph, unable to synthesize its own mycobactin. Hence, this compound must be added exogenously to culture media to cultivate *M. paratuberculosis* in the lab-

oratory. Its dependence for the iron chelator, mycobactin, serves as a key phenotypic characteristic for distinguishing *M. paratuberculosis* from other slowly growing *Mycobacterium* spp. The requirement for mycobactin cannot be replaced by other siderophore compounds (Morrison, 1965). Although some *M. avium*–*M. intracellulare* and wood pigeon isolates may demonstrate mycobactin dependence, this auxotrophic property is the principal criterion for ascribing isolates to the taxon, *M. paratuberculosis*.

Mycobactins are iron-binding compounds produced by mycobacteria under iron-limiting conditions (Snow, 1970). They are a group of closely related, low molecular weight, lipid-soluble compounds that are presumed to be cell wall associated (Macham et al., 1975; Ratledge et al., 1982). The structures and properties of mycobactins have been described in several reviews (Ratledge, 1982 and 1984; Snow, 1970). Their role in iron transport, however, is not clear (Barclay et al., 1985). In the present study we describe the requirement for iron and mycobactin by *M. paratuberculosis* in quantitative terms and examine the influence of pH on these requirements. The lack of an appropriate assay in the past has prohibited studying growth associated with iron assimilation. This problem has been resolved by the development

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of a sensitive and quantitative radiometric bioassay for *M. paratuberculosis*, which can be modified to evaluate growth under a variety of conditions (Lambrecht and Collins, 1988). We define iron and mycobactin dependence in quantitative terms and demonstrate that exogenously added mycobactin rapidly becomes associated with the mycobacterial cell wall and can be carried over with mycobacterial organisms upon subculture, resulting in an apparently artificial loss of mycobactin dependence.

MATERIALS AND METHODS

Bacterial Strains and Growth

The type strain, *M. paratuberculosis* ATCC 19698, had been passed numerous times in Middlebrook 7H9 medium supplemented with 10% OADC enrichment (Difco, Detroit, MI), 0.1% Tween 80 (Sigma, St. Louis, MO) and 1 µg/ml ferrimycobactin J (Allied, Glenwood Springs, CO). *Mycobacterium paratuberculosis* UWIS B025 was isolated from the feces of a cow diagnosed as having clinical Johne's disease, confirmed by pathologic and microbiologic findings, and was passed no more than three times on supplemented Middlebrook 7H9 medium prior to inoculation of iron-deficient medium. Both strains were iron-starved for at least 1 week in a chemically defined medium that was free of iron and mycobactin. Inocula were prepared as single-cell suspensions (Lambrecht and Collins, 1988) for use in all growth experiments and contained $\sim 10^{4.4}$ *M. paratuberculosis* ATCC 19698 or $10^{6.8}$ *M. paratuberculosis* UWIS B025.

Media

Middlebrook 7H12B medium (Becton-Dickinson, Towson, MD) was used in growth experiments where iron concentration was not critical. The concentration of iron in Middlebrook 7H12B is ~ 130 µM. A chemically defined medium (CDM), pH 6.8, was prepared free of iron and contained 4 µM CuSO₄, 3.4 µM ZnSO₄, 3.4 µM CaCl₂, 0.4 mM MgSO₄, 0.34 mM sodium citrate, 17.6 mM sodium phosphate, 7.3 mM potassium phosphate buffer, 1.5 mM ammonium sulfate, 7.5 mM asparagine, 2.9 mM L-glutamate, 4.8 µM pyridoxine, 2 µM biotin, 0.5% bovine albumin fraction V (Sigma), and 2.2 mM dextrose. To minimize iron contamination, disposable plastics or acid-rinsed glassware were used throughout media preparation. Deionized water was further deferated by treatment with the ion-exchange resin Chelex 100 (Sigma). Glassware was sterilized in a hot-air oven. A radiometric CDM (RCDM) was used for all iron-free or low-iron growth experiments and was

prepared as follows: 250 µCi of [1-¹⁴C]palmitic acid in toluene, specific activity 54 mCi/mmol (Amersham, Arlington Heights, IL), was evaporated to dryness under N₂ and dissolved in 0.5 ml 0.05 N KOH at 80°C. The dissolved palmitate was added to 12.5 ml of prewarmed 10% bovine albumin fraction V, gently mixed, and combined with 237 ml of CDM without dextrose or albumin. The medium was filter-sterilized through a 0.22-µm-pore filter (Nal-gene, Rochester, NY) and 2 ml dispensed into 20-ml sterile vials (West, Phoenixville, PA). Each vial contained 2 µCi of ¹⁴C-palmitate under ambient air and was sealed with a sterile rubber septum. Where appropriate, various concentrations of ferrimycobactin J or mycobactin J (the metal-free ligand) were added to RCDM. Mycobactin J was prepared by washing ferrimycobactin J dissolved in chloroform exhaustively with equal volumes of 6 N HCl and the chloroform was evaporated under a stream of air. The iron-free siderophore was then dissolved in 95% ethanol. The potent iron chelator desferrioxamine mesylate (Desferal; Ciba, Summit, NJ) was added (25 µM) to all vials when determining iron dependence so as to bind contaminating trace levels of iron. The concentration of iron in media was confirmed by the ferrozine assay (Stookey, 1970). Mycobactin concentration was determined based on a molecular weight of 841 (Merkal and McCullough, 1982).

Growth Measurement

Iron-stressed, single-cell cultures were inoculated (0.1 ml) into vials containing radiometric media using a Tridak Stepper repetitive syringe (Vanguard, Neptune, NJ). All necessary supplements were also added to the growth vials using the precision syringe device. Inoculated vials were gently swirled and incubated at 37°C without shaking. Growth of mycobacteria in ¹⁴C-palmitate medium produced respired ¹⁴CO₂, which was detected by an automated ionization detector, BACTEC (Becton-Dickinson). One growth unit is equal to 0.25 nCi of ¹⁴C. Growth measurements of individual vials were taken at multiple times during the incubation period (raw growth units) and expressed as the sum of all previous measurements (cumulative growth units). Previous studies have demonstrated that the rate of ¹⁴CO₂ production is strongly correlated to the number of viable organisms during logarithmic growth (Lambrecht and Collins, 1988). Growth of *M. paratuberculosis* was determined in triplicate culture vials for each concentration examined. Organisms were quantified and growth rates determined based on a previously described mathematical growth kinetic model (Lambrecht and Collins, 1988), which took into account the inoculum

size (\log_{10}), growth unit measurements (amount of $^{14}\text{CO}_2$ released) and time of incubation (days). Growth rate was determined by calculating the slope of the linear segment resulting from plotting the natural logarithm of the growth unit measurements over time (days). This log-linear segment represents exponential (maximum) growth.

Demonstration of Iron and Mycobactin Dependence

Growth in the presence of limiting iron was demonstrated by adding calculated amounts of ferric ammonium citrate or ferric nitrate to RCDM containing 25 μM Desferal followed by addition of 0.12 μM mycobactin J a minimum of 3 hr later. Final concentrations of total iron (free and bound) in the RCDM ranged from 0–50 μM . The minimum amount of mycobactin-bound iron required for growth was also determined by partially saturating mycobactin with different levels of iron from ferric ammonium citrate. Calculated amounts of ammonium metavanadate (Sigma) were added to saturate unbound binding sites on mycobactin molecules. The iron–vanadate-saturated mycobactin preparations were diluted and added to RCDM at a concentration of 0.12 μM . The concentration range of mycobactin-bound iron was calculated to be between 0 and 0.1 μM . To determine the minimum and optimum concentrations of mycobactin required for growth of *M. paratuberculosis*, mycobactin and ferrimycobactin were added to RCDM in concentrations ranging from 0 to 6 μM and growth rates compared.

Carryover of Mycobactin with Transfer of Mycobacterial Cells

Iron-stressed, mycobactin-dependent *M. paratuberculosis* ATCC 19698 organisms were incubated for 12 hr in Middlebrook 7H9 broth containing either no mycobactin or 2 μM mycobactin J. Duplicate portions (2 ml) of each culture were filtered through sterile 0.8- μm -pore cellulose ester filters (Millipore, Bedford, MA) and the trapped cells rinsed with 5 ml washes of sterile phosphate-buffered saline to remove excess free mycobactin and culture medium. To control for mycobactin contamination of the filter, sterile medium containing 2 μM ferrimycobactin J was filtered and rinsed. One filter from each pair was carefully removed from the filter holder and placed into a vial containing radiometric medium and ferrimycobactin; the other filter was placed into radiometric medium without ferrimycobactin J. Both vials were resealed and incubated at 37°C. Growth in vials containing mycobactin was compared with growth in vials without mycobactin using an index

of mycobactin dependence described below. In an attempt to remove mycobactin that may have become bound to the cell wall of *M. paratuberculosis*, cells previously exposed to mycobactin were trapped on 3- μm -pore filters and rinsed with various concentrations of ethanol followed immediately by buffered saline to chase away excess ethanol. Filters were removed and placed into vials containing Middlebrook 7H12B medium with and without mycobactin. Growth rates were determined and used to calculate a mycobactin-dependence index.

Definition of Mycobactin Dependence

Mycobactin dependence was measured using a mycobactin-dependence index (MDI), which compared growth rates of strains in RCDM with mycobactin (μ_m) to growth rates in RCDM without mycobactin (μ_0). The MDI was calculated using the equation, $\text{MDI} = (\mu_m - \mu_0)/\mu_m$. When the MDI = 1, the culture was defined to be totally mycobactin dependent (that is, $\mu_0 = 0$). As the MDI approached 0 (μ_0 approximating μ_m), the culture was less dependent on mycobactin for growth.

Electron-Microscopic Evidence of Cell-Wall-Associated Mycobactin

To demonstrate mycobactin association with the mycobacterial cell wall, two cultures of *M. paratuberculosis* were incubated overnight in CDM. One of the cultures was supplemented with 40 mM mycobactin J prior to incubation. After 24 hr, both cultures were cooled to 4°C and made 0.1% with ammonium metavanadate, which binds quickly to mycobactin and is electron dense (Ratledge et al., 1982). A drop of each culture was placed on a collodion copper grid and the whole cells examined immediately with transmission electron microscopy (Phillips 410). As a control, *Mycobacterium smegmatis*, which is capable of mycobactin synthesis, was maintained in CDM and also examined after addition of ammonium metavanadate.

RESULTS

Mycobactin Dependence

The minimum amount of ferrimycobactin required for growth in Middlebrook 7H12B radiometric broth was determined to be 0.006 μM for both strains of *M. paratuberculosis* after 30 days incubation (Table 1). Maximum growth was achieved with ≥ 1.2 μM mycobactin. Both strains of *M. paratuberculosis* also formed visible colonies on Middlebrook 7H9 agar plates (data not shown) containing 0.006 μM mycobactin but not

TABLE 1 Effect of Mycobactin-J Concentration on Growth of *Mycobacterium paratuberculosis*

Mycobactin-J Concentration (μM)	Percent Maximum Growth Rate ^a	
	ATCC 19698	UWIS B025
≤ 0.0006	0	0
0.006	33	22
0.06	76	79
0.6	92	99
≥ 1.2	100	100

^aMean of triplicate determinations calculated from $\frac{\mu_x - \mu_0}{\mu_{\max} - \mu_0} \times 100$ where μ_x = growth rate with x concentration mycobactin, μ_0 = growth rate with no mycobactin J, and μ_{\max} = maximum growth rate observed. (SD < 1%.)

0.0006 μM , which was consistent with growth in radiometric broth. No differences were observed when mycobactin was substituted for ferrimycobactin at the same concentrations in the relatively high iron-containing medium.

Dependence of *Mycobacterium paratuberculosis* on Iron for Growth

Demonstration of iron dependence for *M. paratuberculosis* necessitated using medium that was essentially iron free. Deferration of Middlebrook 7H12B by treatment with cationic chelators (EDTA, 2,2'-dipyridyl, 8-hydroxyquinoline), or chelating resins that removed other essential cations in addition to iron, was toxic to *M. paratuberculosis* or was not efficient in removing iron. Consequently, an iron-free CDM was prepared as described in *Materials and Methods*.

Mycobacterium paratuberculosis ATCC 19698 did not grow to any significant extent at iron concentrations $\leq 10 \mu\text{M}$ even after 9.5 weeks incubation (Figure 1). Slight growth apparent in the virtual absence of iron in the medium was most likely due to our inability to totally removal all iron from mycobactin. At 50 μM iron, growth was significantly stimulated. No difference was observed between vials that received ferric ammonium citrate and those that received ferric nitrate. Iron dependence was also demonstrated with mycobactin to which increasing amounts of iron was bound (Figure 2A). The sites on mycobactin molecules not bound with iron were saturated with the vanadate ion from ammonium metavanadate. Ammonium metavanadate was shown not to affect growth of *M. paratuberculosis* at low concentrations and is known to bind tightly to mycobactin at the site of iron binding. Growth of *M. paratuberculosis* was maximal when mycobactin was completely sat-

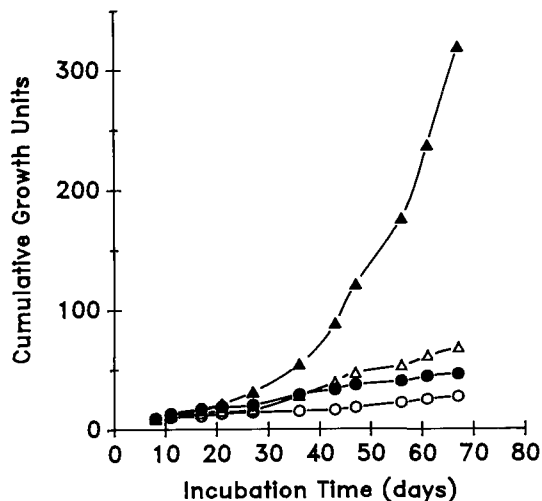


FIGURE 1 Growth of *Mycobacterium paratuberculosis* ATCC 19698 as a function of free iron concentration added as ferric ammonium citrate to radiometric chemically defined medium containing 25 μM deferoxamine mesylate and 0.12 μM mycobactin J. Each point represents the mean growth rate \pm SD of three culture vials: (○) no iron, (●) 1 μM iron, (△) 10 μM iron, and (▲) 50 μM iron.

urated with iron (0.1 μM) and decreased with decreasing amounts of mycobactin-bound iron. We found the expression of growth rate (which is calculated and representative of an entire growth curve) to be an improvement in describing growth over a period of time and in the graphic display of the data rather than showing entire growth curves (Figure 2B). Growth rates of 0.3, 0.2, and 0.1 correspond to generation times of ~ 2.3 , 3.5, and 6.9 days, respectively. Similar findings were observed with the ATCC 19698 strain.

Growth of *Mycobacterium paratuberculosis* in the Absence of Mycobactin

Growth of *M. paratuberculosis* ATCC 19698 in Middlebrook 7H12B medium containing mycobactin J was determined over a wide range of pH and was optimal at pH 5.5–6.0 (Figure 3). Since iron is more soluble at low pH, we decided to examine growth of *M. paratuberculosis* as a function of iron and pH in the absence of mycobactin. Medium (RCDM) without mycobactin and containing $\leq 100 \mu\text{M}$ iron supported growth of *M. paratuberculosis* at pH 5.0 (Figure 4). Only when a relatively high concentration of iron ($>0.1 \text{ mM}$) was added did growth at pH 6.8 in the absence of mycobactin take place. The growth rate of *M. paratuberculosis* at pH 5.0 and 50 mM iron was comparable to the growth rate of the control culture containing 0.12 μM mycobactin and could not be further increased by more iron. In contrast, at pH 6.8, increasing amounts of iron simu-

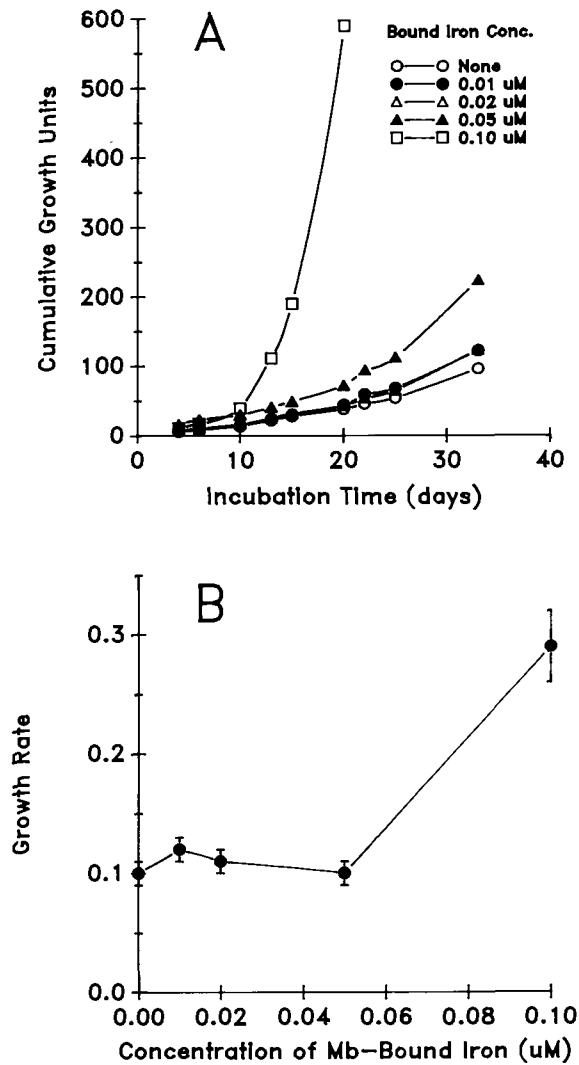


FIGURE 2 Growth of *Mycobacterium paratuberculosis* UWIS B025 as a function of mycobactin-bound iron in radiometrically chemically defined medium containing 25 μM deferoxamine mesylate and 0.12 μM mycobactin J saturated with various amounts of iron. Each point represents the mean growth rate \pm SD of three culture vials. (A) Expressed as growth curves over time. (B) Same data as A, only growth rates were calculated from individual growth curves as explained in text.

lated better growth of *M. paratuberculosis*, and 50 mM iron was required to achieve growth equal to that in control cultures containing mycobactin. Growth in the absence of mycobactin was best at pH 6.8 at the highest concentration of iron tested, but less iron was required to stimulate growth at pH 5.0. Growth of *M. paratuberculosis* was also evaluated on Middlebrook 7H9 agar plates (pH 6.8, containing either 0.1, 1, 5, 10, 50, or 100 mM iron) in the absence of mycobactin. Neither strain formed colonies in media containing ≤ 1 mM iron. However, both strains formed colonies in media containing ≥ 5 mM, with

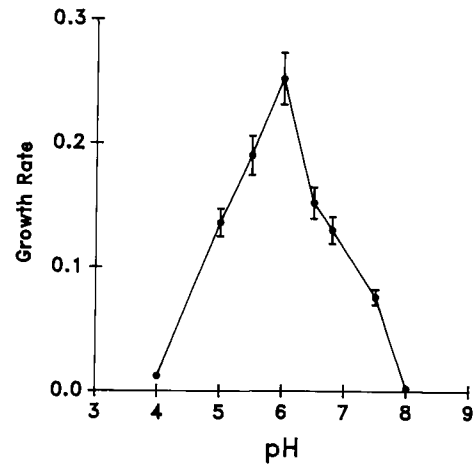


FIGURE 3 Effect of pH on growth rate of *Mycobacterium paratuberculosis* ATCC 19698 in Middlebrook 7H12B medium supplemented with 1 μM ferrimycobactin J. Each point represents the mean \pm SD of three culture vials.

the earliest appearance and highest number of colonies occurring at 50 mM iron.

Loss of Mycobactin Dependence by *Mycobacterium paratuberculosis*

The apparent loss of mycobactin dependence in *M. paratuberculosis* upon subculture from a mycobactin-containing medium was also examined. *Mycobacterium paratuberculosis* was exposed to a mycobactin-containing broth, removed from the broth by filtering, washed with buffered saline, and subcultured to mycobactin-free media. Subsequent growth in mycobactin-free medium resulted in *M. paratuberculosis* appearing to have lost its dependence for my-

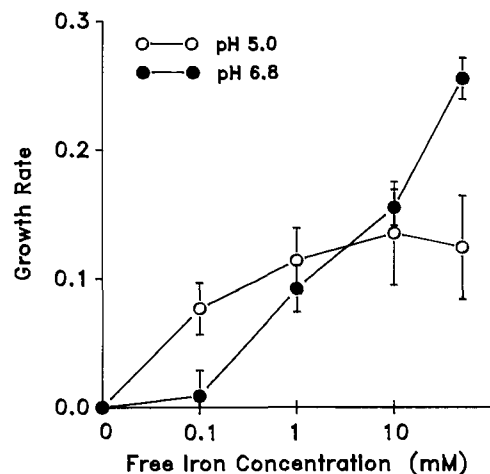


FIGURE 4 Influence of pH and iron concentration on growth rate of *Mycobacterium paratuberculosis* ATCC 19698 in radiometrically chemically defined medium in the absence of mycobactin. Each point represents the mean \pm SD of three culture vials.

TABLE 2 Apparent Loss of Mycobactin Dependence After Exposure of *Mycobacterium paratuberculosis* ATCC 19698 to Medium Containing Mycobactin

Prior Exposure to Mycobactin	MDI ^a	
	ATCC 19698	UWIS B025
No exposure	0.73	0.71
12-hr Exposure	0.01	0.12

^aMDI (mycobactin-dependence index) ranges from 0 to 1, where 1 indicates total dependence on exogenous mycobactin for maximum growth and 0 indicates no exogenous mycobactin required for maximum growth. See the text for calculation.

cobactin. *Mycobacterium paratuberculosis* suspended in broth without mycobactin, filtered and washed in the same manner as above, however, remained mycobactin dependent (Table 2). As a negative control, a sterile solution of 2 μ M mycobactin was filtered and the filter rinsed with saline and placed into RCDM containing mycobactin-dependent *M. paratuberculosis*. The absence of growth stimulation of *M. paratuberculosis* in the bioassay verified that no appreciable amount of mycobactin bound to the cellulose filter membrane.

Because mycobactin is readily solubilized in ethanol, we attempted to remove any mycobactin that may have become associated with the mycobacterial cell wall and thus "carried over" upon subculture so as to convert the no longer mycobactin-dependent culture back to dependency. Rinsing the mycobacteria with ethanol was followed quickly by buffered saline to avoid killing as many mycobacteria as possible. Accordingly, the higher the concentration of ethanol was, the fewer were the number of viable mycobacteria. Treatment with 0–50% ethanol resulted in MDI \leq 0.1 (Table 3). Concentrations of 60%–70% ethanol effectively stripped mycobactin from the cell wall of *M. paratuberculosis* organisms,

TABLE 3 Removal of Cell-Wall-Associated Mycobactin from *Mycobacterium paratuberculosis* with Ethanol

Percent Ethanol	MDI ^a
70	1.0
60	0.7
50	0.1
40	0
\leq 30	0

^aSee the text for explanation of MDI (mycobactin-dependence index) calculation.

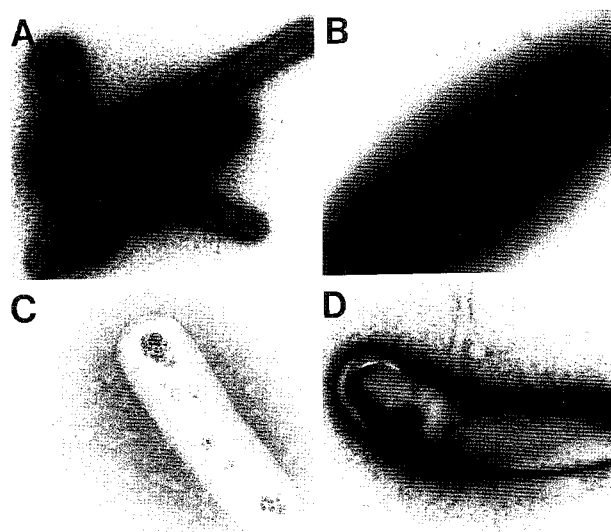


FIGURE 5 Electron micrograph of whole bacterial cells stained with 0.1% ammonium vanadate after incubation in low-iron chemically defined medium (CDM). (A and B) *Mycobacterium paratuberculosis* incubated with mycobactin. (C) *Mycobacterium paratuberculosis* incubated without mycobactin in low-iron CDM. (D) *Mycobacterium smegmatis* incubation in low-iron CDM.

rendering them dependent on mycobactin (MDI = 0.7–1.0). The 70% ethanol resulted in the fewest number of surviving mycobacteria ($10^{4.1}$). Although treatment with ethanol is somewhat harsh, it was effective in removing mycobactin. Perhaps other solvents would be less toxic, but our aim was to use the most effective solvent.

To demonstrate that mycobactin becomes associated with the mycobacterial cell wall, we used electron-dense ammonium metavanadate bound to mycobactin to observe complexing of mycobactin to mycobacterial cells employing transmission electron microscopy. Cells of *M. paratuberculosis* incubated with 40 mM mycobactin–ammonium metavanadate were surrounded by an electron-dense cloud (Figure 5A and B), whereas the majority of cells exposed to ammonium metavanadate alone appeared less dense (Figure 5C). In contrast, mycobacterium smegmatis, which is capable of endogenous mycobactin synthesis, appeared electron dense when incubated with ammonium metavanadate alone (Figure 5D).

DISCUSSION

It has generally been observed that strains of *M. paratuberculosis* and *M. avium* may lose dependency for mycobactin after several passages on laboratory media (Merkal and McCullough, 1982; Merkal and Curran, 1974; Whipple et al., 1987). Because of the

slow growth associated with these species, it is understandable why little has been done to study the influence of media and culture conditions on mycobactin dependence. Growth that occurs only when mycobactin is added exogenously to the medium is currently the most common phenotypic test by which clinical laboratories distinguish *M. paratuberculosis* from other *Mycobacterium* spp. Although genetic phenomenon such as derepression of mycobactin biosynthetic mechanisms or true reversion as a result of mutation might explain reversion of mycobactin-dependent strains to begin synthesizing mycobactin suddenly, we thought it necessary to examine environmental factors that may influence mycobactin dependence. During the past 6 years of maintaining mycobactin-dependent reference strains and clinical isolates, we have observed mycobactin dependence to be a stable phenotypic characteristic. Numerous subcultures of these strains have not resulted in reversion of any strain to produce mycobactin.

Several reports of circumvention of the need for mycobactin by mycobactin-dependent mycobacteria have been described (Morrison et al., 1965; Wheeler and Hanks, 1965; Merkal and Curran, 1974). Unfortunately, it is not clear whether *M. paratuberculosis* grew in the complete absence of mycobactin or whether its growth was simply enhanced in the presence of trace amounts of mycobactin brought into the culture with inoculum previously maintained on mycobactin-containing medium. Furthermore, *M. paratuberculosis* strain 18 (a mycobactin-producing strain from which mycobactin J is derived) produces mycobactin identical to that of *M. intracellulare* M12 (Barclay et al., 1985) and is now considered to be taxonomically misnamed (Collins et al., 1986; Whipple et al., 1987).

The term *mycobactin-dependent* is perhaps misleading in that, outside of an appropriate environment, all mycobacteria are probably dependent upon mycobactin, but most *Mycobacterium* spp. are capable of synthesizing their own mycobactin. At the very least, the term needs to be defined under a set of stringent criteria not influenced by culture conditions. These criteria might include removing cell-

wall-associated mycobactin or conditioning the strain in low-iron, mycobactin-free medium followed by testing for growth in low-iron medium at pH 6.8 with and without mycobactin. This would aid clinical laboratories in identifying an isolate as mycobactin dependent. Under this criteria, a strain could also be critically evaluated for possessing the genetic capacity to synthesize mycobactin.

We have determined minimum concentrations of iron and mycobactin J necessary to stimulate growth of *M. paratuberculosis*. Because of the extreme sensitivity of the radiometric assay, even limited growth could be measured as evidenced by the slight growth of *M. paratuberculosis* in medium containing 0.1 mM iron at pH 5.0 in the absence of mycobactin. Growth of *M. paratuberculosis* in the absence of exogenous mycobactin will occur in media buffered at pH 6.8 only when a relatively high concentration of iron is present. However, we have documented that *M. paratuberculosis* grows optimally at pH 5.5 to 6, and this lower pH may better represent what occurs in vivo when one considers that the pH of the phagolysosome is 5.0–5.5. Studies of mycobacterial growth need to be sensitive to even slight growth under conditions resembling the in vivo environment. Growth of *M. paratuberculosis* in macrophages over a 7- to 10-day period have been shown to represent only a two- to threefold increase in the number of organisms (Zurbrick and Czuprynski, 1987). Thus, radiometric technology is well suited for these sensitive growth measurements, allowing for detecting and quantifying small differences that occur during exponential growth. We were able to take advantage of this attribute in developing the MDI. In addition, we demonstrated that *M. paratuberculosis* binds mycobactin in media to its cell wall and carries it over to new media upon subculture. Stripping mycobactin from the cell wall with ethanol was effective in re-establishing mycobactin dependence of a *M. paratuberculosis* culture. We have been successful in applying this technique for confirming clinical isolates of *M. paratuberculosis* as mycobactin dependent after primary isolation in a mycobactin-containing radiometric broth medium (unpublished data).

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