JOURNAL OF BACTERIOLOGY, Mar. 1983, p. 1138–1146
0021-9193/83/031138-09$02.00/0

Vol. 153, No. 3

Iron-Binding Compounds of *Mycobacterium avium*, *M. intracellulare*, *M. scrofulaceum*, and Mycobactin-Dependent *M. paratuberculosis* and *M. avium*

RAYMOND BARCLAY* AND COLIN RATLEDGE

Department of Biochemistry, The University of Hull, Hull, HU6 7RX, United Kingdom

Received 12 July 1982/Accepted 8 December 1982

Fifty-three strains of *M. avium* and related species all produced one or more exochelins, the extracellular iron-binding compounds of the mycobacteria, when grown iron deficiently. Only those strains which could grow without the addition of mycobactin (i.e., mycobactin independent) produced mycobactin, the intracellular iron-binding compound of the mycobacteria. Exochelins varied from 20 to 2,000 μg per g of cell dry weight; mycobactins were between 1 and 10 mg per g of cell dry weight. *M. paratuberculosis* (13 strains) and 13 strains of *M. avium*, both species dependent upon mycobactin for growth, failed to produce spectrophotometrically detectable amounts of mycobactin (<0.2 μg per g of cell dry weight), although mycobactin could be recognized in one strain of *M. avium* grown with an additional supply of salicylate and examined by a radiolabeling technique. On repeated subculture three of the mycobactin-dependent strains of *M. avium*, but none of those of *M. paratuberculosis*, lost their mycobactin dependence and on reexamination were found to produce their own mycobactin at 0.3 mg per g of cell dry weight. It is concluded that mycobactin biosynthesis is probably strongly repressed in the mycobactin-dependent strains rather than being a genetic deletion. The exochelins, when examined by high-pressure thin-layer chromatography were revealed as being multiples of similar compounds, with up to 20 individual iron-binding compounds being recognizable with some strains. It is argued that the exochelins represent the single most important means of iron acquisition in mycobacteria growing in vitro and in vivo, and their elaboration by the fastidious *M. paratuberculosis* and related species explains how these organisms are able to grow in vivo in the absence of an external supply of mycobactin.

Many mycobacteria produce mycobactins, which are cell-associated iron-binding compounds, to assist in the acquisition of iron from their environment (16). The importance of mycobactin is shown by freshly isolated strains of *Mycobacterium paratuberculosis* and some strains of *M. avium* which, at least in laboratory culture media, require an extraneous supply of iron-chelated mycobactin for growth.

Unless the integrity of the cell envelope is disturbed by a detergent such as Tween 80 (2), mycobactin-producing cells do not release mycobactin into their environment, even though desferrimycobactin can account for up to 10% of the cell dry weight of *M. smegmatis* when grown under iron-deficient conditions (15). This close association of mycobactin with the mycobacterial cell envelope therefore casts doubt on the availability of an extraneous supply of mycobactin, such as from commensal saprophytic mycobacteria, to provide pathogenic mycobactin-dependent organisms with iron in vivo (16). There is, however, the possibility that iron acquisition by these mycobacteria can be explained instead by the production of mycobactin by these organisms when grown in vivo or by the production of extracellular iron chelators. Such extracellular iron-binding compounds could include the exochelins, which have been isolated from the culture filtrates of *M. bovis* BCG (3) and *M. tuberculosis* (H. J. Morgan, personal communication) as well as from several saprophytic species (10).

There are two main classes of exochelin, each being produced in increased amounts under iron-deficient growth conditions, and these can be distinguished by their solubility. Those exochelins which, when bound to iron, cannot be extracted from aqueous solutions into organic solvents are termed water-soluble exochelins, whereas those that can be extracted are termed chloroform soluble. Exochelins can solubilize iron and participate in its uptake into the myco-

bacteria, but exochelin-mediated iron uptake does not necessarily involve the participation of mycobactin (17, 18). The structures of both types of exochelin have yet to be determined, principally due to their occurrence at low concentrations (approximately 1 to 10 μg/ml) and difficulties with purification and because each organism appears to produce more than one exochelin. *M. smegmatis*, for example, may produce up to eight separable water-soluble exochelins (17). However, the exochelins are known to be low-molecular-weight compounds (<1,000) and are probably substituted peptides (10).

This work is concerned with the ability of *M. avium*, *M. intracellulare*, *M. scrofulaceum* (i.e., members of the *M. avium* complex or MAIS complex), and *M. paratuberculosis* to acquire iron by the use or production of mycobacterial iron chelators. Experiments are presented which examine the production of iron-chelating compounds by these organisms and the differentiation of these compounds, and observations concerning their possible metabolic role are made.

MATERIALS AND METHODS

Organisms and growth. The mycobactin-independent *M. avium* strains studied were M1 (NCTC 8559), M2 (NCTC 8562), M3, M4, M5, M6, M7, M8, M9, M10, A8/12, and A5/11. *M. intracellulare* strains studied were M11 (ATCC 13950), M12, M13, M14, M15, M16, M17, M18, M34, M35, M36, 6841, and 17573. Mycobactin-dependent *M. avium* strains used were M19, M20, M21, M22, M23, M24, M25, wood pigeon WP7/74, wood pigeon WP1/77, 2(W), 5(W), 8(W), and 80(W). *M. paratuberculosis* strains studied were M26, M27, M28, M29, M30, M31, M32, S119, C57/2, 12(W), 14(W), 221-0(W), and 465 BC(W). Strains with the designation "M" were obtained from J. L. Stanford, Middlesex Hospital Medical School, London, U.K.; strains designated "W" were from T. W. Little, Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Weybridge, Surrey, U.K. All other strains were from P. R. J. Matthews, Agricultural Research Institute, Compton, Berks, U.K. The strains of *M. scrofulaceum* used were M70 and NCTC 10803. *M. smegmatis* NCIB 8548 and *M. bovis* BCG (batch P691 from Glaxo Laboratories, Greenford, England) were studied. Cultures were grown on an iron-deficient (20 ng of Fe per ml) glycerol-asparagine-mineral salts medium (100 ml) which was prepared as before (13). Mycobactin-dependent strains were supplemented with mycobactin S (i.e., mycobactin from *M. smegmatis*) at 3 μg/ml. *M. avium*, *M. intracellulare*, and *M. scrofulaceum* were grown without shaking at 42°C for 6 to 7 weeks unless stated otherwise. *M. paratuberculosis* was grown in the same way but at 37°C for 3 to 12 months. *M. bovis* BCG was grown for 7 days at 37°C with rotary shaking (100 rpm) as previously described (3).

Isolation of mycobactins and exochelins. Ferrimycobactins were extracted from freshly harvested whole cells with ethanol by the method of Snow (11, 16). Water-soluble and chloroform-soluble exochelins were extracted from cell-free culture filtrates by the methods of Macham et al. (3).

TLC of mycobactins and exochelins. Thin-layer chromatograph (TLC) was as described by Macham et al. (3) but using 250-μm-thick silica gel 60 TLC plates with concentrating zones (E. Merck AG). Solvent system A, which was used for both mycobactins and exochelins, consisted of petroleum ether–*n*-butanol–ethyl acetate (2:3:3, by volume). Solvent system B was methanol-ethyl acetate (4:1, vol/vol), and solvent system C was ethanol-petroleum ether-ethyl acetate (1:4:6, by volume). The solvent systems and the methods for high-performance TLC (HPTLC) were the same as for TLC except that 10- by 10-cm precoated silica gel 60 HPTLC plates with 1-cm concentrating zones were used (Merck).

Radiodetermination of extracellular iron-binding compounds. Cultures (100 ml) of *M. avium* M3, M24, WP1/77, and WP7/74 and *M. intracellulaire* M12 were grown under iron-deficient conditions as described above for 8 weeks at 42°C. *M. paratuberculosis* 465-BC was also grown under iron-deficient conditions, but for 12 weeks at 37°C. In each case the cells were harvested by filtration and extracted with ethanol to remove cell-associated iron-binding compounds. The ethanol was evaporated under vacuum, and the residues were suspended in a known volume (1 ml) of ethanol. To this 0.5 ml of 1.25 μg of $^{55}FeCl_3$ (4.45 μCi/ml) per ml was added at pH 7.0 and left overnight at 20°C.

The cell-free culture filtrates, in which extracellular iron-binding compounds (the exochelins) would be expected, were evaporated in a rotary evaporator, and the residues were taken up in 10 ml of iron-free water (all glass, double distilled). To 5 ml of each culture filtrate suspension 2.5 ml of 1.25 μg of $^{55}FeCl_3$ (4.45 μCi/ml) per ml was added at pH 7.0, incubated overnight at 20°C, and then extracted three times with equal volumes of chloroform to isolate the chloroform-soluble exochelins. The remaining extracted culture filtrate was retained for examination of unremoved exochelins (i.e., the so-called water-soluble exochelins).

Iron-binding compounds from the three extracts, i.e., the cell itself, the culture filtrate, and the chloroform extract thereof, were applied and run on a silica gel 60 TLC plate with solvent system A, as described above. The TLC plates were then autoradiographed (see below) to detect any migrating iron-binding compounds. Iron-solubilizing compounds in the culture filtrates were also assessed by the ultrafiltration method of Macham et al. (3).

Preparation of ^{14}C-labeled mycobactin. Two flasks (250 ml), each containing 100 ml of iron-deficient medium and 6 μmol of [*carboxy*-^{14}C]salicylate (0.84 μCi/ml), were each inoculated with 1 ml of an 18-h (37°C) shake culture of *M. smegmatis* NCIB 8548 and incubated without shaking at 37°C for 7 days. Mycobactin S (238 μg) was extracted by the method of Snow (16) and purified by the method of Ratledge (11) to about 85% purity; a total of 63 μCi was recovered in the mycobactin.

Utilization of ^{14}C-labeled mycobactin and [*carboxy*-^{14}C]salicylate. Mycobactin-dependent and -independent strains of *M. avium* and *M. paratuberculosis* were each inoculated in triplicate into iron-deficient medium as described above. Irrespective of the depen-

dence of the cells on mycobactin, one of the following was added to each of the triplicates: (i) ^{14}C-labeled mycobactin S (12 μg/ml; 0.168 μCi/ml) plus 6 μmol of salicylate; (ii) 6 μmol of [*carboxy*-^{14}C]salicylate (2.1 μCi/ml) plus 12 μg of mycobactin S per ml; (iii) 6 μmol of salicylate plus 12 μg of mycobactin S per ml as a control.

The flasks were then incubated at 42°C without shaking for 8 weeks (*M. avium* strains) or at 37°C for 12 weeks (*M. paratuberculosis*), after which the cells were harvested by filtration and the mycobactins were extracted with ethanol. The ethanol was evaporated and the residues were suspended in a known volume (1 ml) of ethanol. The remaining ethanol-extracted cells were dried at 160°C and then digested overnight at 20°C with 1 ml of Soluene TM-100. The digestion was stopped with 0.8 ml of glacial acetic acid. The exochelins were extracted from the culture filtrates with three 50-ml volumes of chloroform (3). In each case, the chloroform extracts were pooled, the solvent was evaporated, and the residues were suspended in known volumes (1 ml) of ethanol. The extracted culture filtrates were evaporated to dryness under reduced pressure, and the residues were taken up in 10 ml of water.

The amount of radioactivity in each of the four fractions (i.e., the ethanol cell extracts, the digested cells, the chloroform-extracted culture filtrates, and the chloroform extracts) was quantitatively estimated, using 0.5 ml of each, by liquid scintillation counting (see below). The distribution of this radioactivity into different compounds was assessed by TLC (on silica gel 60 with solvent system A), using 50 μl of each sample, followed by autoradiography. In each estimation, ^{14}C-labeled mycobactin and [*carboxy*-^{14}C]salicylate were used as reference standards.

Radioactive counting and autoradiography. Radioactive counting was done by the methods of Ratledge et al. (14) in which aqueous solutions of [*carboxy*-^{14}C]salicylate or ^{55}Fe were counted by adding 0.5 ml of sample to 10 ml of xylene-based scintillant. Non-water-soluble compounds (1 ml) and digested bacterial cells wer counted in the toluene-based scintillant.

Autoradiography of the TLC plates was done with Kodak X-Omat RB X-ray film (18 by 24 cm). After 4-week exposure at room temperature, the film was developed with D163 (Kodak) and fixed.

RESULTS

Production of mycobactin and exochelin. Fifty-three strains of *M. avium* and related species of mycobacteria, selected on the basis of previous reports on other mycobacteria, were examined for the production of mycobactin and exochelin after iron-deficient growth (Table 1). All strains of *M. avium*, *M. intracellulare*, and *M. scrofulaceum* which could grow in laboratory media without the addition of mycobactin (that is, mycobactin-independent strains) produced their own cell-associated mycobactin. Maximum production of mycobactin (1 to 10 mg per g of cell dry weight) by these strains was after 6 to 8 weeks at 42°C. Under the same conditions, or at 37°C for 6 to 12 weeks, those strains which required mycobactin as a growth factor (that is, the mycobactin-dependent strains of *M. avium* and *M. paratuberculosis*) did not produce a mycobactin. The detection limit of mycobactin by the spectrophotometric method used was approximately 0.2 μg per g of cell dry weight, and about 3 g of dry cells was used for the determination. None of the mycobactin S (3 μg/ml), added to the culture media as a growth factor could be extracted from these cells at the end of growth. Prolonged incubation, for up to 1 year, still did not result in detectable levels of mycobactin being produced by any of the mycobactin-dependent strains of *M. avium* and *M. paratuberculosis*.

TABLE 1. Production of mycobactin and exochelin by organisms of the MAIS complex grown under iron-deficient conditions

Organism	No. of strains examined	No. of strains producing:	
		Mycobactin	Exochelin
M. avium (mycobactin independent)	12	12	12
M. avium (mycobactin dependent)	11	0	11
M. avium (mycobactin-dependent wood pigeon strains causing Johne's disease, WP7/74 and WP1/77)	2	0	2
M. intracellulare	13	13	13
M. scrofulaceum	2	2	2
M. paratuberculosis	13	0	13

None of the 53 strains, when grown under iron-deficient conditions, produced a water-soluble exochelin (that is, at more than 1 μg per g of dry cell weight). However, they all produced chloroform-soluble exochelins at between 20 and 600 μg per g of dry cell weight. Maximum production of exochelin varied with the size and age of the inoculum but was at between 6 and 8 weeks of incubation at 42°C for MAIS organisms (Table 2) and between 10 and 12 weeks at 37°C for *M. paratuberculosis*. After reaching a maximum concentration, the exochelin, like that previously reported for *M. bovis* BCG (3), gradually disappeared from the culture filtrates (Fig. 1). Exochelins were not detectable when the test strains were supplemented with 5 μg of $FeCl_3$ per ml (i.e., grown with a sufficiency of iron).

For all strains of *M. avium*, *M. intracellulare*, and *M. paratuberculosis*, the ability to produce exochelin decreased with repeated subculture. In the cases of *M. avium* and *M. intracellulare*, this coincided with an increase in mycobactin production. This increase in mycobactin production was not observed with the two strains of *M. scrofulaceum*.

TABLE 2. Production of chloroform-soluble exochelins by MAIS organisms after 6 weeks at 42°C and by *M. paratuberculosis* after 10 weeks at 37°C under iron-deficient conditions

Mycobacterium species and strain no.	Maxium exochelin production (μg/g of dry cell wt)
M. avium (mycobactin independent)	
M1	1,965
M2	473
M3	200
A8/12	67
A5/11	125
M. intracellulare	
M12	600
6841	148
M. avium (mycobactin dependent)	
M22	1,289
M24	1,101
WP7/74	769
WP1/77	1,583
2(W)	336
5(W)	769
8(W)	197
80(W)	474
M. paratuberculosis	
S119	475
465-BC	20
12(W)	688
14(W)	336
M. scrofulaceum	
NCTC 10803	460

After repeated subculture, some of the mycobactin-dependent *M. avium* strains [*M. avium* 2(W), 8(W), and 80(W)] completely lost their dependence upon mycobactin. The other strains did not. On examination, the former strains were now found to produce their own mycobactin at about 0.3 mg per g of dry cell weight. This mycobactin was identical to the other *M. avium* mycobactins when compared by HPTLC (Table 3). When compared with the same cultures that had been maintained by lyophilization, only the continuously subcultured cells produced a mycobactin. This might be taken to suggest that all mycobactin-dependent strains of *M. avium* have the genetic information needed to synthesize mycobactin but it is strongly repressed.

HPTLC of ferrimycobactins. To distinguish the mycobactins from MAIS organisms in later work from those of other mycobacteria, the mycobactins of those MAIS strains which produced such compounds were compared by HPTLC, using four different solvent systems (Table 3). Mycobactins from *M. smegmatis* and *M. bovis* BCG were used as references (11, 16). With all of the solvent systems used, the mycobactins of the MAIS complex were readily distinguishable from those of other mycobacteria, but not from other members of the MAIS complex. These results may be taken as further confirmation of the close relatedness of the MAIS members.

Separation of the ferriexochelins. To catagorize and distinguish the exochelins produced by all 53 strains of mycobacteria, they were compared by TLC on silica gel 60 developed with solvent system A. The exochelins from all of the

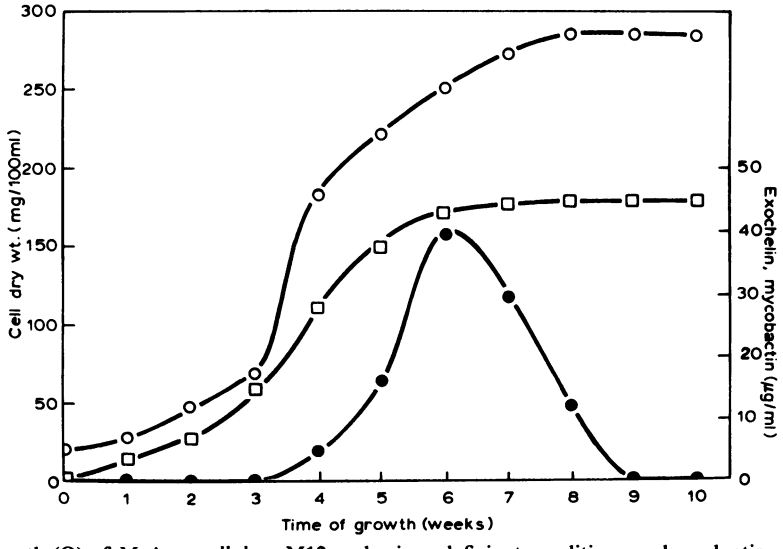

FIG. 1. Growth (O) of *M. intracellulare* M12 under iron-deficient conditions and production of mycobactin (□) and exochelin (●) at 42°C.

TABLE 3. Identification of mycobactins by HPTLC

Source of mycobactin	No. of strains examined	R_f in given solvent system[a]			
		A	B	C	D
M. avium (formerly mycobactin dependent; see text)	3	0.89	0.79	0.50	0.76
M. avium (mycobactin independent)	12	0.89	0.79	0.50	0.76
M. intracellulare	13	0.89	0.79	0.50	0.76
M. scrofulaceum	2	0.89	0.79	0.50	0.76
M. bovis BCG	1	0.57	0.64	0.18	0.27
M. smegmatis	2	0.36	0.75	0.02	0.16

[a] See text for composition of solvent systems.

M. avium and *M. intracellulare* strains were separated into two components, both running as streaks (R_f 0.16 to 0.39 and 0.45 to 0.75). The exochelin from *M. scrofulaceum* remained as a single component and migrated like the exochelin of *M. avium* with the lower R_f value. The exochelin from *M. paratuberculosis* behaved like the faster migrating of the *M. avium* exochelins. These two exochelins could also be separated on a column of basic grade alumina (1 by 10 cm). When the column was eluted with petroleum spirit (bp, 60 to 80°C), the exochelin that migrated slowly on TLC became firmly bound to the alumina, whereas the faster-migrating exochelin was washed through with no retardation. Contaminating materials were removed from the alumina-bound exochelin by successive washes with cyclohexane, petroleum spirit (bp, 60 to 80°C), toluene, diethyl ether, ethyl acetate, and chloroform. The adsorbed exochelin was then released from the column with methanol-formic acid (4:1, vol/vol).

When the unpurified exochelins were chromatographed by HPTLC with solvent system A, the broad bands of exochelin found by TLC were further resolved, and over 20 different components of the *M. avium* and *M. intracellulare* exochelins were now recognized (Fig. 2). The exochelins isolated from *M. bovis* BCG, *M. scrofulaceum*, and *M. paratuberculosis* were also resolved into several components (Fig. 2). Many of these resolved exochelins migrated like those of the *M. avium* and *M. intracellulare* strains.

Taken together, these results suggest that there are two main types of chloroform-soluble exochelin, each comprising several components which presumably arise from minor structural variations in each of the two molecules. As yet we have not obtained enough pure material with which to undertake structural analysis of the exochelins, but by combining the methods of separation and purification of the exochelins on alumina and the further separation by HPTLC, the exochelins might be purified for more detailed studies.

Detection of ^{55}Fe-binding compounds. In the above experiments we were concerned with the mycobactins and exochelins as they are the principal iron-binding compounds produced by mycobacteria. To detect whether any other cell-associated or extracellular iron-binding compounds were present, we added $^{55}FeCl_3$ to the cell extracts and culture filtrates of *M. avium* M3, M24, WP1/77, and WP7/74, *M. intracellulare* M12, and *M. paratuberculosis* 465-BC (see Materials and Methods) and chromatographed the products. After autoradiography we compared the radioactive (iron-binding) areas with their corresponding areas on the TLC plates (see Materials and Methods) and found that all of the cell-associated ^{55}Fe-binding was with the mycobactins. Salicylate, a known precursor of myco-

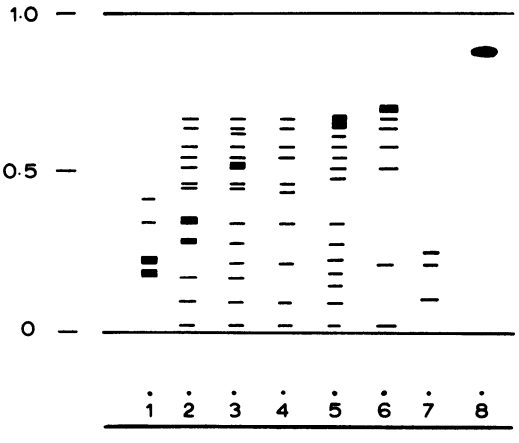

FIG. 2. HPTLC of the chloroform-soluble ferriexochelins, developed with solvent system A. The chromatographed exochelins were (1) *M. bovis* BCG, (2) *M. avium* M3, (3) *M. avium* M24, (4) *M. intracellulare* M12, (5) *M. intracellulare* 17573, (6) *M. paratuberculosis* M31, and (7) *M. scrofulaceum* NCTC 10803. Mycobactin (8) from *M. intracellulare* M12 was run as a marker. (Ferriexochelins were detected visually.)

bactin synthesis (12) and a potential mycobacterial iron-transporting molecule (14), was not detected by this ^{55}Fe-binding experiment. No cell-associated iron-binding compounds were produced by the mycobactin-dependent organisms. Only chloroform-soluble exochelins were detected as iron-binding compounds in the culture filtrates. These were separated into many red bands on HPTLC (solvent system A) as was found above. Autoradiography (Fig. 3) confirmed that these bands corresponded to ^{55}Fe-binding compounds and revealed the presence of even more of these compounds.

The chromatograms of the exochelins (Fig. 2 and 3) suggest that the differences in the production of the exochelins between the MAIS organisms, and also *M. paratuberculosis*, possibly have more to do with the differences in the amount of each component produced than with radical structural differences in the components themselves.

Utilization of ^{14}C-labeled mycobactin and [*carboxy*-^{14}C]salicylate. In the above experiments, the mycobactin-dependent strains of *M. avium* and *M. paratuberculosis* had been supplemented with 3 μg of ferrimycobactin S per ml to enable them to grow. When these cultures were each examined individually, mycobactin could neither be extracted from the cells nor detected in the culture filtrates. To find out what had happened to the mycobactin that had been added, cultures (100 ml) of two mycobactin-dependent strains (*M. avium* M24 and *M. paratuberculosis* 465-BC) and a mycobactin-independent strain (*M. intracellulare* M12) were supplemented with 12 μg of ^{14}C-labeled mycobactin (i.e., more than enough to satisfy the dependence upon mycobactin) plus 8.3 μg of salicylate per ml (see Materials and Methods). At the same time, to see whether mycobactin could be synthesized from salicylate by any of these strains, as had been shown for *M. smegmatis* (12), similar cultures were supplemented with 8.3 μg of [*carboxy*-^{14}C]salicylate per ml plus 12 μg of mycobactin S per ml.

The *M. avium*, *M. intracellulare*, and *M. paratuberculosis* strains were incubated as above and were examined after 8 (*M. avium* and *M. intracellulare*) and 12 (*M. paratuberculosis*) weeks. The cells were extracted three times with ethanol to ensure that all of the mycobactin and salicylate were removed from the cells. The extracted cells were then solubilized with Soluene TM-100 to release their intracellular contents.

With the mycobactin-dependent strain of *M. avium* M24 and *M. intracellulare* M12 about 50% of the total radioactivity added as ^{14}C-labeled mycobactin and about 4% of that added as [*carboxy*-^{14}C]salicylate was measured in the ethanol extracts from the cells (Table 4). No radioactivity remained associated with the cell residue. Thus, despite the fact that the mycobactin was essential for the growth of the dependent strain, there appeared to be no difference between this and the mycobactin-independent strain in the utilization of mycobactin. With *M. paratuberculosis* 465-BC, which also needs mycobactin as a growth factor, about 77% of the total radioactivity added as ^{14}C-labeled mycobactin and about 60% of that added as [*carboxy*-^{14}C]salicylate was recovered from the cells by ethanol extraction. Clearly, the uptake of mycobactin and salicylate was greater for the mycobactin-dependent *M. paratuberculosis* strain than for the similarly incompetent *M. avium* strain, and this might reflect differences in the acquisition of iron by these two species.

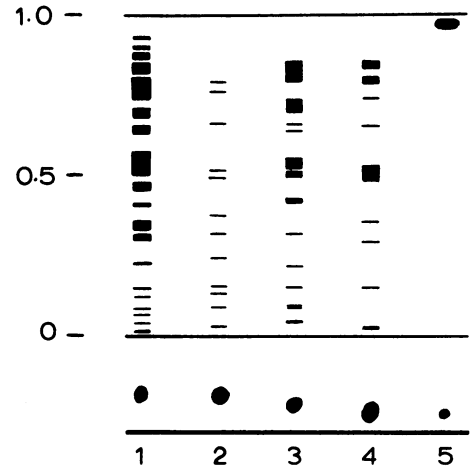

FIG. 3. Autoradiograph after HPTLC (solvent system A) of extracellular ^{55}Fe-binding compounds from (1) *M. avium* M3, (2) *M. avium* M36, (3) *M. scrofulaceum*, and (4) *M. paratuberculosis* 465-BC. Mycobactin (5) from *M. intracellulare* M12 was used as a marker.

The nature of the radiolabels recovered from the cells and culture filtrates was examined by TLC (solvent system A) and autoradiography to assess the possible metabolism of the added compounds. With the three cultures supplemented with [*carboxy*-^{14}C]salicylate, the only radioactive compounds in the culture filtrates, or their chloroform extracts, was salicylate itself. With the ^{14}C-labeled mycobactin-supplemented cultures, most of the label in the culture filtrate remained associated with the mycobactin, but about 0.5% was recovered in a fraction corresponding to salicylate. No radioactivity was recovered in the exochelin, which indicated that these compounds have independent metabolic origins.

TABLE 4. Recovery of ^{14}C after growth of mycobactin-dependent and -independent strains under iron-deficient conditions and supplemented with (i) 12 μg of ^{14}C-labeled mycobactin S (0.168 μCi/ml) per ml plus 6 μmol of salicylate, (ii) 6 μmol of [*carboxy*-^{14}C]salicylate (2.1 μCi/ml) plus 12 μg of mycobactin S per ml, or (iii) 6 μmol of salicylate plus 12 μg of mycobactin S per ml

Organism	Radiolabel added	Cell dry wt (mg)	Total radioactivity recovered, with background subtracted (dpm × 10^4)			
			Cell extract	Extracted cells	Chloroform extract from cultures filtrates	Chloroform-extracted culture filtrates
M. intracellulare M12 (mycobactin independent)	None	104.2	0	0	0	0
	^{14}C-mycobactin	105.5	1.29	0	0.16	1.71
	[*carboxy*-^{14}C]salicylate	107.6	1.96	0	15.4	28.76
M. avium M24 (mycobactin dependent)	None	112.2	0	0	0	0
	^{14}C-mycobactin	102.6	1.70	0	0.10	2.02
	[*carboxy*-^{14}C]salicylate	99.4	1.78	0	0.10	41.13
M. paratuberculosis 465-BC	None	99.3	0	0	0	0
	^{14}C-mycobactin	104.0	2.77	0	0.02	0.80
	[*carboxy*-^{14}C]salicylate	96.6	20.49	0	0.52	18.87

With the cell extracts from these three cultures, most of the [*carboxy*-^{14}C]salicylate that had been taken up remained unmodified and was extracted as unchanged salicylate (Table 5). However, for the *M. avium* and *M. intracellulare* strains an additional radioactive compound was detected which had an R_f value the same as that of an *M. avium* mycobactin. The mycobactin-independent strain (M12) produced more of this compound than the dependent strain (M24), whereas the mycobactin-dependent *M. paratuberculosis* strain did not produce it at all (Table 5).

Of the labeled mycobactin taken up by each of the three strains, some was hydrolyzed to salicylate. With the *M. avium* and *M. intracellulare* strains almost 50% of the added mycobactin S was either hydrolyzed to give free salicylate or metabolized to form an *M. avium* type of mycobactin. The difference between the mycobactin-dependent and -independent strains appeared to be in the ability of the independent strain to synthesize mycobactin, using the salicylate released from the degradation of the added mycobactin S. (It seems unlikely that the added mycobactin S would have been structurally modified to give a new mycobactin type, Av, although this possibility cannot as yet be entirely eliminated.) The *M. paratuberculosis* strain differed from the *M. avium* M24 (mycobactin-dependent) strain in that nearly 90% of the mycobactin taken up by the cells was hydrolyzed to give free salicylate and also no mycobactin, other than that supplied, was detected.

TABLE 5. Distribution of radioactivity from ethanolic extracts of cells of mycobacteria supplemented with ^{14}C-labeled mycobactin S or [*carboxy*-^{14}C]salicylate[a]

Mycobacterium species and strain no.	Marker compound with which R_f of test compound was equivalent	[*carboxy*-^{14}C]salicylate-grown cells		^{14}C-mycobactin-grown cells	
		(dpm × 10^4)	% Distribution	(dpm × 10^4)	% Distribution
M. intracellulare M12	Salicylate	1.40	71.4	0.35	26.8
	Mycobactin S	0	0	0.69	53.8
	Mycobactin Av	0.56	28.6	0.25	19.3
M. avium M24 (mycobactin dependent)	Salicylate	1.75	98.4	0.8	47.0
	Mycobactin S	0	0	0.89	52.5
	Mycobactin Av	0.03	1.6	0.01	0.5
M. paratuberculosis (mycobactin dependent)	Salicylate	20.49	100	2.43	87.8
	Mycobactin S	0	0	0.34	12.2
	Mycobactin Av	0	0	0	0

[a] Data were calculated after TLC of the cell extract.

Exochelin as a growth factor. The production of exochelins by both mycobactin-independent and mycobactin-dependent organisms indicates a biological role for these molecules. In part, this has already been confirmed by showing the ready uptake of iron chelated to exochelin into *M. avium* (18). However, the possibility of exochelin acting as a growth factor in its own right for the mycobactin-dependent strains was also examined. The results of a preliminary study (Table 6) clearly showed that exochelin, extracted from *M. avium* M12 (mycobactin independent), was as effective as mycobactin, taken from *M. smegmatis*, in stimulating growth of two mycobactin-dependent organisms (*M. avium* WP7/74 and *M. paratuberculosis* 465-BC). It was noteworthy that the addition of exochelin and mycobactin together produced a greater stimulation of growth than either separately. (Further details of the studies on exochelin as a growth factor will be presented in due course.)

DISCUSSION

The iron-limiting conditions under which *M. avium* and *M. paratuberculosis* (and indeed all pathogenic bacteria) are found in nature compel these organisms to acquire iron by means of high-affinity iron-binding compounds. That in vitro growth of the mycobactin-dependent mycobacteria requires supplementation of their medium with mycobactin might imply that these organisms do not produce their own iron-binding compounds and would hence find it difficult to grow in vivo. Yet, these mycobacteria cause disease in animals. Certainly, freshly isolated mycobactin-dependent strains do not produce significant amounts of mycobactin but, as we have shown here, they do produce small amounts of exochelin. Exochelins, as iron-binding compounds, are likely candidates for the acquisition of iron both in the extracellular milieu of the organism when growing in vivo and in laboratory culture in vitro (3, 17, 18). However, production of exochelin does depend on the growth and metabolic activity of these organisms, and growth cannot occur without iron. Thus, it might be postulated that the iron needed to initiate growth of these mycobacteria is needed in a specific form and that this would be fulfilled by a small amount of mycobactin. After growth has been initiated, a continuous supply of mycobactin need not be essential as exochelin would then be produced. This could then explain the observation that small inocula of *M. paratuberculosis* are more dependent upon mycobactin than are large inocula (4) as the carry-over of the exochelin with the inoculum would then be higher. It would also explain our own observation (Table 6) that the requirement for mycobactin is diminished if exochelin is added. Indeed, the exochelins can satisfy the mycobactin requirement in their own right.

TABLE 6. Growth stimulation of mycobacteria by mycobactin S and *M. avium* M12 exochelin after 8 weeks at 42°C (*M. avium*) or 12 weeks at 37°C (*M. paratuberculosis*) under iron-deficient conditions

Supplement to culture medium (3 μg/ml)	Cell yield (mg [dry wt]/100-ml culture) of:		
	M. avium		*M. paratuberculosis* 465-BC[a]
	A8/12[b]	WP7/74[a]	
None	79.1	0	0
Mycobactin	80.7	80.4	109.7
Exochelin	80.1	90.9	109.0
Mycobactin plus exochelin	81.6	142.6	120.8

[a] Mycobactin dependent.
[b] Mycobactin independent.

The presence of the exochelins also explains how the mycobactin requirement of *M. paratuberculosis* can be circumvented by using a medium of low pH if ferric ammonium citrate is added (9). At the low pH sufficient iron must be able to gain access to the cell, probably by simple diffusion, to allow growth to be initiated. Exochelins would then be synthesized and quickly take over the role of iron transport. Similarly, the culture media devised by Merkal and Curran (6) for growth of *M. paratuberculosis* without mycobactin is explicable in terms of the medium stimulating initial growth to allow some subsequent expression of exochelin biosynthesis. The success of 1% ferric ammonium citrate in encouraging growth of *M. paratuberculosis* (6) obviously lies in the ability of this organism to take up iron as ferric citrate, a process which has already been recognized in *M. smegmatis* (8) and which could be ubiquitous among most mycobacteria.

The mycobactin-dependent strains of mycobacteria differ from the mycobactin-independent strains by being unable to initiate growth in a conventional growth medium without the addition of mycobactin (or exochelin). Once the initial impediment to growth has been overcome, the organisms are seemingly able to produce their own exochelins and thus elaborate a mechanism for iron acquisition. However, the role of mycobactin in this mechanism of iron uptake is still a matter of conjecture (10), although we do know that mycobactin is not involved in the acquisition of iron via the water-soluble exochelins (17). It may be that the role of mycobactin principally resides in it being able to act as a store of iron rather than functioning as a means of the cell rapidly acquiring iron. (This, however, does not mean that mycobactin does

not act as an iron transport compound, only that its capacity to do so is confined to a narrow range of conditions.)

How the in vitro observations extend into the in vivo situation for the mycobactin-dependent mycobacteria is a matter of speculation. However, with the recognition of the elaboration of the exochelins by this group of organisms, the explanation of how they are able to multiply in vivo now becomes clearer. It is no longer necessary to postulate that these mycobacteria acquire mycobactin from commensal mycobactin-competent mycobacteria in order to grow. It would also seem that at least some of these mycobactin-dependent strains can synthesize small amounts of their mycobactin. Merkal and colleagues (6) have observed that strains of *M. paratuberculosis*, when maintained in the laboratory over long periods, frequently lose mycobactin dependence. A recent examination (7) of one such strain has indeed revealed the presence of a small amount of mycobactin (mycobactin J at approximately 9.5 mg per g of cell dry weight). Although we did not find this with our strains of *M. paratuberculosis*, we nevertheless were able to recover mycobactin (at approximately 0.3 mg per g of cell dry weight) from some of the mycobactin-dependent strains of *M. avium* which had lost their mycobactin dependency. We were also able to show, using radiolabeling techniques, that nonrevertant strains did in fact contain very small amounts of mycobactin. Thus, mycobactin dependency is probably not due to a genetic inability to synthesize mycobactin but rather to strong phenotypic repression. Merkal et al. (7) considered that the repression of mycobactin synthesis in *M. paratuberculosis* occurred because of its usual habitat, being within the macrophage. This, however, seems to beg the question as to why *M. paratuberculosis* should be an exception when this does not seem to occur with other mycobacteria isolated from macrophages.

In conclusion, our results suggest that both mycobactin and exochelin are necessary for good mycobacterial growth and that the exochelins, because of their ubiquity among these organisms, probably represent the single most important means of iron acquisition in vitro and, by extension, also in vivo.

ACKNOWLEDGMENTS

This work was supported by a grant from the Agricultural Research Council.

We thank Pauline Shepherd for fine technical assistance.

LITERATURE CITED

1. **Goodfellow, M., and L. G. Wayne.** 1982. Taxonomy and nomenclature, p. 471–521. *In* C. Ratledge and J. L. Stanford (ed.), The biology of the mycobacteria, vol. 1. Academic Press, London.
2. **Kochan, I., N. R. Pellis, and C. A. Golden.** 1971. Mechanism of tuberculosis in mammalian serum. III. Neutralization of serum tuberculosis by mycobactin. Infect. Immun. **3**:553–558.
3. **Macham, L. P., C. Ratledge, and J. C. Nocton.** 1975. Extracellular iron acquisition by mycobacteria: role of exochelins and evidence against the participation of mycobactin. Infect. Immun. **12**:1242–1251.
4. **Matthews, P. R. J., A. McDiarmid, P. Collins, and A. Brown.** 1977. The dependence of some strains of *M. avium* on mycobactin for initial and subsequent growth. J. Med. Microbiol. **11**:53–57.
5. **McDiarmid, A.** 1948. The occurrence of tuberculosis in the wild woodpigeon. J. Comp. Pathol. Ther. **58**:128–133.
6. **Merkal, R. S., and B. J. Curran.** 1974. Growth and metabolic characteristics of *Mycobacterium paratuberculosis*. Appl. Microbiol. **28**:276–279.
7. **Merkal, R. S., W. G. McCullough, and K. Takayama.** 1981. Mycobactins, the state of the art. Bull. Inst. Pasteur Paris **79**:251–259.
8. **Messenger, A. J. M., and C. Ratledge.** 1982. Iron transport in *Mycobacterium smegmatis*: uptake of iron from ferric citrate. J. Bacteriol. **149**:131–135.
9. **Morrison, N. E.** 1965. Circumvention of the mycobactin requirement of *Mycobacterium paratuberculosis*. J. Bacteriol. **89**:762–767.
10. **Ratledge, C.** 1982. Nutrition, growth and metabolism, p. 185–271. *In* C. Ratledge and J. L. Stanford (ed.), The Biology of the mycobacteria, vol. 1. Academic Press, London.
11. **Ratledge, C.** 1982. Mycobactins and nocobactins, p. 575–581. *In* A. I. Laskin and H. A. Lechevalier (ed.), CRC handbook of microbiology, vol. 4, 2nd ed. CRC Press Inc., Boca Raton, Fla.
12. **Ratledge, C., and M. J. Hall.** 1970. Uptake of salicylic acid into mycobactin S by growing cells of *Mycobacterium smegmatis*. FEBS Lett. **10**:309–312.
13. **Ratledge, C., and M. J. Hall.** 1971. Influence of metal ions on the formation of mycobactin and salicylic acid in *Mycobacterium smegmatis* grown in static culture. J. Bacteriol. **108**:314–319.
14. **Ratledge, C., L. P. Macham, K. A. Brown, and B. J. Marshall.** 1974. Iron transport in *Mycobacterium smegmatis*: a restricted role for salicylic acid in the extracellular environment. Biochim. Biophys. Acta **372**:39–51.
15. **Ratledge, C., and B. J. Marshall.** 1972. Iron transport in *Mycobacterium smegmatis*: the role of mycobactin. Biochim. Biophys. Acta **279**:58–74.
16. **Snow, G. A.** 1970. Mycobactins: iron-chelating growth factors from mycobacteria. Bacteriol. Rev. **34**:99–125.
17. **Stephenson, M. C., and C. Ratledge.** 1979. Iron transport in *Mycobacterium smegmatis*: uptake of iron from ferriexochelin. J. Gen. Microbiol. **110**:193–202.
18. **Stephenson, M. C., and C. Ratledge.** 1980. Specificity of exochelins for iron transport in three species of mycobacteria. J. Gen. Microbiol. **116**:521–523.