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MINIREVIEW

Iron Acquisition and Metabolism by Mycobacteria

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AN OVERVIEW OF IRON METABOLISM

Iron is a prerequisite for in vitro growth of mycobacteria. Iron is an obligate cofactor for at least 40 different enzymes encoded in the *Mycobacterium tuberculosis* genome (8). It is required for the cytochromes involved in electron transport and other hemoproteins involved in oxygen metabolism, such as catalase-peroxidase KatG. Most of the intracellular iron in mycobacteria, however, is in the form of nonheme iron (48). One important form of nonheme iron is the iron-sulfur clusters that are cofactors of proteins involved in amino acid and pyrimidine biogenesis, as well as in enzymes involved in the tricarboxylic acid cycle and electron transport. Iron is also required for DNA synthesis in ribonucleotide reductase, superoxide dismutase, and 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (Rv2178c; DAHP synthase [Every gene in the genome of *M. tuberculosis* H37Rv has been assigned a gene identifier beginning with Rv which can be used to retrieve information on line; see reference 30a]). It has been estimated that 7 to 64 μg of Fe/g of mycobacterial cell mass is required to support growth (39, 48). Iron limitation in vitro to levels below these can result in growth restriction in many species of mycobacteria, including pathogens such as *M. tuberculosis*. Since iron is the fourth most abundant element, difficulty in its acquisition may seem counterintuitive. However, the extremely low solubility of ferric ion in aqueous solution makes obtaining it difficult and so microorganisms have evolved many strategies for acquiring sufficient soluble iron for aerobic growth.

Iron sequestration represents a formidable challenge to in vivo growth of pathogenic mycobacteria. An important component of the mammalian host defense against bacterial pathogens involves restricting access of such organisms to iron (35). *M. tuberculosis* is a highly specialized pathogen of humans and must contend with iron sequestration in order to survive in the human lung. Pulmonary tuberculosis patients are often anemic, suggesting sequestration of available iron by the host (5). The serum of many mammals, including humans, is tuberculostatic because of its ability to sequester iron from the bacilli (32, 34). The addition of iron to such sera relieves the bacteriostatic effect completely. In a tragic attempt to rectify what was perceived as a debilitating iron deficiency in infected patients in Somalia, iron supplementation was found to actually promote the development of active tuberculosis (54). Other clinical studies in Africa have also established a strong correlation between dietary iron overload and an enhanced risk of death from tuberculosis (20, 52). Purified transferrin inhibits the growth of *M. tuberculosis* in vitro (33), and haptoglobin may play a role in restricting access to heme iron (29, 57). Thus, iron availability or sequestration and the bacterium's efforts to circumvent this restriction are critical determinants of the outcome of infection with *M. tuberculosis*.

Infections with mycobacteria of the *M. avium* complex (MAC) occur predominantly in human immunodeficiency virus-infected patients and are a major source of mortality for such patients. Iron storage in macrophages is known to be increased in patients with AIDS (although overall iron levels in serum are lower than those of patients without AIDS), and this observation has been proposed to underlie the predisposition of such patients to MAC infections (11, 12, 22). Animal model experiments support this hypothesis in that mice fed iron-rich diets more rapidly develop disease from MAC (11). In cultured human macrophages, the rate of MAC replication has been shown to be directly correlated with the concentration and iron loading of transferrin (12).

Mycobacterial iron acquisition is mediated by siderophores. The problem of iron acquisition has been solved by many microorganisms, including mycobacteria, by producing small, soluble iron chelators known as siderophores. These substances are generally secreted by the organism to compete with environmental iron binding molecules for the small amount of available iron. Siderophore synthesis is typically controlled by extracellular iron abundance, and these molecules display a high affinity for ferric iron. Four types of structurally distinct molecules are thought to be involved in iron acquisition in mycobacteria, salicylic acid and citric acid being the simplest, followed by two classes of more complex siderophores. The first, the mycobactins (MBs), are distinguished primarily by the presence of a phenyloxazolidine ring, while the second class, the exochelins, are peptidic siderophores whose iron-chelating ability is associated with ornithine-derived hydroxamates. MBs and exochelins are complex molecules with very high affinities for iron and are integral to the bacterial strategy for obtaining iron from the environment.

Early work focused on the dramatic accumulation of salicylic acid secreted into the growth medium coincident with iron starvation of either slow-growing or fast-growing mycobacterial species (60). Salicylate was postulated to be an important extracellular siderophore, but the stability of the salicylate-iron complex appears to be too low to support this hypothesis. However, the amount of salicylate secreted into growth media upon iron starvation reaches as much as 2.28 mg/100 ml of medium and this concentration suggests a secondary physiological role, perhaps in facilitating iron solubilization (60). Citric acid has also been proposed to be involved in iron acquisition in *M. smegmatis* on the basis of cell association of an iron-citrate complex (51). However, the very high concen-

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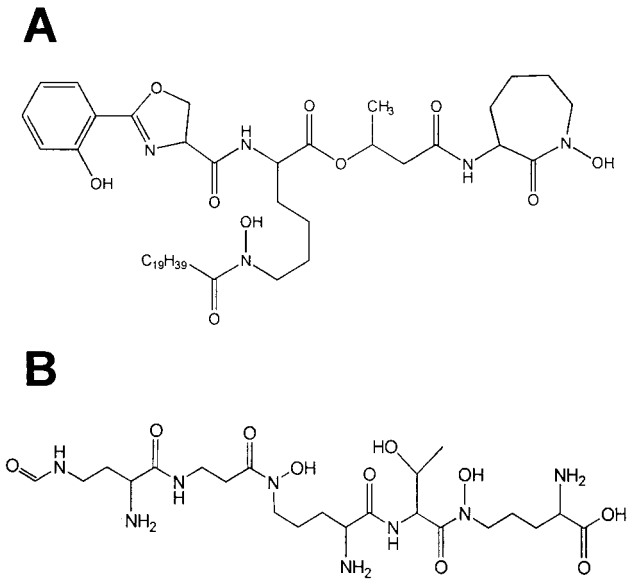


FIG. 1. Siderophores of mycobacterial species. (A) Structure of the major MB of *M. tuberculosis* (MB T). (B) Structure of the major exochelin of *M. smegmatis* (exochelin MS). Peptidic siderophores such as exochelin MS have only been identified from fast-growing saprophytic strains, while MBs such as that shown in panel A have been found widely distributed among mycobacterial species.

trations of citric acid required, the lack of inducibility of citrate synthesis upon iron starvation, and the lack of an energy-dependent specific uptake system for such complexes all argue against such a role.

The MBs and exochelins (Fig. 1A and B) are produced specifically in response to iron starvation and are known to have a high affinity for iron(III). The increase in MB production in iron-limiting medium has been recognized since the very first isolation work (15). Iron sufficiency has been reported to decrease MB content by as much as 10,000 times (45, 59), although a much smaller decrease is generally reported (58). Mycobacterial species fall into four groups based upon the expression of these molecules. *M. tuberculosis* produces only the MB type of siderophore, *M. vaccae* produces only the exochelin type, *M. smegmatis* produces both types, and some strains of *M. paratuberculosis* produce none (50, 60). The real distribution of organisms among these groups is difficult to assess precisely because of substantial confusion over the use of the term exochelin (when what is meant is a water-soluble MB or a mixture of MBs and exochelins in species which produce both). Many authors equate exochelin with water-soluble MB in spite of the fact that exochelin was first proposed to represent the non-salicylate-containing peptidic siderophores of *M. smegmatis* (Fig. 1B) (70, 71). Use of the term exochelin should be limited to those molecules structurally related to the peptidohydroxamate siderophores typified by the structure of exochelin MS (Fig. 1B). Molecules related to the salicylate-containing siderophores which contain chemical functionality rendering them more water soluble should be more properly referred to as MBs (or water-soluble MBs, also referred to as exomycobactins [84]). Some strains of mycobacteria, in particular, of *M. paratuberculosis*, that do not produce any siderophores are dependent for growth upon the inclusion of such molecules in the medium (4, 24, 79). Species, such as *M. bovis*, which produce only MBs are not able to utilize water-soluble peptidic exochelins for uptake of iron (77). In vitro growth of an MB-deficient mutant of *M. tuberculosis* H37Rv was significantly impaired under low-iron conditions compared to the wild-type strain, supporting a critical role for this molecule in iron acquisition and transport (10).

The interplay of cell-associated and secreted siderophores in iron acquisition. In many species of mycobacteria, it has been noted that two forms of siderophore are often present, one primarily cell associated and one secreted or medium associated. This has given rise to a hypothesis that there are two distinct roles for such molecules. The extracellular form competes directly for environmental iron, while the cell-associated form acts as an ionophore, facilitating transport into the cell cytoplasm (43, 64). The water-soluble form of MB from *M. bovis* BCG (confusingly labeled exochelin MB-2) is capable of supporting ferritin-dependent growth of *M. bovis* BCG even when the iron source is separated from the growing cells by a dialysis membrane (43). The restriction of growth which accompanies culture of the tubercle bacillus in serum can be reversed by addition of MB and transferrin or ferritin but not by that of either molecule alone (19). More recently, the water-soluble MBs of *M. tuberculosis* (again confusingly mislabeled exochelins) were shown to acquire iron from 40% iron-saturated transferrin and to transfer this iron to the cell-associated MBs (17). Transferrin receptors, and presumably therefore transferrin, are present in the phagosomal membrane surrounding living *M. tuberculosis* within human monocytes (7). These experiments support the hypothesis that both soluble and cell-associated forms of siderophore are required for iron acquisition from the mammalian host in vivo for mycobacterial pathogenesis. Finally, and definitively, the availability of a knockout in the biosynthetic pathway which produces both forms of MB in *M. tuberculosis* has allowed us to demonstrate that a mutant incapable of producing both types of MBs has lost the ability to grow within macrophages (10). The definitive experiments regarding the hypothetical specific role for cell-associated compared to secreted siderophores must await the identification and specific modification of the acyl transferases which attach the R_5 chain. These bifurcate the biosynthetic pathway and determine the ultimate ratio of MB to water-soluble MB produced by *M. tuberculosis*.

Storage of iron in mycobacterial species. Mycobacterial siderophores are thought to be taken up and transported across the cell membrane by specific receptors and transporters, by analogy to other siderophore uptake mechanisms. Siderophores are transient reservoirs of iron which bind to ferric iron during transport into the cell cytoplasm. Recent experimental evidence shows that very little intracellular iron is contained within these molecules on a long-term basis (31). In situ Mössbauer spectroscopy and electron paramagnetic resonance spectroscopy have been used to confirm these results, as signals associated with siderophore-bound iron are only observed upon short-term labeling of iron pools with ^{57}Fe (46, 47). Long-term labeling experiments have revealed that under iron-sufficient conditions, iron is accumulated in a form with spectral parameters consistent with bacterioferritin (46). Bacterioferritin proteins have been purified from *M. leprae* (55), and a bacterioferritin gene has been cloned from *M. avium* (30). On the chromosome of *M. tuberculosis*, there are apparently two bacterioferritin homologs, *bfrA* (Rv1876) and *bfrB* (Rv3841) (2).

STRUCTURE OF MYCOBACTERIAL SIDEROPHORES

An invariant "core" MB structure. The structure of MB P, isolated from *M. phlei*, was initially established through chemical degradation by Snow (74). Since that time, a large number

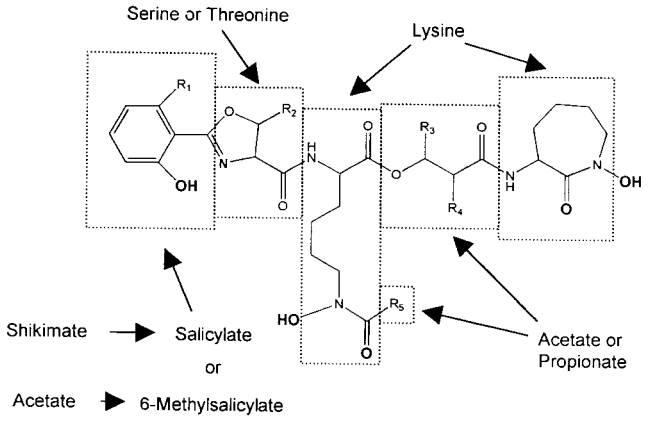
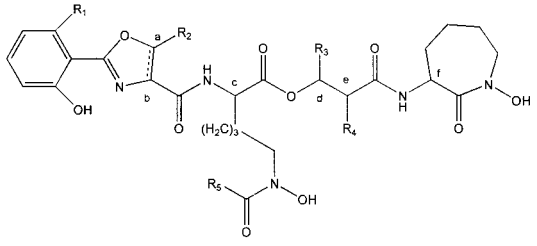


FIG. 2. General structure of MBs. Iron-chelating groups are in boldface. Letters and R groups correspond to the structures listed in Fig. 3.

of MBs have been isolated but their core structure displays remarkably little variation (Fig. 2) (73). They all have a 2-hydroxyphenyloxazoline moiety linked via an amide bond to an acylated ε-*N*-hydroxylysine residue. This lysine is, in turn, esterified at the α-carboxyl with a β-hydroxy acid that forms an amide link with a second ε-*N*-hydroxylysine which has cyclized to give a seven-membered lactam. The absolute configurations of the stereogenic centers derived from an amino acid (where known) all appear to arise from the naturally occurring L series. This basic structure serves to chelate ferric iron very effectively via the two hydroxamic acids of the ε-*N*-hydroxylysines, the N of the oxazoline ring, and the phenolate oxygen atom (25). Although a formation constant of 10^{30} M^{-1} is widely quoted, the only direct measurements have monitored the dissociation of ^{59}Fe-labeled ferri-MB S under strongly acidic conditions. From this data, one can extrapolate a formation constant of approximately 4×10^{26} M^{-1} at pH 7 (41). Coordination to ferrous iron by MB is much less effective, allowing release of iron from a ferri-MB complex by reduction. Computational work suggests that the reason for ferric ion selectivity lies in the confluence of the size of the ferric cation and the cavity created by a sterically favorable organization of the ligand (40).

Within this core of the MB, a methyl group may or may not be present at the position 6 of the phenolic ring (R_1) and/or at the 5′ position of the oxazoline (R_2) (Fig. 3). The presence of both types of oxazoline in *M. tuberculosis* has been reported (18), and MBs containing both aromatic moieties are found in *M. fortuitum* (75). One final core variation seen in some of the MBs from *Nocardia* sp. (the nocobactins) is an aromatic oxazole in place of the oxazoline ring (53, 65).

Variation in alkyl substitution patterns of MBs. In contrast to the relatively subtle variation seen in the nuclei of MBs, the alkyl substituents of the hydroxy acid (R_3 and R_4) and the acyl moiety R_5 are highly variable (Fig. 3) (73). The β-hydroxy acid is usually β-hydroxybutyrate or 2-methyl-3-hydroxypentanoic acid. Both absolute configurations of the β-hydroxybutyrate have been reported from different MBs (75). Similarly, the absolute stereochemistry of the 2-methyl-3-hydroxypentanoic acid has been reported, and although the relative stereochem-



Mycobactin	R_1	R_2	R_3	R_4	R_5	a‡	b	c	d	e	f	Species
A	CH_3	H	CH_3	H	C_{13}	-	?	?	?	-	?	*M. aurum*
Av	H	CH_3	CH_3CH_2	CH_3	$C_{11\text{-}14,18}$	?	?	?	?	?	?	*M. avium*
Water Sol. Av*	H	CH_3	CH_3CH_2	CH_3	$C_{2\text{-}10}$*	?	?	?	?	?	?	"
F	CH_3/H	CH_3	CH_3	H	$C_{9\text{-}17}$	*threo*		?	S	-	L	*M. fortuitum*
H	CH_3	CH_3	CH_3	H	$C_{17,19}$	R	L	L	S	-	L	*M. thermoresistible*
J†	H	H	$(CH_3)_2CH$	CH_3	C_{15}	?	-	?	*erythro*		?	*M. paratuberculosis*
M	H	CH_3	$C_{15\text{-}18}$	CH_3	C_1	?	?	?	*erythro*		?	*M. marinum*
N	H	CH_3	$C_{15\text{-}18}$	CH_3	C_2	?	?	?	?	?	?	*M. marinum*
P	CH_3	H	CH_3CH_2	CH_3	$C_{15\text{-}19}$	-	L	L	S	R	L	*M. phlei*
R	H	H	CH_3CH_2	CH_3	C_{19}	-	L	L	R	S	L	*M. terrae*
S	H	H	CH_3	H	$C_{9\text{-}19}$	-	L	L	S	-	L	*M. smegmatis*
T	H	H	CH_3	H	$C_{17\text{-}20}$	-	?	?	R	-	L	*M. tuberculosis*
Water Sol. T*	H	H/CH_3	CH_3	H	$C_{2\text{-}8}$*	-/?	?	?	?	?	-	"
Formobactin¶	H	CH_3	C_9	CH_3	H	-	?	?	?	?	-	*Nocardia sp.*
NA¶	H	CH_3	$C_{9,11}$	CH_3	C_1	-	?	?	?	?	?	*N. asteroides*

FIG. 3. General structures of known MBs. Symbols: ∗, the terminal carbon in this chain of this water-soluble MB is either a carboxylic acid or its methyl ester; †, the structure as originally reported (49); §, MB that contains an oxazole moiety instead of the usual oxazoline and is found in *Nocardia* sp; ‡, letters refer to the stereocenters shown in the structure above, − indicates that the center is not chiral in this compound, and ? indicates that the stereochemistry is unknown.

istry is always *erythro*, both absolute configurations are seen in different MBs. There are also reports of more complex β-hydroxy acids with branched chains (R_3 = *i*-Pr, R_4 = Me) (49), long-chain alkyl substituents (R_3 = $C_{15–18}$, R_4 = Me) (3, 75), or both (R_3 = C_9, R_4 = Me_2) (53) being employed.

The acyl moiety (R_5) is the source of most of the observed structural variation in the MBs (73). In general, the acyl group is a long-chain fatty acid (C_{10} to C_{21}) that is often unsaturated with an unusual *cis* double bond conjugated to the carbonyl group (73). However, enormous variation on this theme is observed. Ratledge and Ewing detailed 17 different MBs from a single strain of *M. smegmatis* that differ only at R_5; 7 of these compounds were present in significant amounts (61)! Thin-layer chromatography and high-performance liquid chromatography have been employed extensively, with some success, to identify strains of mycobacteria to the species level; however, they give no information on the exact structures of the MBs isolated (38). At least three different structures for MB J (the major MB from *M. paratuberculosis*) have appeared, the final one without comment in the context of other work (4, 18, 49). In addition, recent work on the carboxy-MBs or the chloroform-extractable exochelins has added to the complexity of the situation (18, 36, 37, 81). These compounds are clearly MBs with a modified acyl group (R_5) derived from a dicarboxylic acid. This may be present as the free acid, the methyl ester, or a mixture of the two. Once again, a number of analogues are observed, with 20 different compounds identified from *M. avium* that differ only at R_5 (81).

Nomenclature of extracellular MBs. The small structural difference between the prototypical MBs and the extracellular compounds, i.e., the presence of an additional carboxyl or ester functionality on R_5, leads to a profound increase in water solubility. This prompted the two groups of workers involved to each suggest a new name for this class of compounds, i.e., the carboxy-MBs (37) or the exochelins (18). Unfortunately, both are problematic. The name exochelin has already been applied to structurally unrelated, extracellular, peptidic siderophores from *M. smegmatis* (71) and *M. neoaurum* (70) and belies the structural similarity of these exochelins and the MBs. The name carboxy-MB, while addressing the latter concern, is inappropriate, as both the methyl esters and the free acids of these compounds have been reported (81). It seems best simply to refer to this class of compounds as water-soluble MBs, a term that is indicative of both their structure and their distinguishing physical characteristic. (The actual situation is even more complex. Ratledge and coworkers discovered water-soluble, mycobacterial siderophores and collectively termed them exochelins [42]. It was known that there were at least two types based on solubilities [43], and the first to be structurally characterized were peptidic compounds unrelated to MBs and named exochelins [70, 71]. Subsequently, the water-soluble MBs were identified [18, 37].) The more insightful term exo-MB, which evokes both the putative functional difference of these compounds from and the structural similarity of these compounds to cell-associated MBs, has also been used to describe these extracellular MB analogs (84).

As might be expected for such a complex array of structures, systematic chemical nomenclature is too unwieldy and trivial names are generally employed. The best system still appears to be that suggested by Snow in his excellent review in 1970 (73). Families of MBs are identified by their core structures, each of which is indicated by a letter referring to a previously identified MB. The letter historically indicated the species from which the compound was isolated, e.g., MB P from *M. phlei*.

A second structural class of siderophores: exochelins. The existence of a second family of mycobacterial siderophores has been recognized for some time (42), but only recently have some of its members been characterized (70, 71). Two structures are known, both highly modified peptides with iron coordination sites provided by hydroxamic acids derived from ornithine (71), and in one case, a novel β-hydroxyhistidine (70). Exochelin MS (Fig. 1B), the major exochelin from *M. smegmatis*, reportedly representing 25 to 35% of the total of these compounds in this organism, was shown to be a formylated pentapeptide derived from three molecules of δ-*N*-hydroxyornithine, a β-alanine, and a threonine. Hydroxamic acids derived from the three ornithine moieties provide the iron coordination sites. Exochelin MN, one of the exochelins from *M. neoaurum*, is a hexapeptide consisting of two δ-*N*-hydroxyornithine residues and a β-hydroxyhistidine, which provide the iron coordination sites; two β-alanines; and an ornithine. The differences between exochelin MS and exochelin MN implies that there is a greater variation in structure and iron coordination strategy in this class of siderophore than is seen in the MBs. Thus far, such peptidic siderophores have only been reported from rapidly growing saprophytic mycobacterial species.

BIOSYNTHESIS OF MYCOBACTERIAL SIDEROPHORES

The basic building blocks. Soon after the first structure of an MB was reported, the identities of the basic biosynthetic building blocks were elucidated (Fig. 2). It was shown that L-lysine was the precursor of both ε-*N*-hydroxylysines through radiolabeling studies (1, 78). The β-hydroxy acid moiety was shown to be derived from the condensation of two propionate molecules in the case of the 2-methyl-3-hydroxypentanoic acid of MB P (78). Results obtained with [1-^{14}C]acetate were consistent with the β-hydroxybutyrate and the acyl moiety at R_5 of MB S being derived from acetate (78). The aromatic moiety of MB S from *M. smegmatis* has been shown to arise from salicylate formed via the shikimic acid pathway (27, 62, 63). The aromatic moiety of MB P, with its extra methyl group, is believed to arise from 6-methylsalicylate instead of salicylate. Once again, this acid is a known extracellular metabolite in mycobacteria that forms MBs bearing the methylated aromatic moiety (73). However, the biosynthetic origins of these two aromatic acids are very different. In contrast to salicylate, 6-methylsalicylate is not a shikimate metabolite but rather a polyketide formed from the condensation of four acetate units (28). This provides an interesting example of two very different biosynthetic pathways being utilized to provide the required structural unit, a hydroxyaromatic acid. Even more surprising is the co-occurrence of MBs in *M. fortuitum* (75) that have both a salicylate- and a 6-methylsalicylate-derived aromatic moiety. This implies that both pathways must apparently be employed by this organism (73). No studies have been carried out on the origins of the oxazoline, but serine and threonine are presumed to be the precursors of the unsubstituted and methyl-bearing rings, respectively (73).

The enzymes which assemble MBs belong to the family of nonribosomal peptide synthases. Studies of MB biosynthesis have languished for the last 20 years, only to be reinvigorated by the recent sequencing of the *M. tuberculosis* genome. A gene cluster designated the *mbt* genes was recognized by Cole et al. (8) and by Quadri et al. (56) as having the potential to encode the appropriate enzymes for MB formation (Fig. 4A). Several of the encoded proteins are highly homologous to nonribosomal peptide synthases (NRPSs), which have been implicated in the biosynthesis of other siderophores, as well as a variety of peptide-derived secondary metabolites (44). These NRPSs are large modular enzymes that activate amino acids as their acyl adenylates through an activating domain and covalently link

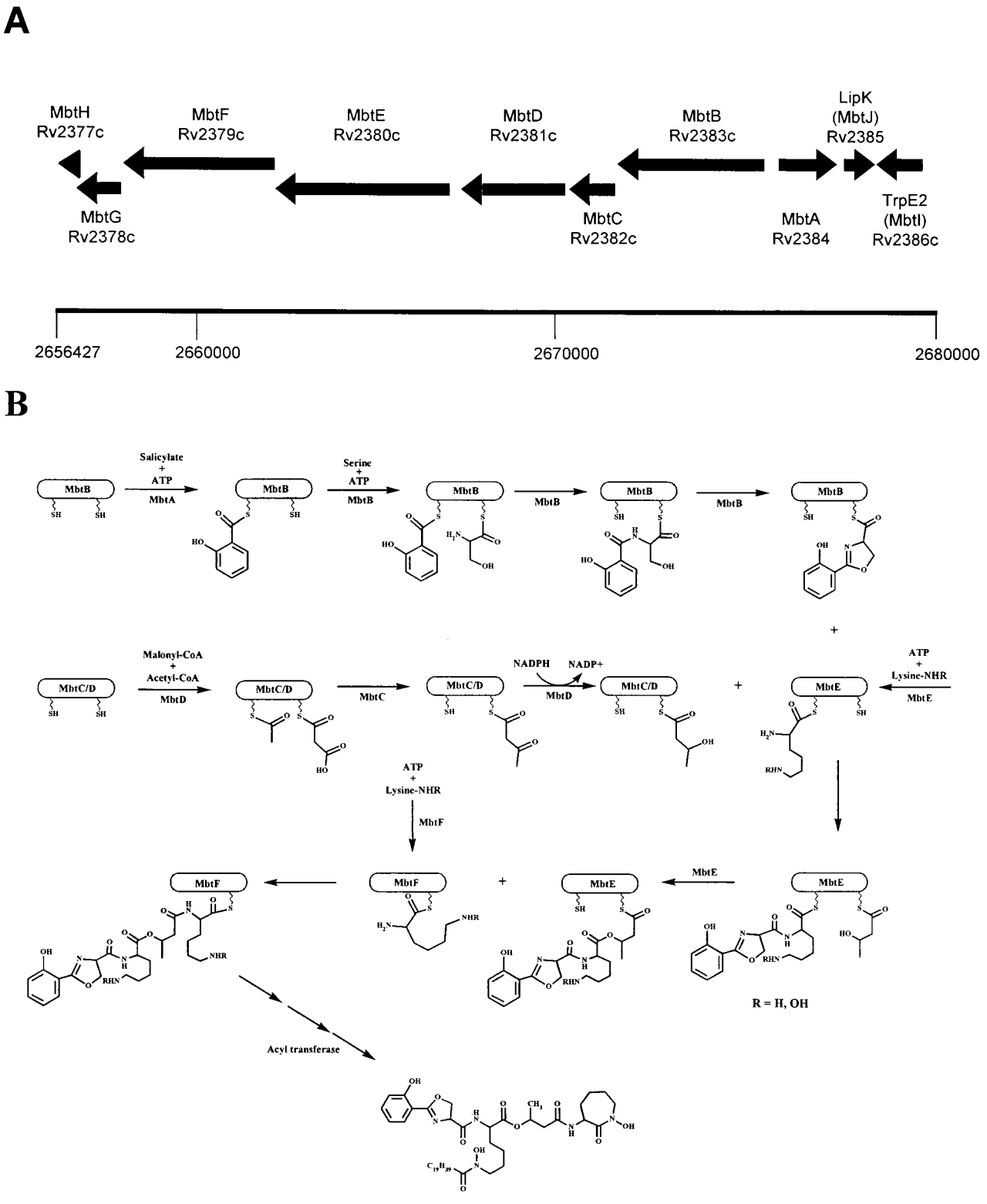


FIG. 4. Proposed biosynthetic scheme for MB T. (A) Region of the genome of *M. tuberculosis* H37Rv containing the putative MB biosynthetic enzymes. Numbering refers to chromosomal positions reported by Cole et al. (8). (B) Proposed biosynthetic cascade catalyzed by the Mbt locus. See the text for details of individual enzymatic predictions. There are significant areas of uncertainty in this model, as suggested by the following questions. (i) Does the ketoacyl synthase domain of MbtC act upon the MbtD-bound acyl carrier protein domains as shown? (ii) Does the second peptidyl/acyl carrier protein domain of MbtE function to attach the β-hydroxybutyrate as shown, or does it transfer the acyl group to the first lysine (the R_5) position in Fig. 2? (iii) Do MbtE and MbtF act in the order in which they appear in the genomic locus, or are they reversed? (iv) Is MbtG involved in N-hydroxylation as shown? (v) What is the nature of the acyltransferase, and how is the specificity determined? When does this act and upon what substrate? Since this determines the ratio of water-soluble to cell-associated siderophore, this is a critical question. (vi) What is the role played by MbtH and MbtJ?

them to the enzyme via a phosphopantetheinyl thioester attached to a peptide carrier protein domain. Two such activated amino acids are then condensed to form a peptide bond in a reaction catalyzed by a condensation domain. The activation domains specify the amino acids incorporated, and their order encodes the sequence of the final peptide. Numerous other domains or activities are also encountered in these proteins, i.e., epimerization domains that convert L amino acids to D amino acids, cyclization domains that form either macrocyclic esters or amides or smaller rings such as oxazolines and thiazolines, and thioesterase domains that cleave the final peptide product from the enzyme.

A unified scheme for the production of MBs. The proposed biosynthetic scheme based upon the known precursor incorporation results and the sequence homologies of the deduced *mbt*-encoded proteins is as follows (Fig. 4B). Salicylate is activated as its acyl adenylate by MbtA (Rv2384) and is then covalently attached to a phosphopantetheine prosthetic group of MbtB (Rv2383c). This activation-attachment appears to be a common motif in the biosynthesis of siderophores which contain a phenolate moiety. Proteins homologous to MbtA have been observed in the biosynthetic pathways of enterobactin (67) from *Escherichia coli* and yersiniabactin from *Yersinia pestis* (16). MtbB is an NRPS that is believed to activate serine, condense it with the salicylate moiety, and cyclize this product to a hydroxyphenyloxazoline. Once again, MtbB has homologues with analogous functions in both the pyochelin (from *Pseudomonas aeruginosa*) (66) and yersiniabactin (16) pathways. There are two other NRPSs, encoded by *mbtE* (Rv2380c) and *mbtF* (Rv2379c), that have the appropriate activation, condensation, and peptide carrier domains for donation of the two lysine-derived moieties of MB. MbtF has a terminal domain that was assigned a role as either an epimerization domain or as a thioesterase responsible for releasing the MB from the enzyme by lactamization of the terminal hydroxylysine residue. The latter function appears much more likely, as all amino acid-derived centers with known absolute configurations in the MBs arise from the naturally occurring L series (25, 73, 75, 76). It should be noted, however, that the absolute stereochemistries of the oxazoline and the acylated lysine (i.e., b and c in Fig. 3) have not been determined unambiguously for MB T, the MB from *M. tuberculosis*. Also in the gene cluster are *mbtC* (Rv2382c) and *mbtD* (Rv2381c), which encode proteins that are homologous to polyketide synthases. The encoded proteins appear to contain the appropriate modules to produce the required β-hydroxybutyrate. Additionally, *mbtG* (Rv2378c) encodes a protein that is homologous to known ornithine and lysine oxygenases, an activity required for the production of the ε-*N*-hydroxylysines. Thus, these seven genes, *mbtA* to *mbtG*, appear to encode sufficient activities for the biosynthesis of the core of the MBs (Fig. 4). There are two other proposed gene products, MbtH (Rv2377c) and MbtJ (Rv2385), to which no clear biochemical role has been assigned.

The genes involved in the formation of salicylate are not clearly defined. Cole et al. (8) suggested that a distinct operon with two genes (*entC* and *entD* [Rv3215 and Rv3214, respectively]) homologous to those implicated in salicylate formation in *P. aeruginosa* (69) is responsible for its formation. Walsh et al. (56) concluded that the product of another gene, *mbtI* (Rv2386c), located at the 5′ end of the *mbt* gene cluster and homologous to salicylate- and anthranilate-forming enzymes was most likely to be responsible for salicylate formation. This gene was originally called *trpE2* and assigned the encoding of an anthranilate synthase (8). Given the similarity between the different biochemical functions postulated for these various enzymes (anthranilate is the amino analog of salicylate), certain assignment will await the expression and characterization of these proteins. It is interesting, however, that the DNA sequence upstream of the *mbtI* gene contains a very convincing match for an IdeR binding consensus sequence, which suggests repression of the expression of this gene under high-iron conditions, consistent with a predicted role in salicylate synthesis (13). It should also be noted that a gene (*acpS* [Rv2523c]) which encodes a phosphopantetheinyl transferase, necessary for the formation of the holoenzyme of the NRPSs, has been identified in the *M. tuberculosis* genome (56).

What proof exists for the function of the above enzymes (MbtA to MbtG), postulated on the basis of homology alone? The protein encoded by *mbtA* has been expressed and shown to form the acyl adenylate of salicylate (56). This, in turn, acylates a fragment of MbtB which was predicted to encode the necessary phosphopantetheinyl carrier domain. This only occurred after treatment of the MbtB protein fragment with the appropriate phosphopantetheinyl transferase (56). We have constructed an *mbtB* mutant strain of *M. tuberculosis* through homologous recombination with a DNA fragment in which a portion of the *mbtB* gene was replaced with a hygromycin resistance marker (10). The resulting Δ*mbtB*::*hyg* strain was shown to be incapable of siderophore production, thus conclusively linking this gene cluster with MB biosynthesis. The absence of siderophore production in this mutant furthermore demonstrates that all of the MBs, including the water-soluble ones, arise through a common biogenic pathway. This latter work (10) also was the first to demonstrate the long-suspected importance of siderophore production to virulence in *M. tuberculosis*, as the *mbtB* mutant strain was avirulent in macrophage infections. Thus, the importance of this operon in MB biosynthesis has been established. The roles of MbtA and MbtB (in part) are as predicted, but the completion of much of the MB biosynthetic story awaits the functional characterization of the remaining enzymes.

The biosynthesis of exochelins. Recently, some results concerning the biosynthesis of the other mycobacterial siderophore, the exochelins, have been reported (14, 83, 84). Complementation studies carried out with *M. smegmatis* mutants defective in exochelin production have identified a cluster of three genes believed to be involved in the biosynthesis of exochelin MS (the exochelin from *M. smegmatis*). Two of these genes encode large NRPSs (83, 84), while the third encodes a protein with homology to phosphoribosylglycineamide formyltransferases (14). This latter enzyme was believed to be responsible for the production of the formylornithine moiety of exochelin MS, while the NRPSs catalyzed the assembly of the peptide backbone. However, the identification of a total of six amino acid activation domains for the production of this pentapeptide indicates that, as with the MBs, much remains to be understood about exochelin biosynthesis. Part of the explanation for this discrepancy may lie in the fact that exochelin MS is only one of the family of exochelins produced by *M. smegmatis*.

THE REGULATION OF IRON METABOLISM

Iron-responsive alterations in protein synthesis. Combination of sophisticated mass spectrometry techniques with the genome sequence to analyze protein patterns on two-dimensional gels has been a useful tool in the identification of proteins that are upregulated or downregulated in response to iron concentration (82). Fifteen proteins from *M. tuberculosis* were upregulated and 12 proteins were downregulated under low-iron conditions. In another study of protein profiles of *M.*

tuberculosis grown in low and high concentrations of iron, several iron-regulated proteins were isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subjected to N-terminal sequencing (6). The gene encoding Irp10, a 10-kDa protein prominent in iron-deficient cell extracts but virtually absent from extracts of iron-replete cultures, was designated *irpA* (Rv3269). This gene is similar to those that encode heat shock proteins or chaperonins and lies immediately upstream of a gene that encodes a putative cation-transporting ATPase named CtpC (Rv3270). This protein is a member of the P-type cation-transporting ATPase family. Both *irpA* and *ctpC* appear to be induced by low iron, although no consensus binding sites for known iron-dependent regulators were identified upstream of either gene. If further studies reveal that Irp10 and CtpC do, in fact, transport iron, it will be the first example of iron transport by a P-type ATPase, rather than an ABC transporter ATPase.

The N-terminal sequences of two other iron-regulated proteins that were upregulated under low-iron conditions were also reported (6). By searching the *M. tuberculosis* genome database, it is now possible to identify these proteins. The N-terminal sequence of the upregulated Irp13 protein corresponds to Acr (Rv2031c), the same protein previously shown by these authors to be downregulated by iron starvation (82). Irp28 contains the sequence found in HupB (Rv2986c), an HU-histone fusion. Interestingly, the same N-terminal sequence was found for both Irp28 and Irp29. While Irp28 is upregulated by iron starvation, Irp29 is downregulated by iron starvation, suggesting that the same histone-like protein is modified differently under the two conditions.

Phenotypic responses to iron deprivation. The effect of iron starvation on stress response-related protein synthesis in *M. smegmatis* was examined (39). Killing by hydrogen peroxide was increased in cultures grown in 0.2 μM or a lower iron concentration. The simplest interpretation is that during iron starvation, iron-containing enzymes, such as catalase, which are required for protection from hydrogen peroxide are not produced in their active, iron-containing form. However, the situation may be more complicated. Patterns of stress-induced proteins were examined by placing *M. smegmatis* under heat, ethanol, or hydrogen peroxide stress while labeling the induced proteins with ^{35}S. When the labeling experiments were performed with iron-sufficient cells, all three stress conditions induced expression of GroEL1 (Rv3417c) and DnaK (Rv0350). Surprisingly, when the labeling experiments were done with iron-starved cells, GroEL and DnaK were not induced by hydrogen peroxide but were induced by heat shock or ethanol, suggesting that the regulatory networks involved in iron metabolism and stress responses are interrelated. The response of iron-starved *M. tuberculosis* to hydrogen peroxide stress may differ from that described for *M. smegmatis*, since *M. smegmatis* displays a broader phenotypic response to oxidative stress than does *M. tuberculosis* (72).

Control of gene expression by iron in mycobacteria. The best-studied iron-responsive transcriptional regulator is the ferric uptake regulator Fur (9). Encoded by the *fur* gene, this protein binds its corepressor, ferric iron, and then binds to operator sequences known as Fur boxes, thereby repressing transcription. Under iron starvation, Fur no longer binds to the Fur box and the corresponding genes become derepressed. *M. tuberculosis* contains as many as four such iron-dependent regulators. IdeR (Rv2711) is the only protein for which experimental evidence of a role in iron binding and DNA binding exists (68). It contains extensive similarity to the DtxR family. This family, named after the diphtheria toxin repressor, is only distantly related to the gram-negative iron-dependent regulator Fur and binds a different DNA operator sequence. Interestingly, *M. tuberculosis* contains two genes, *furA* and *furB*, that encode proteins more similar to *E. coli* Fur than to IdeR/DtxR. FurA (Rv1909c) clusters more tightly with Fur proteins found in other mycobacteria and FurS from *Streptomyces reticuli*. In each case, immediately downstream of the *fur* homolog is a gene encoding a catalase peroxidase. In the mycobacteria, this gene is *katG*; in *S. reticuli*, it is called *cpeB*. In *S. reticuli*, FurS has been shown to negatively regulate the expression of *cpeB* in the presence of iron (85). The sequence similarity of both the regulator and catalase-peroxidase, as well as the conserved gene arrangement, suggests functional conservation between *S. reticuli* and *M. tuberculosis*. Even more similar to *E. coli* Fur is FurB (Rv2359), for which no other mycobacterial homologs have been described. Finally, there is SirR (Rv2788), putatively described as an iron-dependent regulator based on similarity to SirR from *Staphylococcus epidermidis* (23), TroR from *Treponema pallidum* (21), and the IdeR/DtxR family.

The iron-responsive regulatory protein encoded by *ideR*, which is homologous to the *dtxR* gene from *Corynebacterium diphtheriae*, is biochemically the best characterized. The mycobacterial protein has been analyzed both in vitro and in vivo for function. The recombinant protein overproduced in *E. coli* was shown by band shift analysis to bind the *C. diphtheriae tox* promoter-operator sequence in a divalent metal-dependent manner (68). The role of IdeR in the repression of siderophore production was shown with the construction of an *ideR* mutant of *M. smegmatis* (13). This mutant produces siderophore when grown in high- or low-iron media, demonstrating the requirement for IdeR to repress siderophore production under high-iron conditions. Interestingly, but perhaps not surprisingly in light of the presence of *furA*, *furB*, and *sirR* in *M. tuberculosis*, the mutant was still capable of upregulating siderophore production under low-iron conditions, suggesting the presence of a second iron-sensing regulator in *M. smegmatis*. Another phenotype associated with the *ideR* mutant was increased sensitivity to hydrogen peroxide. Native gel activity assays demonstrated a reduction in the activities of the catalase-peroxidase KatG and the superoxide dismutase activity of Mn superoxide dismutase. However, the activity of another catalase, KatE, was unaffected by the *ideR* mutation. These results again demonstrate the link between the responses to iron starvation and oxidative stress in *M. smegmatis*.

CONCLUSIONS

Iron is an essential nutrient whose concentration is critical in determining the outcome of infection with pathogenic mycobacteria. These organisms have evolved a complex web of biosynthetic and metabolic pathways by which to obtain it, store it, and regulate its use. Unraveling of the intricacies of this system may reveal an Achilles heel that can be used to guide and inform the development of new antibiotics. As a precedent for such antibiotics, *p*-aminosalicylic acid is a clinically useful second-line antitubercular agent, the target of which may lie in the enzymes which biosynthesize MBs (80). MB structural analogs have inhibitory potential as well, attracting the interest of synthetic chemists. *M. tuberculosis*, which biosynthesizes and utilizes MB T, is inhibited by synthetic MB S, which only appears to differ from MB T in the absolute configuration of one to three of its stereogenic centers (26, 73). Further progress toward new therapies based upon a rational understanding of iron acquisition and metabolism by mycobacteria requires an even greater understanding of the complexities of the structure, biosynthesis, and functions of sidero-

phores; their uptake into bacterial cells; and the long-term storage and retrieval of iron.

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