Iron acquisition by Mycobacterium tuberculosis: Isolation and characterization of a family of iron-binding exochelins

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ABSTRACT Mycobacterium tuberculosis, the primary agent of tuberculosis, must acquire iron from the host to cause infection. To do so, it releases high-affinity iron-binding siderophores called exochelins. Exochelins are thought to transfer iron to another type of high-affinity iron-binding molecule in the bacterial cell wall, mycobactins, for subsequent utilization by the bacterium. In this paper, we describe the purification of exochelins of M. tuberculosis and their characterization by mass spectrometry. Exochelins comprise a family of molecules whose most abundant species range in mass from 744 to 800 Da in the neutral Fe3+-loaded state. The molecules form two 14-Da increment series, one saturated and the other unsaturated, with the increments reflecting different numbers of CH2 groups on a side chain. These series further subdivide into serine- or threonine-containing species. The virulent M. tuberculosis Erdman strain and the avirulent M. tuberculosis H37Ra strain produce a similar set of exochelins. Based on a comparison of their tandem mass spectra, exochelins share a common core structure with mycobactins. However, exochelins are smaller than mycobactins due to a shorter alkyl side chain, and the side chain of exochelins terminates in a methyl ester. These differences render exochelins more polar than the lipophilic mycobactins and hence soluble in the aqueous extracellular milieu of the bacterium in which they bind iron in the host.

Mycobacterium tuberculosis, the primary causative agent of tuberculosis, infects one-third of humanity and is one of the world’s most important infectious agents. M. tuberculosis is a facultative intracellular pathogen. In the host, it multiplies intracellularly in mononuclear phagocytes, and it also appears to multiply extracellularly at least in lung cavities. Its capacity to infect the host is closely linked to its ability to acquire iron. Serum containing poorly saturated transferrin, such as human serum, is tuberculostatic, and its tuberculostatic effect is neutralized by the addition of iron (1, 2).

Free iron is very limited in the host, particularly in extracellular sites, owing to the high affinity with which it is held by host iron-binding proteins, chiefly transferrin and lactoferrin. To obtain iron at sites where it is limited, many pathogens have developed high-affinity iron-binding molecules of their own called siderophores, which can remove iron from host iron-binding molecules. Mycobacteria have been shown by Macham, Ratledge, Barclay, and colleagues (3–5) to produce small water-soluble siderophores called exochelins. This group of investigators has proposed that exochelins bind iron in the extracellular aqueous environment and transport the metal to another high-affinity iron-binding molecule located in the cell wall of M. tuberculosis—mycobactin (3). Mycobactin is a highly lipophilic molecule thought to facilitate the transport of iron across the cell wall to the interior of the bacterium (6).

Both exochelins and mycobactins are induced by low concentrations of iron in broth medium (4, 7). There are two general types of exochelins, classified according to their extractability in organic solvents (8). The chloroform-insoluble exochelins, produced by saprophytic mycobacteria, are not extractable into any organic solvent. The chloroform-soluble exochelins, produced by slow-growing pathogenic mycobacteria, including M. tuberculosis, are extractable into chloroform (5).

Mycobacteria have been extensively studied and their structures delineated (7). In contrast, individual exochelins have not been purified previously and neither their structure nor composition has been described. In this paper, we describe the purification of exochelins of M. tuberculosis from both a virulent (Erdman) and an avirulent (H37Ra) strain and their characterization by MS.

MATERIALS AND METHODS

Medium and Reagents. Modified iron-deficient Sauton’s broth medium (9) was prepared with 1–10 μM Fe3+ and without Tween. Mycobactin J was purchased from Allied Monitor (Fayette, MO).

Bacteria. M. tuberculosis Erdman (ATCC catalog no. 35801) and H37Ra (ATCC catalog no. 25177) strains were grown on Middlebrook 7H11 agar plates (10), suspended in modified Sauton’s medium at A600 = 0.05, and cultured in 225-cm2 culture flasks (Costar) at 150 ml per flask and at 37°C in 5% CO2/95% air for 3, 6, or 8 weeks.

Purification of Exochelins. The culture supernatant fluid was filtered successively through 0.8- and 0.2-μm low-protein-binding filters and saturated with iron (150 mg of ferric chloride per liter). Ferriexochelins were extracted into chloroform by shaking 1 vol of culture filtrate with 1.5 vol of chloroform. The chloroform layer (containing the exochelins) was removed, dehydrated overnight with anhydrous magnesium sulfate (2 g/liter), filtered through a fritted glass filter, and evaporated by rotary evaporation, yielding a brown compound. The brown extract was suspended in buffer A [0.1% trifluoroacetic acid (TFA)] and exochelins were prepurified on a C18 Sep-Pak cartridge (Waters, Millipore). Visible as a brown band at the top of the column, exochelins were eluted with buffer B (0.1% TFA/50% acetonitrile). This partially purified material was loaded onto a C18 column in buffer A and subjected to reverse-phase high-pressure liquid chromatography (HPLC) with a 0–100% buffer B gradient at a flow rate of 1 ml/min on a Rainin (Woburn, MA) HPX5 system. All peaks exhibiting high 450-nm absorbance, tentatively identified as exochelins, were hand collected and subjected to a final purification on an alkyl phenyl column.

Purification of Mycobactins. The bacterial cells from cultures described above were suspended in 6 ml of 95% ethanol

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Abbreviations: LSIMS, liquid secondary ion mass spectrometry; ESI-MS, electrospray ionization mass spectrometry.
per g of wet cells and stirred for 24 hr at 24°C. The ethanol extract containing mycobactins was then filtered, and the filtrate was saturated with 600 mg of ferric chloride per liter and diluted 1:1 with distilled water. Ferrimycobactins were extracted into chloroform by shaking 1 vol of the ethanol extract with 0.5 vol of chloroform. Mycobactins were purified successively on a C18 Sep-Pak cartridge, C18 column, and alkyl phenyl column using the same procedures to purify exochelins except that buffer B consisted of 0.1% TFA/95% acetonitrile.

**MS Analysis.** Peaks isolated from the HPLC and identified as potential exochelins on the basis of their strong 450-nm absorbance were first subjected to mass analysis by liquid secondary ion mass spectrometry (LSIMS) on a Kratos Analytical Instruments MS505 mass spectrometer. Samples were concentrated under vacuum and small aliquots (1–5 μl) were transferred to the LSIMS probe along with 1 μl of thioglycerol/glycerol (1:1, vol/vol). A Cs+ beam energy of 10 keV was used to bombard the sample and resulting secondary ions were accelerated to 8 keV. Scans were taken in the positive ion mode at 100 sec per decade and recorded with a Girdel (Cleveland) ES-1000 electrostatic recorder. Mass assignments were made to an accuracy of ±0.2 Da by manual calibration using Ultramark 1621 as an external reference. For exact mass measurements, the peptide YSPTPS with an exact 13C monoisotopic mass of m/z 738.3309 for the (M + H)⁺ ion was added to the LSIMS probe along with the exochelin samples and used as the internal reference mass under peak matching conditions. The resolution of the spectrometry was ~7000 (M/ΔM).

Electrospray ionization mass spectrometry (ESI-MS) was conducted in the positive ion mode using a VG/Fison (Manchester, U.K.) BioQ triple quadrupole mass spectrometer and a Vydac (Hesperia, CA) narrow bore C18 reverse-phase HPLC column (1 mm × 10 cm). Conditions for HPLC separation were similar to that described for the analytical separation of exochelins with the exception of a lower flow rate of 70 μl/min and a postcolumn split ratio of ~20:1.

Tandem MS was performed with a four-sector Kratos Analytical Instruments tandem mass spectrometer. The collision cell was filled with He and floated at 2 keV for a collision energy of 6 keV. Samples were ionized using a LSIMS source operating with Cs⁺ in the positive-ion mode, and all spectra were recorded and mass assigned using an array detector and Mach3 data system (11).

**RESULTS**

The *M. tuberculosis* Exochelin Family. We purified the exochelins of *M. tuberculosis* from culture filtrates by chloroform extraction and reverse-phase HPLC. To enhance the production of exochelins, we cultured the bacteria in iron-deficient medium. To allow their ready detection during the purification procedure, we first loaded the exochelins with iron. Preliminary studies using ⁵⁹FeCl₃-saturated culture filtrates demonstrated efficient extraction of the label into chloroform. Iron-binding compounds were subsequently detected in the HPLC eluate by simultaneously monitoring the UV absorbance at 450 nm, a wavelength at which iron compounds generally exhibit relatively high absorbance, and at 220 nm, a wavelength at which amide and aromatic groups absorb. There were approximately 5 major and 10 minor peaks exhibiting a high 450-nm absorbance eluting off a C18 column (Fig. 1A). We tentatively identified all of these peaks as exochelins, and we subsequently confirmed their identity as exochelins by MS (see below). Virtually all of the exochelins in the culture filtrate were extracted with a single chloroform extraction. When we performed two chloroform extractions on culture filtrate and then quantitated the 5 major exochelin peaks in each extract, the amount of each peak in the second extract was <5% of the amount in the first extract.

All major exochelin peaks were purified further by a second reverse-phase HPLC run on an alkyl phenyl column. The final products were highly pure, as evidenced by elution off the column of a single major absorbance peak (Fig. 1B) and by the finding of a single mass species of exochelin on subsequent MS analysis.

The HPLC pattern of exochelins released into the culture medium by *M. tuberculosis* Erdman strain was very similar after

![Fig. 1.](image-url)
3, 6, and 8 weeks of incubation (data not shown). Subsequent LSIMS analysis of the major peaks revealed that exochelins of the same mass were released at each of those times. Quantitatively, the highest amount of exochelins was recovered from 6-week cultures, which had 6.7 and 1.5 times the amount of exochelins in 3- and 8-week cultures, respectively (n = 3). The decline in quantity after 6 weeks likely reflects a decreased rate of production of exochelins by stationary-phase organisms, whose metabolism is slowed, and degradation of exochelins, which degrade slowly even at 4°C in the purified state. The set of exochelins produced by the highly virulent Erdman strain of M. tuberculosis was similar to that produced by the avirulent H37Ra strain on the basis of the HPLC elution pattern and mass determination.

Characterization of Exochelins of M. tuberculosis. MS analysis confirmed that all peaks exhibiting a high 450-nm absorbance were exochelins. LSIMS of each peak revealed an ion pair differing in mass by 53 Da (Fig. 2), corresponding to protonated (M + H)+ and iron (M - 2H + FeIII)+ adducts of the same molecular ion species. Consistent with this, ESI-MS of each exochelin revealed only one ion for each exochelin species representing the Fe3+ adduct (M - 2H + FeIII)+ (data not shown). This indicated that the protonated form seen on LSIMS is an artifact of that technique and that all the exochelins are Fe3+ loaded. On LSIMS analysis, the major exochelin had peaks at m/z 773 (iron adduct) and 720 (protonated adduct).

High-resolution peak matching of exochelins with (M + H)+ at m/z 720 and 748 (see corresponding neutral Fe3+ adduct peaks of 772 and 800 labeled with an asterisk in Fig. 3) yielded exact m/z values of 720.345 (M_exp = 719.337) and 748.373 (M_exp = 747.365) for their corresponding isotopically pure 12C-containing molecular ions. These exact masses are within ±3-5 ppm for an elemental composition of neutral non-iron-containing exochelins of M = C34H49N5O12 (MCalc = 719.3378) and M = C36H50N6O12 (MCalc = 747.3691), respectively (12). Exochelins comprised a large family of molecules. The major species ranged in mass from 744 to 800 Da for the neutral Fe3+ adduct based on LSIMS and ESI-MS analysis (Fig. 3). Exochelins differed from each other in mass by multiples of 14 Da, reflecting different numbers of CH2 groups, and/or by 2 Da, reflecting the likely presence of a double bond in some exochelins. Thus, on the basis of mass alone, exochelins formed two 14-Da-increment series, one saturated [masses (M - 3H + FeIII) of 744, 758, 772, 786, and 800 Da] and one unsaturated [masses (M - 3H + FeIII) of 770, 784, and 798 Da]. The exact location of the double bond in the unsaturated series remains to be determined but is clearly contained in the R1 alkyl side chain on the basis of tandem MS analysis (see below).

The different numbers of CH2 groups are also located primarily on the R1 alkyl side chain. However, the presence or absence of a methyl group at R3 further distinguishes exochelins as serine-containing (R3 = H) or threonine-containing (R3 = CH3) species, as confirmed by amino acid analysis. Therefore, we propose a nomenclature for exochelins that identifies them as members of the saturated or unsaturated series and as members of the serine or threonine series.

In addition to the major exochelin species identified by LSIMS, a number of less abundant species were subsequently identified by on-line HPLC/ESI-MS. These additional exochelin species were 14-Da (∆CH2) increment downward and upward extensions of the two exochelin series described above with masses for the neutral Fe3+ adduct of 716, 730, 814, and 828 Da (saturated series) and 742, 756, 812, and 826 Da (unsaturated series).

Structure of Exochelins and Mycobactins of M. tuberculosis. As noted above, M. tuberculosis produces two high-affinity iron-binding molecules—exochelins and mycobactins. To compare these molecules, we isolated both from the same strain of M. tuberculosis (Erdman strain) and obtained their UV absorbance spectra (Fig. 4). The spectra were very similar, with a major peak of absorbance at 220 nm and a peak at 450 nm.

To characterize the structure of exochelins of M. tuberculosis further, we studied individual exochelins by high-energy tandem MS (Fig. 5A) and compared their structure with mycobactins of M. tuberculosis and with mycobactin J from Mycobacterium paratuberculosis (Fig. 5B), whose structure has been studied (12, 13). This analysis revealed that exochelins have a core structure similar to that of mycobactins, a conclusion evident from the similarity of the fragmentation pattern of the two molecules. Fragment ions of both molecules were assigned to one of six structural moieties (A-F) within the compounds arising from one or more cleavages about the amide or ester bonds (Fig. 5 Upper). Some lower mass peaks were identical for the two compounds, such as m/z 100, 145, and 171, which are derived from conserved structural units A, C, and/or D. For the most part, however, analogous fragment ions were generated for both compounds but were shifted in mass between the
two compounds based primarily on differences in alkyl substituents present in the B, E, and/or F moieties. For example, the phenolic group in mycobactin contained a meta-substituted methyl group, and peaks containing this residue were shifted 14 Da higher in mass relative to the exochelins, which did not contain this methyl group [e.g., (DE - CO)\+ at m/z 176 vs. 162]. Likewise, peaks containing B and F in the mycobactin MS/MS spectrum were shifted up in mass relative to the exochelin spectrum by 28 (\Delta M = C\text{\small 2}H\text{\small 4}) and 80 (\Delta M = C\text{\small 6}H\text{\small 16}, -O\text{\small 2}) Da, respectively.

In addition to the saturated serine-containing exochelin shown in Fig. 5A, nine other exochelins were analyzed by tandem MS and shown to belong to the saturated serine series (MH\+ = 706, 734, 748, and 762), saturated threonine series (MH\+ = 720 and 734), unsaturated serine series (MH\+ = 718 and 732), and unsaturated threonine series (MH\+ = 732). Acid hydrolysis of mycobactin J and the exochelin described in Fig. 5A followed by methylation with diazomethane resulted in the formation of salicylic acid and 2-hexadecanoic acid (mycobactin J), and salicylic acid and pimelic acid (exochelin), which were identified as their corresponding methyl esters by GC/MS analysis. This provided further proof of the structures of moieties E and F in both compounds. Furthermore, it was clear from the tandem mass spectrum of the exochelin that pimelic acid is present as a methyl ester, a fact that is supported by the peaks at m/z 688 (MH - CH\text{\small 2}OH\+) and 661 (MH - COOCH\text{\small 3}\+) and also by the series of alkyl chain cleavages at m/z 647, 653, 619, and 605 [MH\+ - (CH\text{\small 2})\text{\small 1},COOCH\text{\small 3}].

Thorough comparisons of the tandem spectra obtained from individual exochelins and mycobactins provided tentative structure assignments for exochelins as shown for the non-iron-bound saturated serine-containing exochelin (M\text{\small 7}, 719) in Fig. 5A. The precise substitution pattern of the two linker carbons in the B group of both the exochelin and the mycobactin J could not be determined from the MS/MS data and is only postulated.

The general structure for the exochelins and mycobactins of M. tuberculosis is shown in Fig. 6. The core molecule is circular with iron in the center. It contains 3 amino acid moieties—2 N-hydroxylsines and 1 serine or threonine depending on whether R\text{\small 3} is a hydrogen or a methyl group. However, the two types of molecules differ from each other in two significant respects. First, exochelins (M\text{\small 7}, 744–800) for the major neutral Fe\text{\small 3}\+ adducts) are smaller than mycobactins (M\text{\small 7}, 882–924 for the neutral Fe\text{\small 3}\+ adducts) owing to a long-chain rather than a short-chain alkyl side group at R\text{\small 1}. Second, the shorter alkyl chains in the exochelins terminate in methyl ester moieties.

**DISCUSSION**

Exochelins and mycobactins, the two high-affinity iron-binding molecules of M. tuberculosis, share a common core structure (Fig. 6). However, exochelins are smaller than mycobactins owing to a long rather than a short alkyl side chain which, in exochelins, terminates in methyl ester moieties. These differences render exochelins more polar than mycobactins and likely explain their water solubility. The less polar mycobactins, in contrast, are virtually insoluble in water. The water solubility

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**Fig. 4.** Absorbance spectra of the major exochelin [M - 3H + Fe\text{\small 3}\+] = 772] and major mycobactin from M. tuberculosis Erdman strain.

**Fig. 5.** Tandem MS under collision-induced dissociation of the major saturated serine-containing exochelin with (M + H\+)\+ at m/z 720.3 (A) and commercial mycobactin J. (B) (Upper) Fragment ions of both the exochelin and mycobactin are assigned to one of six structural moieties (A-F) within these two compounds as described in the text. (Lower) On the mass spectra, hydrogen transfers relative to the neutral molecule are indicated by +1, +2, or +3.
of exochelins allows these molecules to function as iron-binding molecules in the aqueous extracellular milieu of the organism in the host.

It is possible that the methyl ester moieties of the exochelins are converted to their corresponding carboxylic acids through the action of human esterases. Although some exochelins have been identified by tandem MS as their carboxylic acids, we suspect they are formed by non-specific hydrolysis during purification.

That exochelins and mycobactins are so closely related suggests that their core structure is synthesized by a common set of enzymes and that subsequent reactions at R1 determine whether the final product is an exochelin or mycobactin. Possibly, one of the molecules is derived from the other.

Our ability to purify exochelins allows analysis not only of their composition and structure but also of their role in the physiology of M. tuberculosis and the pathogenesis of tuberculosis. Among other issues, the role of exochelins in the infected host remains to be clarified. It seems likely that exochelins are required for multiplication of M. tuberculosis in extracellular sites of the host such as lung cavities where free iron is undoubtedly very limited, and the organism must scavenge iron from high-affinity iron-binding compounds of the host. In support of this concept, we have recently determined that desferriexochelins rapidly remove iron from human transferrin, whether it is 95% iron saturated or 40% iron saturated, its approximate percentage saturation in human serum. Moreover, we have demonstrated that desferriexochelins acquire iron from iron-saturated lactoferrin and ferrotransferrin. Preliminary studies in this laboratory indicate that ferriexochelins donate iron to desferriexochelins in the cell wall of live M. tuberculosis organisms. If confirmed, these studies will provide evidence for the concept that exochelins function by transferring iron from human iron-binding proteins to mycobactins in the M. tuberculosis cell wall.

Whether exochelins are required for intracellular multiplication of M. tuberculosis in host mononuclear phagocytes is less certain. Legionella pneumophila, another intracellular bacterial parasite and lung pathogen, evidently lacks siderophores and yet multiplies readily in its phagosome in mononuclear phagocytes (14). L. pneumophila derives its iron from the intermediate labile iron pool of the mononuclear phagocyte, which in turn obtains iron from iron–transferrin via transferrin receptors, iron–lactoferrin via lactoferrin receptors, and intracellular ferritin, which recycles iron to the pool (14–16). Possibly M. tuberculosis, which has a relatively low requirement for iron for optimal multiplication on artificial medium in comparison with L. pneumophila (1 vs. 20 μM) also obtains iron intracellularly without the need for a siderophore. However, L. pneumophila and M. tuberculosis reside in markedly different phagosomes within the host cell (17) and the availability of iron in these two compartments may differ.

Knowledge of the composition and structure of exochelins may point to new strategies for interfering with their iron-acquiring function and hence growth of M. tuberculosis in the host. This may in turn provide a rational basis for the design of drugs to combat tuberculosis.

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