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Characterization of Exochelins of *Mycobacterium avium*: Evidence for Saturated and Unsaturated and for Acid and Ester Forms

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Mycobacterium avium **secretes iron-binding siderophores called exochelins. The exochelins from *M. avium* have previously been reported to have unsaturated side chains that terminate in carboxylic acid. In contrast, our data show the side chains to be both saturated and unsaturated and to terminate with either a carboxylate or methyl ester.**

Mycobacterium avium is a slowly growing mycobacterium and an important human pathogen. Because *M. avium* is one of the most common opportunistic pathogens associated with AIDS, the prevalence of *M. avium* infection has skyrocketed in recent years (5).

Like many pathogens, *M. avium* produces high-affinity iron-binding molecules known as siderophores to help it acquire iron in the host (9). Free iron is extremely limited in the host because of the high affinity with which it complexes to host iron-binding proteins. Other investigators (2, 7, 8, 10) have shown that mycobacteria produce small water-soluble siderophores called exochelins. They have proposed that exochelins bind iron in the extracellular environment and deliver the iron to mycobactin, another high-affinity iron-binding molecule, which is located in the cell wall (or envelope) of the mycobacterium (8). Gobin and Horwitz have recently demonstrated that the exochelins of *Mycobacterium tuberculosis* remove iron from human transferrin and lactoferrin and donate iron to mycobactins in living intact organisms (3).

Exochelins can be classified into two general categories depending on their extractability into organic solvents (11). Saprophytic mycobacteria produce exochelins which cannot be extracted into any organic solvent. In contrast, slowly growing, pathogenic mycobacteria, like *M. tuberculosis* and *M. avium*, produce exochelins that are extractable into chloroform (2). Mycobactins have been extensively studied (14), but because of purification problems, physiological and structural studies of exochelins have only recently been performed. The structures of chloroform-insoluble exochelins from the nonpathogens *Mycobacterium smegmatis* (13) and *Mycobacterium neoaurum* (12) have recently been described. These structures differ greatly from those of the chloroform-soluble exochelins isolated from the pathogens *M. tuberculosis* (4) and *M. avium* (6). More precisely, the exochelins of the nonpathogens are peptides, whereas the exochelins from the pathogenic mycobacteria resemble mycobactins and contain both amino acid and non-amino acid moieties. Although both our group and Lane et al. (6) found that the core structure of the exochelins of the mycobacteria resembles that of the mycobactins, the results of our two groups differ with respect to the R_1 alkyl side chain, which critically influences the water solubility of exochelins and allows them to function in the extracellular environment. Whereas we previously had reported that the R_1 alkyl side chain of the exochelins of *M. tuberculosis* exists in both saturated and unsaturated forms that terminate predominantly with a methyl ester but additionally with a free carboxylic acid, Lane et al. (6) reported that the R_1 group of the *M. avium* exochelins exists exclusively in unsaturated forms terminating with a carboxylic acid moiety. In this paper, we describe the purification of exochelins from the type strain of *M. avium* (ATCC 25291) and their characterization by mass spectrometry (MS). We show them to exist in both saturated and unsaturated forms that terminate in either a free carboxylate or methyl ester but are otherwise identical to the mycobactins produced by the same strain. In addition, we demonstrate the ready conversion of exochelins from the methyl ester to the carboxylic acid form by esterase treatment.

The *M. avium* type strain (ATCC 25291) was cultured in modified iron-deficient Sauton's broth medium (1 μM iron, no Tween) in 1.9-liter tissue culture flasks, 300 ml per flask, without shaking at 37°C in 5% CO_2–95% air for 3, 6, 8, or 10 weeks (4). The bacteria were grown in iron-deficient broth to enhance exochelin production. Exochelins and mycobactins were purified as previously described (4). For exochelins, the culture supernatant fluid (final pH 6.5) was saturated with iron and ferriexochelins were extracted into chloroform at the same pH, unless otherwise specified. The chloroform extract was dried, and the exochelins were dissolved in 0.1% trifluoroacetic acid and purified by reverse-phase high-pressure liquid chromatography (HPLC) on a Vydac C_{18} column (4.6 mm by 25 cm; Western Analytical, Temecula, Calif.) with a 50 to 100% buffer B (0.1% trifluoroacetic acid, 50% acetonitrile) gradient at a flow rate of 1 ml/min. Individual exochelins were further purified on an alkyl phenyl column (Waters, Bedford, Mass.). Ferri-exochelins were identified in the HPLC eluate by their high A_{450}, and their presence was confirmed by MS.

For structural characterization, peaks isolated from the HPLC were first subjected to mass analysis by liquid secondary

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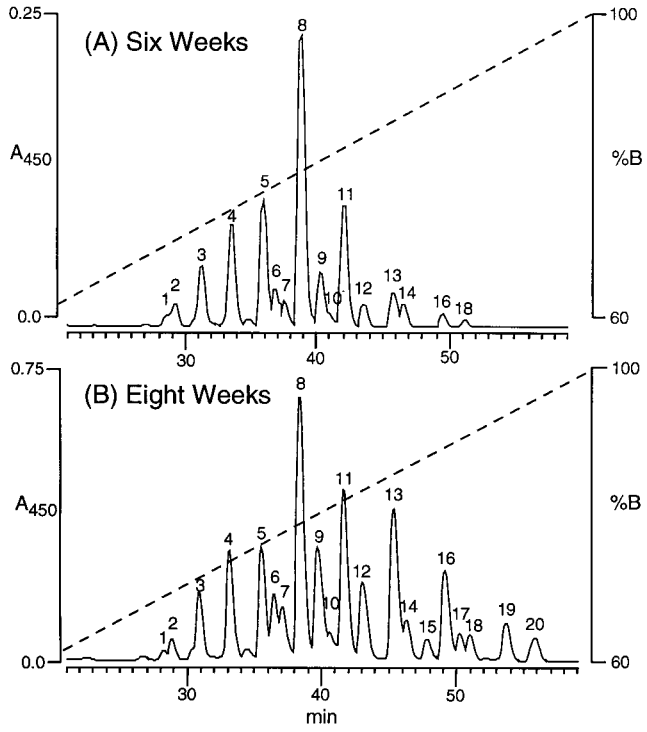


FIG. 1. Elution profiles of 6-week and 8-week culture filtrates from the *M. avium* type strain, ATCC 25291. Each preparation was obtained by chloroform extraction of a 400-ml culture filtrate and separated on a C_{18} HPLC column. Iron-binding molecules were monitored at 450 nm. The dashed lines represent the concentrations of buffer B. Peaks are numbered in the order of their elution off the HPLC column and are further characterized in Table 1.

ion MS by using MS-1 on a four-sector mass spectrometer (Concept II HH; Kratos Analytical, Manchester, England) as described in detail elsewhere (4). Tandem MS was performed on the desferri molecular ion forms, $(M + H)^+$, of exochelins as well as on mycobactins from the same strain by using both MS-1 and MS-2 of a four-sector Kratos mass spectrometer. All spectra were recorded and mass was assigned by using a scanning array detector and a Mach3 data system. Electrospray ionization MS was conducted in the positive-ion mode with a VG Platform II (Manchester, England) quadrupole mass spectrometer. HPLC fractions containing exochelins were dried down under vacuum and dissolved in the running buffer (50% acetonitrile, 1% acetic acid), and 4 μl was injected via a Rheodyne injector into a constant stream of running buffer flowing at a rate of 20 μl/min.

The presence of methyl ester forms of exochelins was confirmed by treating them with rabbit liver esterase (Aldrich). For these experiments, exochelins (≈100 μg) were dissolved in 50 μl of 50% acetonitrile, and then 0.5 ml of 50 mM Tris (pH 8.5) was added. Rabbit liver esterase (6.8 μg of protein, 0.74 U) was added and the mixture was incubated for 2 h at 37°C. Additional rabbit liver esterase (6.8 μg of protein, 0.74 U) was added for an additional 3 h. The reaction mixture was purified on a C_{18} Sep-Pak cartridge (Waters, Millipore). Exochelins were eluted with 50% acetonitrile–0.1% trifluoroacetic acid. The sample was concentrated under vacuum and then loaded onto a Vydac C_{18} column and separated by HPLC with a 15 to 70% buffer B (70% acetonitrile, 0.08% trifluoroacetic acid) gradient at a flow rate of 4 ml/min.

Reverse-phase HPLC analysis revealed a large family of *M. avium* exochelins in the chloroform extracts of culture filtrates. The approximately 20 peaks that were eluted off of the C_{18} column with a high A_{450} (Fig. 1) were tentatively identified as exochelins from their absorbance spectra. As with the exochelins of *M. tuberculosis*, liquid secondary-ion MS analysis of each peak revealed an ion pair differing in mass by 53 Da, corresponding to protonated $(M + H)^+$ and iron $(M - 2H + Fe^{III})^+$ adducts of the same molecular species. The exochelins can be grouped into two distinct saturated and unsaturated series and further subdivided into acid and methyl ester forms, on the basis of their masses, HPLC retention times, and tandem MS spectra (discussed below). Within a series, exochelins differed from one another in mass by 14 Da ($\Delta M = CH_2$), and between series, saturated and unsaturated exochelins of the same form and side chain length differed from one another in mass by 2 Da (CH_2–CH_2 versus CH=CH) (Table 1).

Culture filtrates were extracted under both neutral and acidic conditions (pH 7 and 4) to evaluate the possibility that exochelins undergo degradation or preferential extraction under these different pH regimens. However, no significant differences were found in the chromatogram patterns of exochelins extracted under the two pH conditions (data not shown). The HPLC pattern of exochelins released into the culture medium changed significantly between weeks 6 and 8 of incubation. The major change was a relative increase in the quantity of later-eluting exochelins. Subsequent mass-spectrometric analysis of the HPLC peaks from each harvest revealed a significant increase in the amount of exochelins terminating in methyl esters. More exochelins were recovered from the 8-week culture (8.3 mg/liter) than from the 6-week culture (1.2 mg/liter) (Fig. 1).

To characterize their structures further, we analyzed individual exochelins by tandem MS (Fig. 2). The similar fragmentation patterns of the *M. avium* and *M. tuberculosis* exochelins confirmed that they have a common core structure, similar to

TABLE 1. Molecular masses of iron-bound exochelins associated with HPLC peaks

HPLC peak no.[a]	Exochelin mass (Da)[b] and R_1 *n* value			
	$R_1 = (CH_2)_nCOOR$		$R_1 = CH{=}CH(CH_2)_nCOOR$	
	R = H	R = CH_3	R = H	R = CH_3
1	744, 1			
2	758, 2			
3	772, 3			
4	786, 4			
5			784, 2	
6	800, 5			
7		772, 2		
8			798, 3	
9		786, 3		
10	814, 6			
11			812, 4	
12		800, 4		
13				798, 2
14			826, 5	
15		814, 5		
16				812, 3
17	842, 8			
18			840, 6	
19				826, 4
20	856, 9		854, 7	

[a] See Fig. 1 for HPLC elution positions of peaks 1 through 20.

[b] Masses of iron-loaded exochelins are reported as their nominal masses as determined from mass spectrometric data (see text). Each exochelin peak was observed as an ion pair, $(M + H)^+$ and $(M - 2H + Fe^{III})^+$, differing in mass by 53 Da. Assignments were confirmed by tandem MS analysis (as shown in Fig. 2).

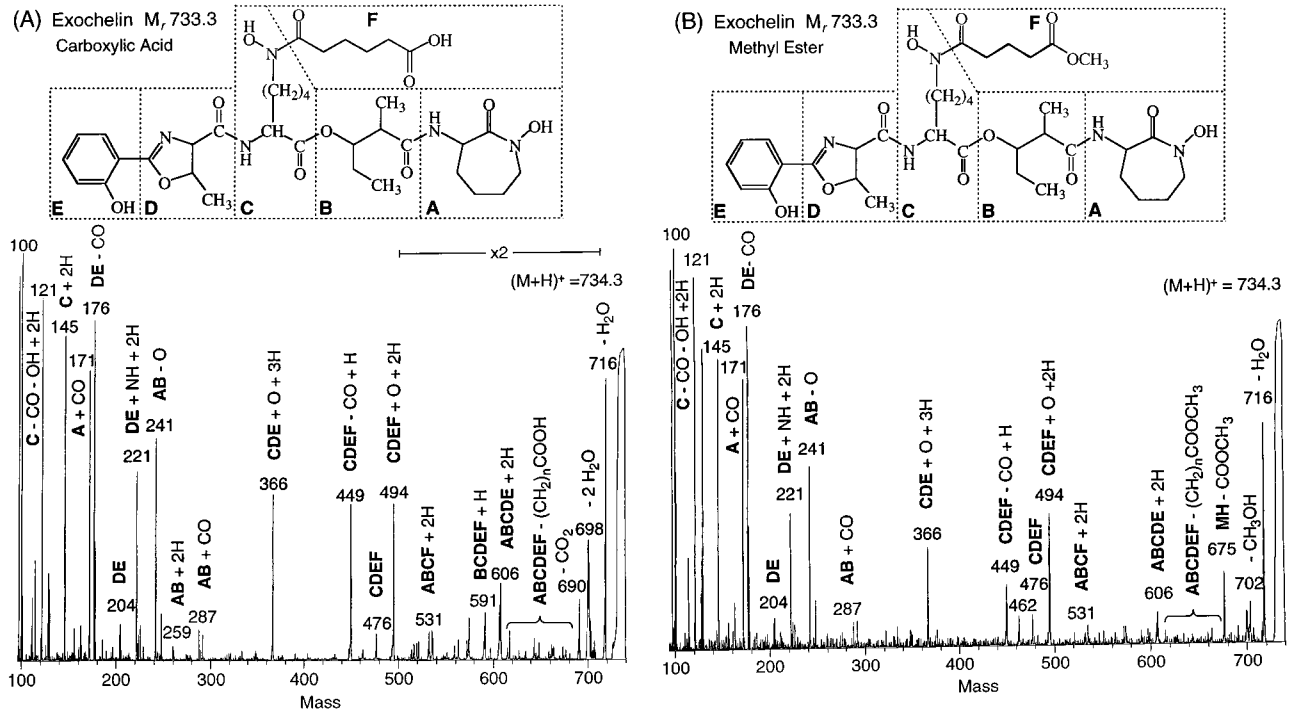


FIG. 2. Tandem MS under collision-induced dissociation conditions of isobaric *M. avium* exochelins with $(M + H)^+$ at *m/z* 734.3 terminating in carboxylic acid and methyl ester. These exochelins correspond to peaks 4 and 9 (Table 1). Fragment ions of the exochelins have been assigned to one of six structural moieties (designated A through F) within these two compounds as described in the text. On the mass spectra, hydrogen transfers relative to the neutral molecule are indicated by +1H, +2H, or +3H.

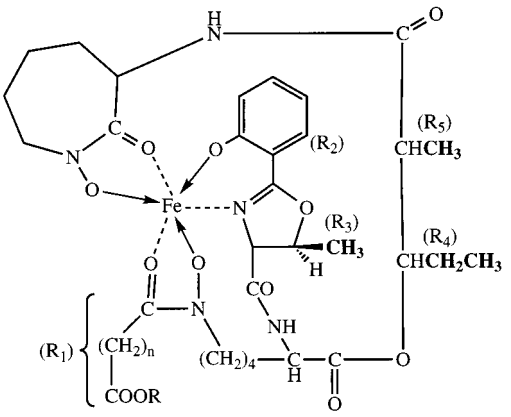
the core structure of mycobactins (Fig. 3). The fragment ions resulted from cleavages about the amide or ester bonds and were assigned to the structural moieties A through F. The main differences between the exochelins of *M. avium* and those of *M. tuberculosis* are in the alkyl substituents present in the β-hydroxy acid (B), oxazoline (D), and acylalkyl acid or methyl ester (F) moieties. As a result, the various exochelins have fragment ions analogous to ions containing the different alkyl moieties shifted in mass. Unlike *M. tuberculosis*, which has both serine- and threonine-containing exochelins (R_3 = H or CH_3), the exochelins from *M. avium* contain only threonine in the oxazoline ring state ($R_3 = CH_3$), an observation confirmed by amino acid analysis. Similarly, the peaks containing the ester-linked B moieties were shifted up in mass by 28 Da ($\Delta M = C_2H_4$).

Although tandem mass spectrometric analysis of the $(M + H)^+$ peak yielded information only about the intact salicylic acid-substituted oxazoline moiety (DE), tandem MS analysis of the $(MH + H_2O)^+$ peak, corresponding to the exochelin with the oxazoline ring hydrolyzed, yielded information about the individual D and E moieties. The loss of 121 Da, corresponding to salicylic acid (E + carbonyl), confirmed the structure of fragment E as a phenolic group and that of fragment D as a cyclized threonine, eliminating the possibility that the D moiety was a cyclized serine and the E moiety was a phenolic group with a metasubstituted methyl, as seen in some mycobactins.

From the tandem MS analysis, the exochelins could be further categorized as acids or esters. Exochelins with an R_1 chain terminating in a methyl ester always demonstrated a loss of 32 Da, $(MH - CH_3OH)^+$, and a loss of 59 Da $(MH - COOCH_3)^+$. In contrast, exochelins with an R_1 chain terminating in a free acid showed a loss of 44 Da, $(MH - CO_2)^+$. Within each of these classes, the exochelins differed in mass by multiples of 14 Da, reflecting different numbers of CH_2 groups on the R_1 alkyl side chain of the molecule. Tandem MS spectra of the two exochelins shown in Fig. 2 indicate that the exochelins have the same mass and differ only in that one has a terminal-acid and the other has an ester R_1 side chain.

The existence of exochelins with R_1 chains terminating in methyl esters was confirmed upon treatment of several of those exochelins with rabbit liver esterase. Over a period of several hours, the conversion of an ester-containing exochelin to a free-acid form was monitored by HPLC (Fig. 4). Mass analysis revealed a mass difference of 14 Da between the original iron-loaded exochelin and the esterase-treated form, i.e., $(M - 2H + Fe^{III})^+$ at *m/z* 798 and 784, respectively. Tandem MS spectra of the desferri molecular ions of these two forms showed a distinct difference in the high-mass neutral fragments associated with the R_1 group, loss of methanol and $COOCH_3$ for the original ester, and loss of CO_2 for the lower-mass esterase-generated form, thus confirming that the 14-Da loss was due to the conversion of the R_1 methyl ester to the free carboxylate.

Therefore, the exochelins of *M. avium* are a complex group of siderophores whose structures are clearly related to those of the mycobactins (Fig. 3). This similarity strongly suggests a common biosynthetic pathway for the exochelins and mycobactins. As previously shown for *M. tuberculosis* (4), the major structural difference between the exochelins and mycobactins of *M. avium* is the chemical composition of the R_1 group that is attached to the ε-nitrogen of the ε-*N*-hydroxylysine. In mycobactins, the R_1 group consists of a simple long-chain fatty acid that is saturated or unsaturated. An examination by tandem MS of the mycobactins produced by the same *M. avium* strain indicates that R_1 is a mixture of saturated and unsaturated fatty acids containing 14 to 19 carbons (Fig. 3). In exo-



R_1		R_3	M_r
Exochelins of *M. avium* Type strain:			
$(CH_2)_nCOOCH_3$	n = 2-5	CH_3	772-814
$(CH_2)_nCOOH$	n = 1-9	CH_3	744-856
$CH{=}CH(CH_2)_nCOOCH_3$	n = 2-4	CH_3	798-826
$CH{=}CH(CH_2)_nCOOH$	n = 2-7	CH_3	784-854
Mycobactins of *M. avium* Type strain:			
$(CH_2)_nCH_3$	n = 14,18	CH_3	896,924
$CH{=}CH(CH_2)_nCH_3$	n = 11-14	CH_3	880,894,922

FIG. 3. General structure of exochelins and mycobactins from the *M. avium* type strain, ATCC 25291. Exochelins contain an R_1 moiety that is either a saturated alkyl methyl ester and/or free acid or a singly unsaturated alkyl methyl ester and/or free acid. In a study of the exochelins of *M. avium* CR1/69, Lane et al. (6) found only the unsaturated, free-acid form with an *n* of 3 to 9 and an M_r of 798 to 882. Tandem MS data of the exochelins of the *M. avium* type strain are consistent with R_4 as ethyl and R_5 as methyl, as reported for *M. avium* CR1/69 (6). The preliminary structures of *M. avium* mycobactins from the type strain are listed on the basis of tandem MS data. In an earlier study of *M. avium* mycobactins, Barclay et al. (1) found R_2, R_4, and R_5 to be the same as noted here but found R_3 to be H and R_1 to be an unsaturated straight-chain fatty acid of undetermined length. *M. tuberculosis* exochelins contain a similar short R_1 group that also terminates in a carboxylic acid or methyl ester but differ from *M. avium* exochelins in the distribution and length of the R_1 group, the oxazoline substituents (R_3 = H or CH_3), and the substituted β-hydroxy acid (R_4 = CH_3 and R_5 = H) (4).

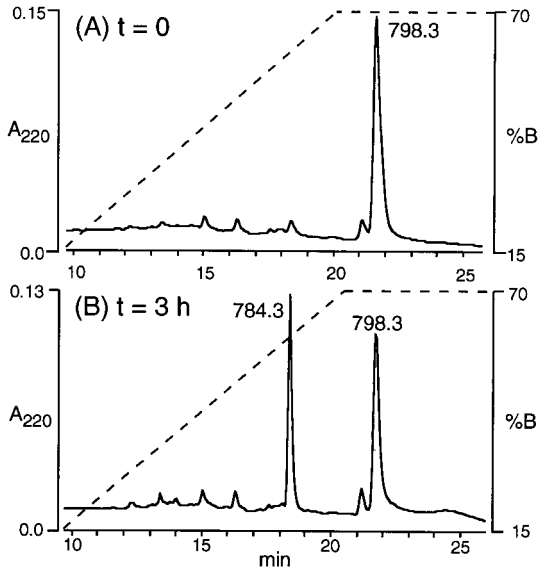


FIG. 4. Esterase treatment of an *M. avium* exochelin. An exochelin with a mass of 798 Da (peak 13 [Table 1]) was treated with rabbit liver esterase for 5 h. The progress of the reaction was monitored at 220 nm by reverse-phase HPLC. The conversion of the exochelin from the methyl ester to the free-acid form was confirmed by MS (see text). The dashed lines represent the concentrations of buffer B.

chelins, the R_1 group is a shorter-chain fatty acid that similarly terminates in either a free acid or the corresponding methyl ester. Thus, in the two cases for which structural data have been obtained for both exochelins and mycobactins isolated from the same strain (this study and our previous study on the exochelins of *M. tuberculosis* [4]), the only difference between the exochelins and mycobactins is in their R_1 moieties.

In a recent study, Lane et al. isolated exochelins from *M. avium* CR1/69 under acidic conditions and found that the R_1 group terminates exclusively in a free carboxylic acid (6). These investigators suggested the name "carboxymycobactins" for this group of exochelin compounds. Our data, however, clearly show that the R_1 group of *M. avium* exochelins exists in both saturated and unsaturated forms that terminate as both free acids and methyl esters, analogous to the exochelins of *M. tuberculosis* (4). Therefore, we propose that the original exochelin name remain the sole descriptor of these compounds and that they simply be referred to as saturated or unsaturated forms or free-acid or methyl ester forms.

Whether the free-acid forms of exochelins exist only as degradative products of the methyl ester analogs is still unclear, although we have observed that methyl ester hydrolysis occurs less readily than the hydrolysis of the oxazoline moiety (D), which we have not observed under neutral extraction conditions. When we extracted the exochelins under the acidic conditions used by Lane et al. (6), we did not observe a change in the relative proportion of free-acid to ester forms. Nonetheless, the methyl ester forms of exochelins will convert to their carboxylic acid analogs during prolonged storage (4) or after brief treatment with esterases. The biological significance of the ester-to-acid conversion and whether both forms are present during disease remain intriguing but unanswered questions. We are currently investigating these issues, as well as the degradative or metabolic fate of exochelins in the host.

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