Exochelin-mediated Iron Acquisition by the Leprosy Bacillus, *Mycobacterium leprae*

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Exochelins, water-soluble siderophores of mycobacteria, were isolated and partially purified from culture filtrates of iron-deficiently grown cultures of *Mycobacterium neoaurum* NCTC 10439 and an armadillo-derived *Mycobacterium* (ADM 8563). Two biologically active fractions mediating iron uptake were isolated from each bacterium which not only were able to transport iron into the producing organism but also into suspensions of *Mycobacterium leprae* isolated from armadillo liver. The rate of exochelin-mediated iron uptake into *M. leprae* was about 1.5% of the rate observed into the producing organisms. The process of iron uptake appears to be by facilitated diffusion as it was not inhibited by HgCl₂, NaN₃, KCN, dinitrophenol or carbonyl cyanide *m*-chlorophenylhydrazone. Since no uptake of iron occurred into iron-sufficient ADM cells, this may indicate that *M. leprae*, as recovered from an animal tissue, had been growing iron-deficiently in order for iron uptake to have been demonstrated *in vitro*.

INTRODUCTION

Although some 15 to 20 million people world-wide suffer from leprosy, our detailed knowledge of *Mycobacterium leprae*, the causative organism of this disease, remains incomplete since no bacterium showing a close chemical, pathological or immunological similarity to it has ever been grown on artificial media (Stewart-Tull, 1982). A major step forward in experimental leprosy occurred with the demonstration that the nine-banded armadillo (*Dasypus novemcinctus* Linn.) could be used for the cultivation *in vivo* of *M. leprae* (Storrs, 1971, 1974; Storrs *et al.*, 1978). However, in addition to the leprosy bacillus infected armadillo tissue may contain other mycobacteria which can be grown *in vitro* (Portaels & Pattyn, 1982; Portaels *et al.*, 1982, 1985). These are the so-called 'armadillo-derived mycobacteria' (ADMs). Their role in leprosy, if any, is unknown as is their origin and possible relationship to *M. leprae*, though it appears unlikely that ADMs are derived from *M. leprae*.

The acquisition of iron from host tissues probably occupies a central role in the development of a bacterial pathogen *in vivo* (Barclay, 1985; Griffiths, 1985; Neilands *et al.*, 1985). As little unchelated iron exists within the host, invading bacteria must obtain their iron from circulating iron-containing proteins, such as transferrin or lactoferrin, or from the iron-storage protein ferritin. Like many other bacteria, mycobacteria synthesize low molecular mass siderophores, termed exochelins, which can remove iron from these molecules (Macham *et al.*, 1975) and thus provide a means for iron acquisition in mycobacteria growing both *in vitro* and *in vivo* (Barclay & Ratledge, 1983). Exochelins are of unknown structure but are probably pentapeptides with *N*-ε-hydroxyornithines providing part or all of the chelating centre (A. J. M. Messenger, unpublished work). Although exochelins are isolated from the culture medium of mycobacteria grown under

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Abbreviation: ADM, armadillo-derived *Mycobacterium*.

conditions of iron-limitation (about $0.05 \mu\text{g Fe ml}^{-1}$) (Macham *et al.*, 1975; Messenger *et al.*, 1986; Stephenson & Ratledge, 1980), it is impossible to assess the production of such compounds by *M. leprae* because of its failure to grow *in vitro*. Already, we have tested the ability of the leprosy bacillus isolated from armadillo tissue, to acquire iron from exochelins isolated from four other species of mycobacteria: *M. vaccae*, *M. bovis* BCG, *M. smegmatis* and *M. neoaurum*, and have found that the process was relatively specific in that only the exochelin from *M. neoaurum* was active in this capacity (Hall *et al.*, 1983). Although *M. leprae* might be related to a rapidly growing mycobacterium (Stanford *et al.*, 1975), it seems highly unlikely that a soil saprophyte, such as *M. neoaurum*, could come into contact with, and supply iron to, *M. leprae in vivo*. Consequently, we suggest that the leprosy bacillus must either itself synthesize exochelins or have a more direct access to a supply of exochelins than chance contact with a soil mycobacterium would permit. We have, therefore, extended our previous work with the exochelins of *M. neoaurum* (Hall *et al.*, 1983) and are now able to show that similar if not identical exochelins are elaborated by the ADMs which also function in iron uptake into *M. leprae*. The process of iron acquisition by *M. leprae* has been studied in more detail and we are able to advance a hypothesis for the mechanism by which the leprosy bacillus is able to acquire iron.

METHODS

Organisms and growth. Armadillo-derived mycobacterium (ADM) no. 8563 was kindly supplied by Dr F. Portaels, Antwerp, Belgium. It appears to be a representative strain of a new species of *Mycobacterium* (Hall & Ratledge, 1985; Portaels *et al.*, 1986). Both it and *M. neoaurum* NCTC 10439 were grown in a rotary-shaking incubator in glycerol/asparagine liquid medium under either iron-deficient conditions ($0.05 \mu\text{g Fe ml}^{-1}$) or iron-sufficient conditions ($2 \mu\text{g Fe ml}^{-1}$) for 8 d (Ratledge & Hall, 1971). *M. leprae* was isolated in the laboratory from frozen (-80°C) armadillo livers, kindly provided via LEPR and the Medical Research Council, following the procedure of Draper (World Health Organization, 1980) and the organisms were then used immediately.

Exochelins: isolation and purification. Exochelins were isolated from culture filtrates of iron-deficiently grown *M. neoaurum* and ADM, and then partially purified by anion-exchange chromatography following the procedures of Macham *et al.* (1975). These are referred to as partially purified, unfractionated ferri-exochelins. Further purification was achieved by cation-exchange chromatography using a $250 \times 15 \text{ mm}$ column packed with BioRad AG50W-X4 (200–400 mesh; NH_4^+ -form) and a gradient of $0.1 \text{ M-NH}_4\text{OH}$ with acetic acid, pH 6.0, to $1.0 \text{ M-NH}_4\text{OH}$ with acetic acid, pH 9.0, as eluant. The absorbance of the eluate was monitored continuously at 260 nm and 3 ml fractions collected. A 2 ml sample of each of the two major fractions (see Results) was made into the desferri-form by the method of Macham *et al.* (1977), the desferri-exochelin being evaporated to dryness and subsequently labelled with $200 \mu\text{l } ^{55}\text{FeCl}_3$ [specific activity $1 \mu\text{Ci} (\mu\text{g Fe})^{-1}$] in water. After allowing the mixture to stand for 16 h, unchelated iron was removed by passage through Sephadex G-10 ($180 \times 10 \text{ mm}$). Subsequent purification was by applying approximately 1 ml samples (approximately 340000 c.p.m.) to a molecular sieve column ($850 \times 20 \text{ mm}$) of Biogel P₄ and eluting with water. Fractions of approximately 2 ml were collected and the radioactivity in $500 \mu\text{l}$ of each fraction was counted in a scintillation counter. Further purification of purified ferri-exochelins was by thin-layer chromatography using cellulose sheets (Eastman Kodak), $200 \times 200 \text{ mm}$, with butanol/glacial acetic acid/water (12:5:2, by vol.) as solvent. Exochelins were detected by spraying the sheets with ninhydrin (0.1% in acetone) before heating at 95°C for 2 to 3 min.

Uptake of ^{55}Fe -exochelins. Procedures for the uptake of ^{55}Fe -exochelins into washed cells of *M. neoaurum* and ADM suspended in $50 \text{ mM-KH}_2\text{PO}_4/\text{NaOH}$ buffer, pH 7.1, were described previously (Macham *et al.*, 1977; Stephenson & Ratledge, 1980). Uptake into similar suspensions of *M. leprae* was essentially the same though with precautions being taken to prevent microbial contamination and evaporation over the longer incubation times; the protocol was described by Hall *et al.* (1983). Experiments for the uptake of iron into *M. neoaurum* and ADM were done at least twice and results are quoted for a typical experiment; experiments with *M. leprae* were usually only done once due to severe limitation in supply of bacteria.

Preparation of other iron chelates. Ferri-salicylate and ferri-EDTA were prepared as 1:3 complexes; ferri-citrate was prepared as a 1:200 complex to achieve soluble iron at pH 7.0 as previously described (Messenger & Ratledge, 1982).

RESULTS

Biological activities of unfractionated exochelins

Initial examination of the biological activity of the partially purified, unfractionated exochelins from ADM 8563 and *M. neoaurum* showed that these were able to donate iron (as

Fig. 1. Exochelin-mediated iron uptake. (a) Uptake of ^{55}Fe , at $1\ \mu\text{M}$, complexed to unfractionated partially purified exochelins isolated from ADM 8563, by suspensions of iron-deficiently grown cells of the same organism (●) and by *M. neoaurum* NCTC 10439 grown similarly (○); uptake of ^{55}Fe exochelins (also $1\ \mu\text{M}$) isolated from *M. neoaurum* by *M. neoaurum* (□) and by ADM suspensions prepared similarly (■). (b) Uptake of ^{55}Fe , at $10\ \mu\text{M}$, complexed to the same exochelins, by a suspension of *M. leprae* recovered from infected armadillo liver: ●, exochelins from ADM; ■, exochelins from *M. neoaurum*. (Note that different concentrations of ^{55}Fe -exochelin were used in the two sets of experiments.)

^{55}Fe) to both the producing cells and to each other at equal rates (Fig. 1a). Also, the exochelins from ADM and *M. neoaurum* both donated iron at equal rates to *M. leprae* cells recovered from armadillo tissue, though over a longer period (Fig. 1b) than had been taken with the producing organisms. (It should be noted that experiments with *M. leprae* were done with $10\ \mu\text{M}$ - ^{55}Fe -exochelin, compared to $1\ \mu\text{M}$ for *M. neoaurum* and *M. vaccae*.)

The process of exochelin-mediated iron uptake into *M. neoaurum* was shown by Hall *et al.* (1983) not to be inhibited by HgCl_2 ($0.1\ \text{mM}$), NaN_3 ($30\ \text{mM}$) or dinitrophenol ($2\ \text{mM}$). Similarly, uptake into ADM was not inhibited by NaN_3 ($30\ \text{mM}$), KCN ($10\ \text{mM}$), carbonyl cyanide *m*-chlorophenylhydrazine ($0.1\ \text{mM}$) or HgCl_2 ($0.1\ \text{mM}$). Both processes therefore appear to be mediated by facilitated diffusion rather than by active transport.

Fractionation of exochelins

Subsequent fractionation of the exochelins from ADM 8563 and *M. neoaurum* was done on non-radioactive ferri-complexes by cation exchange chromatography (Fig. 2a). Each mixture had two components with equivalent retention volumes; a third, smaller component was also recognized in the siderophores from *M. neoaurum*. Of the two similar fractions, the one eluting first (fraction 1) in each case was about four times more active for ^{55}Fe uptake into *M. neoaurum*, as test organism, than the second fraction. The third, minor fraction from *M. neoaurum* was not active for the uptake of iron into *M. neoaurum* and was consequently not examined further. Further purification of the exochelins was therefore continued only with the first fraction from each organism. Each fraction 1 was chromatogrammed as the ferri-complex by gel filtration which separated it into three further components (as shown by absorbance at $260\ \text{nm}$), only two of which were chelators of Fe(III) (see Fig. 2b). These components are termed 1A and 1B. Further cellulose thin-layer chromatography failed to differentiate them, though it did reveal that both fractions were still not single components as there were two ninhydrin positive materials in each fraction 1A, and four (two major and two minor) similarly reactive compounds in each fraction 1B.

Activities of exochelins 1A and 1B

Both exochelin 1A and 1B were biologically active siderophores in that they both transported ^{55}Fe into ADM and *M. neoaurum*, irrespective of the origin of the exochelin, at the same rate

Fig. 2(a)

Fig. 2(b)

Fig. 2. (a) Fractionation of the partially purified ferri-exochelins used in Fig. 1 by cation-exchange chromatography (BioRad AG50W-X4) using a gradient of 0.1 M-NH₄OH/acetic acid, pH 6.0, to 1.0 M-NH₄OH/acetic acid, pH 9.0. A_{260} was monitored continuously. Fraction 1 is the one eluting first in each case; fraction 2 is the one eluting second. (b) Resolution of Fraction 1 (from Fig. 2a) after conversion to the ^{55}Fe -form, into components 1A and 1B by chromatography through a Biogel P₄ column eluting with water as solvent.

Fig. 3. Uptake of purified exochelins into ADM 8563 and *M. neoaurum*. (a) Uptake of ^{55}Fe , at 1 μM , complexed to exochelin fraction 1A; (b) uptake from fraction 1B. Exochelin isolated from *M. neoaurum* by washed suspensions of iron-deficiently grown cells of *M. neoaurum* (■) and of ADM (□); exochelin isolated from ADM by similar suspensions of ADM (●) and of *M. neoaurum* (○). Also included in (a), uptake of ^{55}Fe , at 1 μM , complexed to purified exochelin (fraction 3) from *M. smegmatis* by ADM (△) and *M. neoaurum* (▲) cell suspensions.

(Fig. 3a, b). The rate of uptake of iron was similar to that observed with the initial unfractionated exochelins (see Fig. 1).

Although the process of iron uptake as mediated by the exochelins appears to be by facilitated diffusion, it was, nevertheless, specific for the exochelins from *M. neoaurum* and ADM as

Fig. 4

Fig. 4. ^{55}Fe uptake, at $10\ \mu\text{M}$, complexed to exochelin fraction 1 (A and B) – see Fig. 2(a) – prepared from ADM 8563 into iron-deficiently grown cells of ADM (●) and into iron-sufficiently grown cells (○) ($2.0\ \mu\text{g Fe ml}^{-1}$).

Fig. 5

Fig. 5. ^{55}Fe uptake, at $1\ \mu\text{M}$, complexed to purified exochelin fractions, 1A and 1B (see Fig. 2a) prepared from ADM 8563 (● and ○, respectively) and *M. neoaurum* (■ and □, respectively) by *M. leprae* cell suspensions isolated from armadillo liver.

uptake of ^{55}Fe from the biologically active exochelin isolated from *M. smegmatis* (termed MS-3; see Macham *et al.*, 1977) was almost negligible into both organisms (see Fig. 3a). Furthermore, the status of the recipient cells was important as no uptake of ^{55}Fe from exochelin fraction 1 (A + B) could be demonstrated into iron-sufficiently grown cells of ADM (Fig. 4).

Iron uptake into *M. leprae*

Exochelin 1A and 1B from either ADM or *M. neoaurum* were each capable of donating iron to *M. leprae*; the process was essentially complete after 6 h (Fig. 5). Although both the exochelin 1B fractions gave greater uptakes than the 1A fractions, this might not be significant. (Due to the severe shortage of infected armadillo liver tissue, it was not possible to confirm this difference by further experiments but if the exochelins from ADM and *M. neoaurum* are identical, then the results given in Fig. 5 may be taken as arising from duplicate experiments.) Uptake of ^{55}Fe from both exochelins 1A and 1B (from *M. neoaurum*) into *M. leprae* was not affected by sodium azide (at 30 mM).

When other iron complexes were presented to a suspension of *M. leprae* and ^{55}Fe uptake followed over 3 h, the amount of ^{55}Fe taken up from ferri-salicylate was 15% of that attained with exochelin 1 (A + B combined) over the same period; from ferri-citrate, uptake was about 10% of the exochelin-mediated value and uptake from ferri-EDTA was virtually nil. However, citrate and salicylate, but not EDTA, could donate iron into iron-deficiently grown ADM cells at about the same rate as observed with exochelin 1A.

DISCUSSION

The exochelins described here from *M. neoaurum* and ADM 8563 appear to be distinct from those described earlier from *M. smegmatis* and *M. vaccae* (Macham *et al.*, 1975; Messenger *et al.*, 1986) and from BCG, *M. tuberculosis* and *M. avium* (Stephenson & Ratledge, 1980; Barclay & Ratledge, 1983). The exochelins from *M. smegmatis* and *M. vaccae* participate in an active iron transport process, i.e. a process inhibitable by uncouplers of oxidative phosphorylation and energy poisons, whereas the slow-growing species (BCG, *M. tuberculosis* and *M. avium*) yield

exochelins which are soluble in chloroform and mediate iron uptake by a facilitated diffusion process. The ADM and *M. neoaurum* exochelins, on the one hand, are similar in their water-solubility to the former siderophores, but on the other, mediate iron uptake in a manner similar to that of the slow-growing species. Moreover, exochelins from *M. smegmatis* are not used by ADM or *M. neoaurum* (see Fig. 3*b*) or indeed by *M. leprae*, a point that was established by Hall *et al.* (1983).

The two biologically active exochelin fractions (1A and 1B) were each elaborated by *M. neoaurum* and ADM 8563 and are probably the same materials being produced in each organism. Although the exochelins from the two organisms could not be differentiated either biologically or chromatographically, the two bacteria are readily distinguished by conventional taxonomy (Portaels *et al.*, 1986) as well as by differences in their mycobactins (Hall & Ratledge, 1985).

Perhaps of considerable significance was the observation that exochelin-mediated iron uptake into ADM was dependent upon the cells being previously grown under iron-deficient conditions (Fig. 4). As the same process of iron uptake could be successfully demonstrated to occur with *M. leprae* isolated from armadillo tissue, this would suggest that *M. leprae* itself had probably grown iron-deficiently within the host tissue. The evidence that other pathogenic bacteria also grow *in vivo* in an iron-deficient state has been recently reviewed by Griffiths (1985).

Although the requirements of *M. leprae* for iron are not known, its ability to take up iron from exochelins synthesized by an ADM strain that had been isolated as a commensal Mycobacterium along with *M. leprae* is intriguing. This is especially so since these mycobacteria might be present in numbers exceeding 10% of the total acid-fast bacilli isolated from an infected armadillo liver (Portaels & Pattyn, 1982; Portaels *et al.*, 1982). They would therefore be sufficiently numerous to provide enough exochelin, and thus iron, for not only themselves to grow *in vivo* but also the leprosy bacillus itself. Indeed, the hypothesis has already been put forward by Segal (1984) that *M. leprae* might be growing 'as a mixed infection, consisting of definite fixed species existing in commensal or symbiotic association'. Although our results could support this proposal, it is equally tenable that *M. leprae* synthesizes its own exochelins and we have merely detected similar siderophores in other mycobacteria.

The exochelin-mediated uptake of iron into *M. leprae* would appear to be by a facilitated diffusion process with there being little evidence that uptake can be sustained by other iron-complexing agents such as citrate or salicylate. Citrate and salicylate, however, appear to be effective donors of iron not only into ADM 8563 but also into *M. vaccae* (Messenger *et al.*, 1986) and *M. smegmatis* (Messenger & Ratledge, 1982).

The ability of exochelins from *M. neoaurum* to mediate iron uptake into *M. leprae* is possibly fortuitous although the organism was originally chosen for examination (Hall *et al.*, 1983) as it is taxonomically close to *M. vaccae* (Goodfellow & Wayne, 1982), which itself had once been considered as taxonomically close to *M. leprae* (Stanford *et al.*, 1975; Stanford & Rook, 1976). *M. vaccae* does not, however, show any similarity to *M. leprae* with regard to its iron uptake processes. Whether the newly isolated strain of ADM, which probably represents a new species or taxon of *Mycobacterium* (Portaels *et al.*, 1986), is related to *M. leprae* is unknown.

Although the inability to culture *M. leprae in vitro* is most probably a multifunctional problem, we would propose that it would probably be an advantage to incorporate the exochelins from ADM or *M. neoaurum* into any future *in vitro* growth experiments with the leprosy bacillus since the acquisition of iron is of such undeniable importance to pathogens (Barclay, 1985; Griffiths, 1985). Also, with respect to iron metabolism, both ADM 8563 and *M. neoaurum* can act as excellent model systems for future experimentation as supplies of *M. leprae* are never plentiful. A deeper understanding of the mechanism of iron uptake – including the participation of other components in the process – may therefore be usefully unravelled in the more accessible and non-pathogenic strains.

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