Iron Uptake Processes in *Mycobacterium vaccae* R877R, a *Mycobacterium* Lacking Mycobactin

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The production of exochelins (MV) was established in *Mycobacterium vaccae* R877R under iron-deficient conditions in concentrations about five times greater than in *Mycobacterium smegmatis*. *M. vaccae* does not produce mycobactin nor is salicylic acid secreted into the medium. A simple method is described using ^{55}Fe -labelled culture filtrates for assessing exochelin production and which would be applicable to other mycobacteria. One of the exochelins produced (MV3) is part of an active iron uptake system and another (MV1) is responsible for a passive uptake system. MV3 exochelin has similar chromatographic properties and biological activity to the major exochelin produced by *M. smegmatis*: iron uptake from MV3 exochelin was inhibited by dinitrophenol, NaN_3 and HgCl_2 , and was judged to be an active transport process. This process was not inhibited by equimolar amounts of ferri-salicylate or ferri-citrate both of which could be used separately as sources of iron for the organism. Uptake from these latter sources was insensitive to metabolic inhibitors and uncouplers. The multiplicity of pathways for iron uptake in a single organism is discussed.

INTRODUCTION

Studies on iron transport in mycobacteria have so far concentrated on *Mycobacterium smegmatis* due to its rapid growth and lack of pathogenicity. Four systems of iron transport have been reported. Exochelin, which is produced as a bacterial siderophore in the culture filtrate under iron-deficient conditions, complexes with any available iron to form ferriexochelin (Macham *et al.*, 1977) and transports iron into the cell by two uptake processes: (i) a high-affinity system operating at low concentrations of iron and (ii) a low-affinity system which operates at higher ($> 10\ \mu\text{M}$) concentrations of iron. The first process is energy-dependent, the second is passive (Stephenson & Ratledge, 1979). The third system for iron uptake is via ferric salicylate, which can transport iron into the cells under iron-deficient conditions, but its role is restricted, because this system does not operate in the presence of phosphate (Ratledge *et al.*, 1974). Nevertheless, salicylate (or 6-methylsalicylate) has been recognized as an extracellular product in iron-deficient mycobacteria (see Ratledge, 1982) though its occurrence may be due to overproduction as a precursor of the aromatic moiety of mycobactin (see below). The fourth process which has been described is via ferric dicitrate (Messenger & Ratledge, 1982). In addition to exochelin and salicylic acid, *M. smegmatis* also produces large quantities of mycobactin (up to 10% of the dry cell weight) within the cell under iron-deficient conditions. Mycobactin is a lipid-soluble siderophore and its role in iron uptake is considered to be that of a storage mechanism for iron, but it is able to donate iron into the cells by an unspecified process (Ratledge, 1982; McCready & Ratledge, 1979).

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Our interest in *Mycobacterium vaccae* was first aroused during a survey of mycobactin production in rapidly-growing mycobacteria (Hall & Ratledge, 1984). Unlike other species it produced no discernible mycobactin under a variety of different growth conditions and so the question of how its iron uptake differed from mycobactin-producing strains arose. The nutritional disadvantage that may therefore accrue to *M. vaccae* not possessing mycobactin has been considered separately (Hall & Ratledge, 1986).

Iron transport is considered to be important in determining the pathogenicity of micro-organisms (Weinberg, 1978; Bullen, 1981). Since many mycobacteria are pathogenic, the study of their iron transport may have some bearing on control of diseases caused by these species. *M. vaccae* is of particular interest because of a suggested taxonomic link with *Mycobacterium leprae*, which cannot be cultivated on synthetic medium (Stanford *et al.*, 1975). The study of *M. vaccae* may, therefore, add to our knowledge of *M. leprae* and of the reasons for the difficulty in cultivating this species in the laboratory.

In this paper we describe the production of exochelin siderophores in *M. vaccae* R877R and discuss their similarity to those produced by *M. smegmatis*. The method used to find exochelins in *M. vaccae* was a simple radiolabelling technique which could be used as a rapid method for screening other mycobacterial cultures for exochelin production. The possible roles of citrate and of salicylate, even though the latter compound is not produced by *M. vaccae*, are also investigated to complete our knowledge of iron transport processes in this species.

METHODS

Organisms and growth. *M. vaccae* strain R877R (from Dr J. R. Stanford, Middlesex Hospital Medical School, London, UK) and *M. smegmatis* NCIB 8548 were grown with shaking at 37 °C on glycerol/asparagine/mineral salts medium as previously described (Ratledge & Hall, 1971). Iron-deficient cultures contained 0.02 µg Fe ml⁻¹ and iron-sufficient cultures 2.0 µg Fe ml⁻¹.

Isolation of exochelin and estimation of exochelin production. Exochelins were isolated as described previously (Macham *et al.*, 1977) with certain minor modifications. The medium, after addition of FeCl₃, was mixed with Zerolit 225 (SRC 16), and filtered through Whatman no. 1 filter paper. The exochelins were extracted from the resin by adding batches of eluting buffer (NH₄OH/NH₄Cl buffer, pH 9.5), stirring for 10 min and filtering again.

Time course ⁵⁵FeCl₃-labelling of iron-binding compounds in culture medium. Samples of culture were filtered through GF/C filter paper and to 1 ml of the filtrate was added 10 µl ⁵⁵FeCl₃ (Amersham) containing 2.75 µg Fe [1 µCi (37 kBq) per µg Fe] or 50 µl ⁵⁵FeCl₃ containing 41.2 µg Fe [1 µCi (37 kBq) per µg Fe]. The lower amount of iron was insufficient to saturate all iron-binding potential of the culture filtrate and would therefore bind to the compound with the highest affinity for iron, whereas the higher amount of iron was calculated to be sufficient to saturate all iron-binding compounds. In this way the strongest iron-binding compound in the exochelins present could be identified.

The labelled samples were fractionated by cation-exchange column chromatography using AG50W-X4 (BioRad), 200–400 mesh, NH₄⁺-form, 250 × 15 mm, eluting with a gradient of 0.1 M-NH₄OH/acetic acid, pH 6, to 1 M-NH₄OH/acetic acid, pH 9 (cf. Macham *et al.*, 1977). ⁵⁵Fe was assayed in 500 µl samples taken from each fraction.

Uptake studies of ⁵⁵Fe from ferriexochelin. Desferriexochelins were prepared, and labelling with ⁵⁵FeCl₃ was done, as previously described (Macham *et al.*, 1977). Uptake studies were done as described by Stephenson & Ratledge (1979) using cells suspended in Tris/HCl (50 mM) pH 7.1 buffer. Radioactive ⁵⁵Fe was assayed as described below.

Assay of ⁵⁵Fe. ⁵⁵Fe was estimated by liquid scintillation counting using one of two systems. (i) The 500 µl samples from the time course study were placed in plastic scintillation vials and 10 ml scintillation fluid, containing per litre 724 ml xylene, 176 ml Triton N101 and 10 g butyl PBD, was added. (ii) Filter papers from uptake studies were oven-dried in glass scintillation vials and 1 ml Soluene (Packard) was added; the vials were incubated at 50 °C for 5 h and then 50 µl glacial acetic acid was added, followed by 10 ml scintillation fluid (100 g naphthalene, 10 g PPO made up to 1 litre with toluene).

Standard solutions of ⁵⁵Fe were used to calculate the amounts of iron present.

Iron uptake with salicylate and citrate. These were done as described by Stephenson & Ratledge (1979) and Messenger & Ratledge (1982) with some minor modifications. In uptake studies with ⁵⁵Fe-salicylate, 0.1 M-Tris/HCl buffer was used instead of phosphate buffer, which precipitates iron in this system. ⁵⁵Fe-Salicylate was prepared at an Fe:salicylate molar ratio of 1:3; ⁵⁵Fe-dicitrate was prepared at an Fe: citrate ratio of 1:200. ⁵⁵Fe was assayed by liquid scintillation counting as described previously (Stephenson & Ratledge, 1979).

RESULTS

Time course study of siderophore production in M. vaccae

Using the saturation $^{55}\text{FeCl}_3$ -labelling technique of culture filtrates taken from an iron-deficient culture of *M. vaccae*, it was possible to separate three main iron-binding peaks (see Fig. 1) using a cation-exchange chromatography system similar to that used previously in fractionating exochelins from *M. smegmatis* (Macham *et al.*, 1977). These fractions were designated 1 (5 to 30 ml), 2 (90 to 120 ml) and 3 (190 to 240 ml). Production of each siderophore was detected at day 3 of growth; the production of fraction 3 reached a maximum at day 7 and stayed at a steady concentration until day 10. Increased production of fraction 1 did not begin until day 7 and continued to rise to day 10. Fraction 2 also increased from day 7 to 10.

When similar samples of culture filtrates were labelled with non-saturating amounts of $^{55}\text{FeCl}_3$, peak 3 predominated throughout the time course, showing that it had a higher affinity for iron than the other peaks (see Fig. 1, which gives the results for the samples taken after 7 and 10 d; earlier samples are not shown only for the sake of simplicity).

No siderophores of any description were seen when *M. vaccae* was grown under iron-sufficient conditions.

When a similar experiment was done with *M. smegmatis*, ^{55}Fe was associated with similar fractions although the same amount of culture filtrate bound about only 20% of the ^{55}Fe . The fraction in *M. smegmatis* which corresponded to fraction 3 in *M. vaccae* was the major exochelin which was active in the transport of iron into the cell (Stephenson & Ratledge, 1979).

Identification of ^{55}Fe -binding peaks from M. vaccae as exochelins

Once the presence of ^{55}Fe -binding compounds under iron-deficient growth conditions had been established, the procedure for exochelin extraction and fractionation was done in bulk using 7-d-old cultures. A_{430} for such culture filtrates was between 0.44 and 0.51, approximately five times the maximum value observed with culture filtrates from *M. smegmatis*.

Peaks of absorbance at 430 nm characteristic of exochelins were found to correspond, on cation-exchange chromatography, to the ^{55}Fe peaks found during the time course study. Fraction 1 not only bound much iron (see Fig. 1) but also had a high A_{260} value compared with its A_{430} value (approximately 10:1). This is not characteristic of other exochelins and this fraction was considered to be a mixture of neutral and anionic compounds as it eluted in the void volume. It was not fractionated further before beginning biological studies. The three fractions of exochelin were designated MV1, MV2 and MV3 exochelins, following the nomenclature devised for prefixing the exochelins from *M. smegmatis* as 'MS' (Macham & Ratledge, 1975). The elution values of fractions 2 and 3 MV exochelins corresponded to the MS exochelins and may be similar or even identical in each organism.

Uptake of ^{55}Fe from MV exochelins

Because of the strong chromatographic similarities of the exochelins from *M. vaccae* and *M. smegmatis*, uptake studies were done using both types of exochelin into both species. The emphasis of this work was, though, with the MV exochelins.

Uptake of ^{55}Fe from MV1 exochelin at $1\ \mu\text{M}$ (Fig. 2*a*) occurred at similar rates into both *M. vaccae* and *M. smegmatis*. Metabolic inhibitors, DNP ($200\ \mu\text{M}$), HgCl_2 ($100\ \mu\text{M}$), or NaN_3 ($30\ \text{mM}$) had no effect on either process (results not shown). There was no uptake of ^{55}Fe from MV2 exochelin at $1\ \mu\text{M}$ or $10\ \mu\text{M}$ into *M. vaccae*. This exochelin was judged to be biologically inactive and was not examined further. Uptake of ^{55}Fe from MV3 exochelin occurred at an appreciable rate into *M. vaccae* when it was at $10\ \mu\text{M}$ (Fig. 2*b*) though no significant uptake was observed at $1\ \mu\text{M}$ (results not shown). This uptake was much more rapid into cells grown with iron-deficiency than ones grown with a sufficiency of iron, and was, moreover, strongly inhibited by DNP, NaN_3 and HgCl_2 (Fig. 2*c*). When the equivalent exochelin from *M. smegmatis* (i.e. MS3) was used in iron uptake in *M. vaccae*, the rate of uptake was the same as that of MV3 (Fig. 2*b*).

Fig. 1. Time course study of the iron-binding capacity of culture medium from *M. vaccae* grown with iron-deficiency. Iron-binding in 1 ml samples of culture medium was estimated with $41.2 \mu\text{g } ^{55}\text{Fe}$ added ($\circ$, 7 d; $\triangle$, 10 d) and $2.75 \mu\text{g } ^{55}\text{Fe}$ added ($\bullet$, 7 d, $\blacktriangle$, 10 d). Fractionation was by ion-exchange chromatography with a gradient of 0.1 to 1 M- NH_4^+ , pH 6 to 9. Results are from a representative experiment of 2 determinations.

Fig. 2. (a) Uptake of ^{55}Fe from MV1 exochelin ($1 \mu\text{M}$) into *M. vaccae* ($\circ$, $\triangle$) and *M. smegmatis* ($\bullet$, $\blacktriangle$) cells grown with iron-deficiency ($\circ$, $\bullet$) or iron-sufficiency ($\triangle$, $\blacktriangle$).

(b) Uptake of ^{55}Fe from MV3 exochelins ($10 \mu\text{M}$) into *M. vaccae* cells grown with iron-deficiency ($\circ$) or iron-sufficiency ($\bullet$). Uptake was also measured from MS exochelin ($10 \mu\text{M}$) into iron-limited *M. vaccae* cells ($\blacksquare$).

(c) Effect of potential inhibitors, DNP ($200 \mu\text{M}$) ($\triangle$), sodium azide (30 mM) ($\blacktriangle$) and mercuric chloride (0.1 mM) ($\square$), on ^{55}Fe uptake from MV3 exochelins ($10 \mu\text{M}$) into iron-limited *M. vaccae* cells. Results are from a representative experiment of 3 determinations.

This parallel between MS3 and MV3 exochelins was reinforced when both were used at $1 \mu\text{M}$ with comparable rates for iron uptake observed into *M. smegmatis* (Fig. 3), though the absolute rates were much higher than those into *M. vaccae*, where it had not been possible to observe a significant uptake using exochelins at $1 \mu\text{M}$.

The uptake of ^{55}Fe from MV3 exochelin (at $10 \mu\text{M}$) was not affected by the addition of equimolar ferri-salicylate or ferri-citrate though when these were added at $100 \mu\text{M}$ there was about 75% inhibition of uptake as measured over 30 min.

Fig. 3

Fig. 4

Fig. 3. Uptake of ^{55}Fe from MV3 exochelins ($1\text{ }\mu\text{M}$) into *M. smegmatis* cells grown under iron-limitation (○), and effect of a range of potential inhibitors, DNP ($200\text{ }\mu\text{M}$) (△), sodium azide (30 mM) (▲) and mercuric chloride (0.1 mM) (□). ^{55}Fe uptake was also measured from MS3 exochelin ($1\text{ }\mu\text{M}$) (■) into iron-limited *M. smegmatis* cells. Results are from a representative experiment of 3 determinations.

Fig. 4. Uptake of ^{55}Fe from ^{55}Fe -salicylate into *M. vaccae* cells grown with iron-deficiency (○) and iron-sufficiency (●), and also in the presence of a range of potential inhibitors, DNP ($200\text{ }\mu\text{M}$) (△), sodium azide (30 mM) (▲), sodium cyanide (10 mM) (□), CCCP (0.1 mM) (■) and mercuric chloride (0.1 mM) (▽). ^{55}Fe was present at $1\text{ }\mu\text{M}$ in the uptake system at a molar ratio of 1:3 with salicylate. Results are from a representative experiment of 3 determinations.

Other uptake systems for iron acquisition by *M. vaccae*

M. vaccae R877R, when previously examined for mycobactin content (Hall & Ratledge, 1984), was devoid of mycobactin. The bacterium also failed to produce salicylate, which constitutes the aromatic nucleus of mycobactin (Snow, 1970), and which occurs in the free state in culture filtrates of other mycobacteria up to $30\text{ }\mu\text{g ml}^{-1}$. Nevertheless, iron was taken up from ^{55}Fe -salicylate (Fig. 4) as well as from ^{55}Fe -citrate (Fig. 5) into *M. vaccae* with higher rates of uptake being observed with cells grown with iron-deficiency than with those grown with iron-sufficiency. Iron uptake from both salicylate and citrate was insensitive to a range of metabolic inhibitors: NaN_3 ; DNP, CCCP, NaCN and HgCl_2 . Uptake from citrate was slowed when the assay was done at $4\text{ }^\circ\text{C}$ (Fig. 5a): this experiment was not done with ^{55}Fe -salicylate. There was no uptake of iron from ^{55}Fe EDTA into either organism.

When uptakes from ^{55}Fe -salicylate or ^{55}Fe -citrate were assayed at $1\text{ }\mu\text{M}$ in the presence of equimolar amounts of each other, but with unlabelled iron, there was no significant diminution of the rates of uptake (results not shown). Competition experiments involving higher concentrations of the unlabelled iron complex were not done.

DISCUSSION

The production of exochelins by *M. vaccae* under iron-deficient growth conditions in much greater quantities than are found with *M. smegmatis* is offset by the lower efficiency of iron transport into *M. vaccae* cells. As the principal exochelin of both organisms (MV3 and MS3) appears to be similar, judging by their identical chromatographic behaviour and by their interchangeability between uptake assays with *M. smegmatis* and *M. vaccae*, it was concluded that the difference in the rates of iron uptake was due to a difference in cell composition between the two organisms. The fact that *M. vaccae* does not possess mycobactin is probably of no significance in this respect, as mycobactin is not involved in uptake of iron from exochelin in *M. smegmatis* (Stephenson & Ratledge, 1979). One might expect mycobactin, as a potential store of

Fig. 5. (a) Uptake of ^{55}Fe from ^{55}Fe -citrate into *M. vaccae* grown with iron-deficiency (○) and iron-sufficiency (●) and also the former at 4 °C (▼). ^{55}Fe was present at 1 μM in the uptake system at a molar ratio of 1:200 with citrate.

(b) Uptake of ^{55}Fe from ^{55}Fe -dicitrate into *M. vaccae* cells grown with iron-deficiency and in the presence of a range of potential inhibitors, DNP (200 μM) (△), sodium azide (30 mM) (▲), sodium cyanide (10 mM) (□), CCCP (0.1 mM) (■) and mercuric chloride (0.1 mM) (▽).

Results are from a representative experiment of 3 determinations.

iron, to become increasingly involved in iron assimilation as the concentration of iron presented to the cells was increased. Instead a very slow rate of iron uptake was observed with ferriexochelin at 1 μM when compared to that at 10 μM . A more likely explanation is that the components of the active transport system for exochelin MV3 are at a lower activity or amount in *M. vaccae* cells than in *M. smegmatis* (that it is an active transport system is shown by its sensitivity to various inhibitors). This is reflected in the considerable differences seen between cells of both species grown with iron-deficiency and iron-sufficiency with regard to exochelin-mediated iron transport. Such differences would again argue in favour of a minimal involvement of mycobactin as there is no mycobactin present in *M. vaccae* under any growth conditions, and so if lack of mycobactin was a key factor, there should be only a slight difference between the two types of cell. Instead, we deduce that the various components involved in iron uptake, including the proteins for exochelin biosynthesis, are strongly repressed in cells grown with iron sufficiency and this is the reason for the diminished rate of iron uptake in these cells.

It is unlikely that the lower rate of iron uptake into *M. vaccae*, when compared with that into *M. smegmatis*, causes it to have a slower growth rate. No evidence has yet been obtained with any mycobacterium that iron uptake is a growth rate-controlling process (Ratledge, 1982, 1984). A more probable explanation is that the slower growth rate of *M. vaccae* has led to the evolution of a slower iron uptake rate: the components of the exochelin uptake system becoming adjusted to acquiring iron for cells at a rate approximately matching their demand. The question of whether *M. vaccae* suffers any nutritional disadvantage because of its lack of mycobactin under any condition is a separate issue to the mechanisms by which it acquires iron, and has been investigated separately (Hall & Ratledge, 1986).

The number of iron uptake mechanisms found here in *M. vaccae* and previously in *M. smegmatis* (Stephenson & Ratledge, 1979; Messenger & Ratledge, 1982) is comparable with those in *Escherichia coli*, where at least five independent mechanisms exist (Hartmann & Braun, 1981). This diversity may be necessary for adaptation so that the micro-organisms can acquire iron under varied growth conditions. Each uptake system may function only under a narrow set of conditions.

The multiplicity of exochelins has already been commented on with respect to *M. smegmatis* (Stephenson & Ratledge, 1979) and there is clearly more than one present in culture filtrates of *M. vaccae*. However, it is possible that there is only one biologically active molecule in each cell, with the others arising by various biochemical modifications, such as acetylations, as growth progresses. With *M. vaccae*, for example, formation of MV1 and MV2 exochelins was

appreciable after the amount of MV3 had reached a plateau. Exochelin-mediated iron transport may be by one or both of two routes according to the concentration presented to the cell (Stephenson & Ratledge, 1979), but under physiological conditions, the high-affinity active process may be assumed to be the key one.

The uptake of iron from ferri-citrate could be of possible significance for the *in vivo* uptake of iron where citrate could be available from, and within, host tissues and macrophages. The significance of iron uptake from ferri-salicylate is less certain to have a role *in vivo*, although salicylate appears to be over-produced by all mycobacteria which elaborate a mycobactin. However, the presence of such an uptake system in *M. vaccae*, which produces neither salicylate nor mycobactin, is an unexpected finding and may suggest that a novel uptake route exists in *M. vaccae*. Our previous work indicated that the recipient molecule for iron uptake from salicylate was mycobactin itself (Marshall & Ratledge, 1972). The ferri-salicylate route of iron uptake is not a general iron-specific process, since a complex such as ferri-EDTA is not utilized by this organism. However, micro-organisms can acquire iron from various sources which they have not elaborated: for example, *E. coli* can acquire iron from ferri-ferrichrome and produces specific outer membrane proteins for such a function (Luckey *et al.*, 1975).

In conclusion, *M. vaccae* appears to be similar to *M. smegmatis* in elaborating exochelins with similar characteristics. The principal biologically active exochelin from each organism cannot be distinguished and may be identical. Alternative routes for iron assimilation, via salicylate and citrate, occur in *M. vaccae* as they do in *M. smegmatis* although the exact mechanism of uptake remains uncertain in view of the absence of mycobactin in *M. vaccae*. The lack of mycobactin does not appear to have produced any radical change in iron uptake mechanisms, and the necessity for a mycobacterium to elaborate a mycobactin must be presumed to be an environmental one (which would include the ability to grow within animal tissues) rather than it fulfilling any useful function under laboratory growth conditions.

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