Identification of a 29 kDa protein in the envelope of *Mycobacterium smegmatis* as a putative ferri-exochelin receptor

Lynn G. Dover† and Colin Ratledge

Author for correspondence: Colin Ratledge. Tel: +44 1482 465 243. Fax: +44 1482 465 458.
e-mail: c.ratledge@appbiol.hull.ac.uk

Department of Applied
Biology, University of Hull,
Hull HU6 7RX, UK

Evidence of a direct association between ferri-exochelin, the major extracellular siderophore of *Mycobacterium smegmatis*, and a 29 kDa protein has been obtained by three separate methods. (1) Direct binding of $^{55}\text{Fe(III)}$ -exochelin by the 29 kDa protein in an envelope preparation from iron-deficient cells was demonstrated by the extraction of a complex with the non-denaturing detergent CHAPS, and subsequent CHAPS-PAGE and autoradiography. (2) Affinity chromatography on a chemically synthesized ferri-exochelin-Sepharose 4B matrix led to the retention of the 29 kDa protein and a 25 kDa protein. The smaller protein was partially eluted with 1 mM ferri-exochelin although it did not form a stable complex with ferri-exochelin. The 29 kDa protein could not be eluted from the affinity matrix with 1 mM ferri-exochelin either alone or with 1 M NaCl. Only 2% (w/v) SDS could do this, but resulted in protein denaturation. (3) Incubation of ^{55}Fe -exochelin with CHAPS-solubilized envelope proteins in free solution followed by ion-exchange chromatography resolved three radioactive peaks; subsequent analysis by SDS-PAGE showed that the peak with the highest ^{55}Fe -binding activity per unit protein contained both the 29 and 25 kDa proteins. A direct association was demonstrated between the 29 kDa protein and ^{55}Fe -exochelin by gel filtration. The evidence suggests that the 29 kDa iron-regulated envelope protein of *M. smegmatis* is a ferri-exochelin-binding protein and that the 25 kDa protein, which corresponds in size to a previously reported iron-regulated envelope protein in this bacterium, may have a role in the formation or maintenance of this complex. Proteins extracted from the cell envelope of iron-deficient *M. smegmatis* with CHAPS were dialysed to remove the detergent, incorporated into liposome suspensions and then incubated with $^{55}\text{Fe(III)}$ -exochelin. This increased the retention of ^{55}Fe by 133-fold compared to proteins not placed in liposomes. Retention of ^{55}Fe was dependent upon the protein loading of the liposomes. Gel filtration confirmed that the iron was retained by these vesicles and even after dialysis the majority of ^{55}Fe was still retained by the vesicles. Re-solubilization of the labelled proteo-liposomes in various detergents gave limited recovery of a ferri-exochelin-protein complex. Attempts to resolve this complex by Triton X-100 PAGE led to separation of the two entities. The complex was stable, however, in a CHAPS-PAGE system.

Keywords: exochelin, iron, *Mycobacterium smegmatis*, receptor protein, siderophore

INTRODUCTION

Most aerobic bacteria acquire iron via high-affinity uptake systems based on siderophores – low-molecular-mass

chelating agents that are highly specific for ferric ions. Siderophores vary widely in their structure but are all able to mobilize iron from ferric oxyhydroxide polymers and from complexes that may occur in animal tissues such as

†Present address: Department of Physiology, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

Abbreviations: IREP, iron-regulated envelope protein; PEI, polyethyleneimine.

transferrin or ferritin (Neilands *et al.*, 1987). The synthesis of these siderophores and also of the proteins involved in the uptake of their ferric complexes is derepressed by low intracellular iron concentrations which, in *Escherichia coli*, is via the action of the ferric uptake regulation, Fur, protein (Guerinot, 1994). On entering iron limitation, microbial cells begin synthesis of their siderophores, which are secreted into the extracellular milieu. Once they have bound ferric iron, they are available for uptake by the cell.

The onset of iron deficiency in mycobacteria causes the derepression of the synthesis of three types of iron-binding compound: the exochelins, the mycobactins and either salicylic or 6-methylsalicylic acid (6MSA). The mycobactins are envelope-associated, lipid-soluble siderophores (Ratledge *et al.*, 1982). The exochelins are typical extracellular siderophores. The function of salicylic acid or 6MSA is not fully understood, but they may act as an intracellular acceptor for Fe(II) released from ferri-exochelin by reduction (Wheeler & Ratledge, 1994).

The extracellular siderophore of *Mycobacterium smegmatis*, exochelin MS, is a water-soluble, linear, formylated pentapeptide: N-(δ N-formyl, δ N-hydroxy-R-ornithyl)- β -alaninyl- δ N-hydroxy-R-ornithyl-R-*allo*-threoninyl- δ N-hydroxy-R-ornithine (Sharman *et al.*, 1995). Ferri-exochelin MS is transported intact into the cell by an active uptake mechanism (Stephenson & Ratledge, 1979) and several envelope proteins have been identified as putative ferri-exochelin transport proteins (Dover, 1995; Hall *et al.*, 1987) on the basis of their elaboration as a response to iron starvation.

Hall *et al.* (1987) observed that when suspensions of *M. smegmatis* that had been grown iron deficiently were pre-incubated with polyclonal antisera raised against a 29 kDa iron-regulated envelope protein (IREP), ferri-exochelin-mediated iron uptake decreased to 30 % of the control rate in iron-starved cells, suggesting that the 29 kDa protein was exposed at the cell surface and that it may well be the principal receptor for the uptake of ferri-exochelin in this bacterium.

Scrutiny of the promoter region of the gene encoding a 28 kDa antigenic protein of *Mycobacterium leprae* (Cherayil & Young, 1988) revealed a sequence which exhibited significant homology with the operator site of the Fur protein in the promoters of iron-regulated operons in *E. coli* (Dale & Patki, 1990). The possibility of transcriptional regulation by a Fur homologue and the antigenicity of this protein suggest that it could be involved in iron uptake. Sritharan & Ratledge (1990) also recognized 29 kDa proteins in the envelope of both *M. leprae* and *M. avium* recovered after infections in experimental animals.

In this study we attempted to isolate a receptor for ferri-exochelin from *M. smegmatis* using direct ligand-binding techniques.

METHODS

Culture conditions and preparation of envelope fraction. *Mycobacterium smegmatis* NCIMB 8548 was cultured as previously described (Ratledge & Hall, 1971). All low-iron cultures were

grown with an initial iron concentration of 0.04 $\mu\text{g ml}^{-1}$ and high-iron cultures with 2.0 $\mu\text{g ml}^{-1}$. Cells were harvested at 6000 g (4 °C, 10 min), washed twice in 0.05 M Tris/HCl, pH 6.8, re-centrifuged and resuspended in approximately 15 ml of the same buffer containing 1 mM phenylmethylsulphonyl fluoride and 1 mM benzamidinium.HCl. The cell suspension was disrupted at 0 °C by ultrasonication for 10 $\times$ 30 s periods with 15 s cooling intervals using a Dawe Soniprobe type 7533A. Undisrupted cells and large fragments of cell debris were then removed by centrifugation (6000 g, 10 min, 4 °C). The envelope fraction was obtained as the pellet after centrifugation at 105000 g (90 min, 4 °C) of the supernatant. The envelope material was washed twice with 0.05 M Tris/HCl, pH 6.8, and centrifuged as above.

Solubilization of envelope material in the non-denaturing detergent CHAPS. Envelope fractions were resuspended on ice in a small volume of 8 mM CHAPS in 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1, by agitation with a glass rod. The material was transferred to a vial and the suspension was mixed on a magnetic stirrer overnight at 4 °C. Insoluble material was sedimented by centrifugation at 4 °C at 105000 g for 30 min at 4 °C and the supernatant fluid containing the CHAPS-solubilized envelope proteins was removed.

Protein determination. The BCA (bicinchoninic acid) assay (Pierce) was used, following the manufacturer's instructions exactly.

Polyacrylamide gel electrophoresis (PAGE). Three PAGE systems were employed. In all cases the resolving gels cast were 10 % T (total monomers), 2.67 % C (bisacrylamide).

CHAPS-PAGE. The method of Cavinato *et al.* (1988) was followed, the only departure from the method being the use of 10 % T, rather than 8 % T (2.67 % C), resolving gels. The gels were run at a constant current of 35 mA at 4 °C for 3 h. Protein bands were visualized by silver staining or by autoradiography.

SDS-PAGE. Standard protocols were used. Gels were electrophoresed at a constant current of 35 mA for 3 h at 4 °C. Protein bands were visualized by silver staining.

Triton X-100 PAGE. The electrophoretic conditions were the same as used for SDS-PAGE except that 0.1 % Triton X-100 was substituted for 0.1 % SDS. Protein bands were visualized by silver staining and autoradiography.

Silver staining of gels. Gels were fixed by soaking for 2 $\times$ 10 min in 10 % (v/v) ethanol/5 % (v/v) acetic acid. The oxidizing solution, 3.4 mM $\text{K}_2\text{Cr}_2\text{O}_7$ in 0.0032 M HNO_3 , was applied to the gel for 10 min. The gel was then washed in distilled water until it was no longer yellow, soaked for 30 min in freshly prepared 0.012 M AgNO_3 in the dark, and washed for 2 min in distilled water. Gel development was standardized by using strictly timed washes in 0.28 M Na_2CO_3 /0.005 % formaldehyde for 1, 5 and 3 min each with fresh developing solution. The developing reaction was stopped by the addition of 5 % (v/v) acetic acid.

Quantification and labelling of desferri-exochelin with radio-active iron. Ferri-exochelin was isolated, purified and, when necessary, converted to desferri-exochelin as described by Sharman *et al.* (1995). Desferri-exochelin was quantified by titration with a known concentration of FeCl_3 : complex formation was measured by monitoring A_{430} on a dual-beam spectrophotometer and equal amounts of FeCl_3 were added to a

water blank and a desferri-exochelin sample until the relative A_{430} of the exochelin sample became constant. For labelling, $^{55}\text{FeCl}_3$ in 100 mM HCl was added to the required amount of desferri-exochelin in a phosphate buffer strong enough (~ 0.1 M) to maintain the pH close to 7.1 or 7.5. Sufficient non-radioactive FeCl_3 in water was added after 2 h incubation to 95 % saturate the siderophore. This mixture was held at 4 °C overnight. Any ferric phosphate precipitate was removed by centrifugation at 8000 g for 20 min.

Analysis of ferri-exochelin binding activity in a native envelope preparation by CHAPS-PAGE. An envelope preparation derived from low-iron cells was resuspended in 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1, on ice with a Potter homogenizer. A sample containing 100 μg protein ($< 40 \mu\text{l}$) was taken. This material was pre-incubated with 5 μl 100 mM ferric salicylate at room temperature for 10 min and 15 nmol (4.2 μl) $^{55}\text{Fe(III)}$ -exochelin (~ 29 kBq) was then added. Incubation was continued for a further 20 min at room temperature, after which 44 μl 16 mM CHAPS in 100 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1, was added, followed by solubilization of proteins as above. After centrifugation (105000 g for 30 min at 4 °C), the solubilized protein was diluted with 50 μl CHAPS-PAGE sample buffer. The proteins were separated by CHAPS-PAGE. The gel was subsequently dried under vacuum and visualized by autoradiography on Hyperfilm ^3H (Amersham).

Immobilization of ferri-exochelin on Sepharose 4B. An affinity matrix of 6-aminohexanoic acid *N*-hydroxysuccinimide ester-Sepharose 4B (Sigma) was used to immobilize ferri-exochelin. This matrix incorporates a C_8 spacer arm to improve the accessibility of small ligands and will react with any free NH_2 groups of a peptide or related material. The exochelin from *M. smegmatis* has three available NH_2 groups (Sharman *et al.*, 1995) and was immobilized on to this support following the manufacturer's instructions. After five sets of consecutive high- and low-pH washes, the matrix remained red, indicating the retention of iron in its siderophore complex.

Isolation of envelope proteins by affinity chromatography on ferri-exochelin-Sepharose 4B. Samples of CHAPS-solubilized envelope proteins (1 mg protein) derived from both high- and low-iron-cultured cells were loaded onto small (bed volume 0.4 ml) ferri-exochelin-Sepharose columns. The flow of the solution was stopped for 90 min once the solution had entered the column, to increase the extent of the binding of proteins to the affinity matrix. Non-binding proteins were eluted by washing the columns with 10 bed volumes of 8 mM CHAPS in 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1.

Various solutions were used to elute retained proteins: 1 mM ferri-exochelin, 1 mM ferri-exochelin in various concentrations of NaCl, and a 1 mM mixture of ornithine and glutamine as a non-specific control. The eluents were applied to the column in 0.5 ml volumes and allowed to elute for 30 min. The column was then centrifuged at 3000 g at room temperature for 5 min to remove the final traces of the eluent. Each eluate was stored at -20 °C. After the completion of each elution scheme any proteins remaining bound were eluted with 2 % (w/v) SDS in 50 mM Tris/HCl pH 6.8. Eluates were analysed either by gel-filtration chromatography on Sephadex G-100 or by SDS-PAGE following lyophilization to concentrate the sample. After the final SDS elution the columns were washed with 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1, to remove any residual SDS and to allow the column to be reused.

Ion-exchange chromatography on polyethyleneimine (PEI)-cellulose. Samples of CHAPS-solubilized envelope proteins (1 mg protein) were mixed with 50 nmol $^{55}\text{Fe(III)}$ -exochelin (8333 Bq nmol $^{-1}$) in 1 ml final volume and held at 37 °C for

30 min (final detergent and buffer concentrations were 8 mM CHAPS in 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.5). This mixture was then loaded on to a small PEI-cellulose (Sigma) column (5 $\times$ 1 cm), pre-equilibrated with 8 mM CHAPS in 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.5, which was then washed with 30 ml of the same solution to elute any non-protein-bound ferri-exochelin. The adhered envelope proteins were then displaced with NaCl: (i) 20 ml 0.1 M NaCl in 8 mM CHAPS; 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.5, (ii) 10 ml 1.0 M NaCl in 8 mM CHAPS; 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.5. Fractions of 1 ml were collected and 100 μl of each fraction was used for measurement of radioactivity by scintillation counting. The remainder of each fraction was lyophilized to concentrate the protein for subsequent analysis by SDS-PAGE.

Incorporation of CHAPS-solubilized envelope protein into liposomes. A large sample of CHAPS-solubilized envelope proteins derived from low-iron-cultured cells was dialysed against 400 vols 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1, overnight at 4 °C to remove the detergent. This protein was then used for binding analyses in liposomes.

The liposomes were constructed using dipalmitoylphosphatidic acid, phosphatidylcholine and cholesterol (Sigma, approx. 99 % pure) in molar ratios 1:10:10. These were mixed in chloroform, then 300 μg was dried on to the walls of a test-tube under N_2 unless otherwise stated. Dialysed envelope proteins in buffer were added to the lipid-coated test-tubes and the volume was made up to 1 ml with 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1. Crude proteo-liposomes were formed by shaking the mixture vigorously for 30 s. A cloudy suspension of proteo-liposomes was formed. This suspension was incubated with 50 μM $^{55}\text{Fe(III)}$ -exochelin (1.09×10^5 Bq nmol $^{-1}$) for 30 min at 37 °C. Bound and unbound ^{55}Fe was separated by filtering through Millipore GVWP filters (0.22 μm pore size), on which the proteo-liposomes were retained, and subsequent washing with 2 ml of the buffer. The filters were then taken for scintillation counting. Bound and unbound ^{55}Fe was also separated by gel filtration on a Sephadex G 200-120 column (6 $\times$ 1.5 cm), collecting fractions of approximately 0.65 ml. Samples were assayed for turbidity (OD_{628}), protein content and ^{55}Fe retention by scintillation counting in Ecoscint A.

Complex release from liposomes by digestion with various detergents. Larger preparations of proteo-liposomes were made containing 4 mg lipids/1 mg protein and labelled as before. Unbound ^{55}Fe was removed by overnight dialysis against 400 vols 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1, at 4 °C. Specific ^{55}Fe retention was assessed by scintillation counting. Various detergents were used to assess the degree of solubilization of protein-ferri-exochelin complex release: (i) 8 mM CHAPS, (ii) 8 mM CHAPS + 1 mM taurodeoxycholic acid, (iii) 8 mM CHAPS + 0.5 M NaCl, and (iv) 0.5 % (w/v) Triton X-100, all in the above buffer. Proteo-liposomes were incubated with the detergents overnight at 4 °C. Solubilization was quantified by scintillation counting of a sample of the supernatant fluid after centrifugation at 105000 g for 30 min at 4 °C. The diffusibility of the released label was assessed by recounting after overnight dialysis against 100 vols of the relevant detergent solution.

RESULTS

Resolution of envelope proteins by CHAPS-PAGE

To preserve the activity of the envelope proteins during electrophoretic separations we employed the non-denaturing PAGE system of Cavinato *et al.* (1988) in which

Fig. 1. Separation of $^{55}\text{Fe(III)}$ -exochelin-labelled envelope proteins from *M. smegmatis* by CHAPS-PAGE. A cell envelope fraction of *M. smegmatis*, grown iron-deficiently, was prepared by sonication and was separated from intact cells and the cytoplasmic fraction by differential centrifugation. The envelope fraction was incubated for 16 h at 4 °C with 8 mM CHAPS in buffer to extract protein. Soluble and insoluble material were then separated by centrifugation (105000 g, 30 min, 4 °C). The soluble extract was analysed by CHAPS-PAGE chromatography on a gel (14 × 16 cm) with composition 10% T (total monomer concentration) at 35 mA constant current for 2–3 h. The gel was fixed and visualized by silver staining. Lane A, 100 µg extracted envelope protein from iron-starved cells; lane B, 100 µg extracted envelope protein from iron-replete cells. Lane C: a ferri-exochelin-receptor protein complex was formed by incubation of a sample of native envelope, containing 100 µg protein, with $^{55}\text{Fe(III)}$ -exochelin (8332 Bq nmol⁻¹). The complex was then extracted using 8 mM CHAPS for 16 h at 4 °C. The complex was separated by CHAPS-PAGE on a 10% resolving gel at 35 mA for 2–3 h. The gel was then dried and visualized by autoradiography.

the non-denaturing detergent CHAPS, a zwitterionic bile salt derivative, stabilizes the hydrophobic proteins in solution. Proteins were extracted from the envelope of *M. smegmatis*, using 8 mM CHAPS as the solubilizing agent, and were then separated by CHAPS-PAGE. The performance using this gel system was not ideal and the mobility of samples in the gels was poor (Fig. 1, lanes A and B). Silver staining revealed clear bands above R_F 0.47 but below R_F 0.47 no bands were visible. The majority of the loaded protein was located near the boundary between the stacking and resolving gels. This low penetration into the resolving gel is presumably due to the aggregation of these envelope proteins. Increasing the CHAPS concentration in the reservoir buffer by 10- and 100-fold did not improve resolution.

Analysis of ferri-exochelin binding in native envelopes

The best environment for the formation of a ferri-exochelin-receptor complex was expected to be a native envelope preparation where protein conformation would be preserved. As the native envelope fraction from low-iron-cultured cells might contain mycobactin, the lipid-soluble, envelope-associated siderophore which would remove iron from the ferri-exochelin, the envelope material was pre-incubated with ferric salicylate before presenting ^{55}Fe -exochelin, in order to saturate any mycobactin with unlabelled iron. After incubation with ^{55}Fe -exochelin and subsequent extraction with 8 mM CHAPS, the solubilized material was analysed by CHAPS-PAGE. The tendency for *M. smegmatis* envelope proteins to aggregate in this system is evident on analysis of the autoradiograph (Fig. 1, lane C). Bound and unbound ^{55}Fe , however, were separated. A small amount of unbound $^{55}\text{Fe(III)}$ -exochelin migrated at the dye front with R_F 1 but the majority was retained by protein and appeared on the autoradiograph as a dark streak with a finite limit. The limit of mobility of this bound label was very sharp at R_F 0.56, corresponding to a protein of 29 kDa. [Molecular sizes were calculated according to Cavinato *et al.* (1988).] The limited solubility of proteins in this gel system, and the precipitation which ensued, obscured detail in the upper part of the lane, making recognition of any larger complexes which may have formed during the labelling reaction impossible. However, the lower limit corresponding to a 29 kDa protein would indicate that this must be the non-aggregated form and all material above would be the various non-specific aggregates. This result demonstrates that a possible iron-binding activity is resident on a 29 kDa envelope protein.

Analysis of ferri-exochelin binding by extracted envelope proteins

Affinity chromatography of envelope proteins on immobilized ferri-exochelin. CHAPS-solubilized envelope proteins from both high- and low-iron-cultured cells were purified using a ferri-exochelin-Sepharose 4B affinity column (see Methods). Of the proteins eluted with ferri-exochelin (Fig. 2, lanes A–D), only the 25 kDa protein corresponded to the size of a previously reported IREP in *M. smegmatis* (Dover, 1995). The elution of other proteins (typically 22, 19 and 18 kDa) may be due to their non-specific interactions with the matrix. Repetition of the experiment showed that these other proteins were not always present. Only the 25 kDa protein was observed reproducibly.

Although this protein was also present in the affinity eluates derived from high-iron-cultured cells, it stained more intensely in samples from iron-deficient cells, which is consistent with the observation that the synthesis of the 25 kDa protein is indeed regulated by iron availability.

To assess the specificity of the elution of this protein by 1 mM ferri-exochelin, a mixture of two amino acids, ornithine and glutamine, at 1 mM in the same buffer, was used as a control for affinity elution. The 25 kDa protein

Fig. 2. Purification of a 29 kDa envelope protein by affinity chromatography on ferri-exochelin-Sepharose. Protein samples (1 mg) extracted with CHAPS from the cell envelopes of iron-starved and iron-replete *M. smegmatis* were loaded on ferri-exochelin-Sepharose columns. After the removal of non-binding proteins by washing with buffered CHAPS, 1 mM ferri-exochelin was used as an affinity eluent. Eluates were analysed by SDS-PAGE: lane A, first affinity elution of samples from iron-replete cells; lane B, second affinity elution of samples from iron-replete cells; lane C, first affinity elution of samples from iron-starved cells; lane D, second affinity elution of samples from iron-starved cells. After removing these proteins by affinity elution, 2% (w/v) SDS was used to remove the remaining proteins. Eluates were analysed by SDS-PAGE: lane E, first elution with 2% SDS of iron-starved samples; lane F, second elution with 2% SDS of iron-starved samples; lane G, first elution with 2% SDS of iron-replete samples; lane H, second elution with 2% SDS of iron-replete samples. In further experiments, 1 mg CHAPS-solubilized envelope protein, from iron-starved *M. smegmatis*, was loaded onto ferri-exochelin-Sepharose and washed with 1 mM ferri-exochelin in buffered CHAPS with up to 1 M NaCl. The remaining protein was eluted using 2% SDS. The SDS-eluate was dialysed, concentrated by lyophilization and then run on a 10% SDS-PAGE gel (lane I). All gels were silver stained.

was not eluted with the 1 mM ornithine/glutamine mixture (instead ones at 20.5, 47, 39, 37 and 33.5 kDa were found), suggesting specificity for ferri-exochelin as an eluent.

When 1 mM $^{55}\text{Fe(III)}$ -exochelin in phosphate-buffered CHAPS was used as an eluent, analysis of the eluate by gel filtration on Sephadex G-100 showed that most of the radioactivity eluted as a single peak coinciding with the position of free ferri-exochelin. Thus, no stable ferri-exochelin-protein complexes were formed during this elution.

The lack of a stable association between the 25 kDa protein and ferri-exochelin was, however, compatible with the result demonstrated in Fig. 1, lane C, where the limit of mobility of bound ^{55}Fe in a CHAPS-PAGE system coincided with the mobility of a 29 kDa protein. No bound ^{55}Fe was detectable at R_F values corresponding to smaller proteins.

Proteins that remained bound to the immobilized ferri-exochelin after the affinity elution steps were eluted with 2% SDS, lyophilized and then analysed by SDS-PAGE (Fig. 2, lanes E-H). Bands corresponding to a 29 kDa protein stained to a similar intensity in samples derived from both high- and low-iron-cultured cells. This result demonstrates the high affinity of this protein for ferri-

exochelin and is also consistent with its now apparent lack of regulation by iron (Dover, 1995), although in earlier work it had been reported as being iron regulated (Hall *et al.*, 1987). The 25 kDa protein noted above was present in samples from low-iron cultures but not in the high-iron counterparts. Again this observation is consistent with the regulation of its synthesis by iron availability. The fact that this protein was only partially eluted with 1 mM ferri-exochelin may indicate that it associates not with the siderophore but with the 29 kDa protein.

Other attempts were made to elute the 29 kDa protein from the affinity column matrix. As ferri-exochelin purification is time-consuming and yields are low, it was not feasible to use it above 1 mM. When ferri-exochelin at 1 mM was supplemented with NaCl up to 1 M the 29 kDa protein still could not be eluted. Only 2% SDS could dislodge this protein from the affinity matrix (see Fig. 2, lane I), emphasizing that it had remained tenaciously attached to the siderophore until it was finally denatured with the SDS.

Implication of the 25 and 29 kDa proteins as siderophore-receptor complex components by ion-exchange chromatography. Incubation of $^{55}\text{Fe(III)}$ -exochelin with CHAPS-solubilized envelope proteins from iron-deficient cells and their subsequent loading onto a PEI-cellulose column at pH 7.5 resulted in the retention of a small

Fig. 3. Separation of ^{55}Fe -labelled envelope proteins by anion-exchange chromatography on PEI-cellulose. CHAPS-extracted envelope proteins were incubated at 37°C for 30 min with 50 nmol $^{55}\text{Fe(III)}$ -exochelin (8332 Bq nmol^{-1}) before application to the anion-exchange column. Unbound ferri-exochelin was eluted from the column in the void volume. NaCl, 0.1 M and 1 M in buffer, was used to elute labelled proteins from the PEI-cellulose. Fractions of 650 μl were collected and ^{55}Fe elution was quantified by scintillation counting of a 100 μl sample from each fraction.

Fig. 4. Fractionation by gel-filtration chromatography of the components of peak II of the PEI-cellulose eluate. The fractions in peak II from the PEI-cellulose fractionation experiment were loaded on to a Sephadex G-100-Superfine column and were eluted with 8 mM CHAPS in 50 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$, pH 7.1, collecting 2 ml fractions. ^{55}Fe elution was monitored by liquid scintillation counting of 100 μl samples from each fraction. A, B and C represent the elution volumes of marker enzymes of 87, 40, 22 kDa respectively; D represents the elution volume of ferri-exochelin (0.64 kDa). Peak I corresponds to a protein of 28.8 ± 2.5 kDa; peak II corresponds to a molecule of 5.7 ± 0.3 kDa.

amount of radioactive iron. Ferri-exochelin is positively charged at this pH and was eluted in the void volume. Elution of retained material with 0.1 M and then 1 M NaCl gave three radioactive peaks (Fig. 3). Protein determinations revealed that peak II had the highest specific activity, at $4.1\text{ nmol Fe (mg protein)}^{-1}$ (the activities of peaks I and II were 2.7 and 0.17 nmol Fe

mg^{-1} , respectively). Analysis by SDS-PAGE of the proteins eluted in the fractions showed that the 29 and 25 kDa proteins were only present in the eluate of peak II. Other proteins (at 46, 39 and 26 kDa) were also present, however.

Further analysis of peak II eluate using gel filtration through Sephadex G-100 gave two peaks of radioactivity plus ferri-exochelin (Fig. 4). Repetition of the experiment revealed that this elution profile, although noisy, was reproducible. Calibration of the column with marker proteins indicated that these two peaks (I and II in Fig. 4) represented proteins of 28.8 ± 2.5 kDa and 5.7 ± 0.3 kDa, assuming a globular nature of the proteins. The smaller molecule has not been identified, but a direct association between a protein of about 29 kDa and ferri-exochelin was once again demonstrated. No evidence was found of a labelled molecule larger than 29 kDa. Although the co-elution of the 25 kDa protein from the ion-exchange column along with the ferri-exochelin-29 kDa protein complex in peak II does not prove the existence of a larger complex, it is consistent with the formation of a complex involving the 25 kDa IREP which was not stable in the 0.1 M NaCl used as eluent in the initial PEI-cellulose chromatography step.

Studies using envelope extracts in liposomes

Proteins extracted with CHAPS from the envelope of iron-deficient cultures of *M. smegmatis* were dialysed to remove the detergent and were then incorporated into a simple liposome suspension in order to study their ferri-exochelin binding properties in an artificial membrane system. The possibility of porin-mediated entry into the interior of the liposomes, giving the appearance of ferri-exochelin binding rather than a less specific retention, was also investigated by adding an overnight dialysis step to the end of the standard protocol. Porin-mediated retention would theoretically be reversible under this revised protocol and any ^{55}Fe -exochelin so taken up would then be released.

Dependency on protein content and heat-lability of ^{55}Fe retention, assessed by ultrafiltration assays. Proteo-liposomes were formed with protein contents varying from 0 to 75 μg in a 300 μg lipid suspension in 1 ml deionized water. The liposome preparation technique used was unrefined, giving rise to a suspension of vesicles which, on microscopic analysis, were seen to vary markedly in size and to be multi-lamellar (data not presented).

The proteo-liposomes were labelled by incubation with 50 nmol $^{55}\text{Fe(III)}$ -exochelin ($1.09 \times 10^5\text{ Bq nmol}^{-1}$). Non-bound iron was removed by filtering through Millipore GVMP filters (0.22 μm) and washing with 2 ml deionized water. The degree of label retention, quantified by scintillation counting, increased with the protein content of the proteo-liposomes (Table 1). However, as ^{55}Fe retention increased non-linearly, this may suggest that some cooperativity was occurring between the proteins to improve the orientation of the putative receptors in the vesicles.

When 75 µg protein samples were heated at 100 °C for 5 min before incorporation into liposomes there was a marked decrease in the iron-binding capacity of those liposomes, to 25% of that exhibited by the unheated samples (Table 1); this is in keeping with iron-binding activity being protein-mediated. As some ^{55}Fe retention did occur with denatured protein in the liposomes, this was considered to be non-specific.

Separation of proteo-liposome-bound and free $^{55}\text{Fe(III)}$ -exochelin by gel filtration. In preliminary filtration assays, high background counts were recorded for liposomes which contained no *M. smegmatis* envelope proteins and therefore subsequently, when proteins were incorporated, the bound and unbound ^{55}Fe were always separated by gel-filtration chromatography using a Sephadex G-200 Superfine column.

The elution characteristics of each of the components of the liposome-based ferri-exochelin binding system, that is liposomes, free protein, proteo-liposomes and ferri-exochelin, were defined by running each separately through the Sephadex G-200 Superfine column. These components were then mixed in different combinations in order to analyse the requirements of ferri-exochelin binding by the envelope proteins of *M. smegmatis*.

When 'blank' liposomes containing no envelope proteins were mixed with ^{55}Fe -exochelin, ^{55}Fe eluted from the gel filtration column as free ferri-exochelin. No evidence of ^{55}Fe -binding by the liposomes was observed (Fig. 5). However, when liposomes containing envelope proteins from an iron-deficient culture of *M. smegmatis* were mixed with ^{55}Fe -exochelin, a radioactive peak eluted in the fractions corresponding to proteo-liposome fraction (Fig. 5; fractions 6–8). When an equal amount of free envelope proteins was treated similarly, the ^{55}Fe eluted as free ferri-exochelin and no evidence of ferri-exochelin binding by the envelope proteins was observed (Fig. 5).

These data indicate that ferri-exochelin binding by the envelope proteins of iron-deficient *M. smegmatis* was significant only when they were incorporated into the lipid matrix of the liposomes, where it is likely that the relevant proteins may adopt a near-native conformation.

When the ^{55}Fe -exochelin-proteo-liposomes were dialysed the ^{55}Fe retained again eluted in the fraction corresponding to the proteo-liposomes, although the activity had now decreased to 70% of that measured before dialysis (Fig. 5). This decrease could have been due either to the diffusion of ^{55}Fe -exochelin that had been retained unbound within the liposomes, presumably after entering via porin, or to the dissociation of the ferri-exochelin-receptor complex. Nevertheless, a significant proportion of liposome-associated ^{55}Fe was retained, presumably as a specific ferri-exochelin-protein complex.

Enhancement of binding of ferri-exochelin-administered iron by *M. smegmatis* envelope proteins incorporated into liposomes. The sum of the ferri-exochelin retained between fractions 3 and 10 (Fig. 5) of the gel-filtration analysis of the dialysed, labelled proteo-liposome containing 75 µg envelope protein was 3.5 nmol. If this is compared to the ferri-exochelin retained and eluted with

Table 1. Dependency of iron retention upon the non-denatured envelope protein content of proteo-liposomes

Measurements were made of the retention of iron, administered as ^{55}Fe -exochelin, by a proteo-liposome suspension in water after labelling at 37 °C for 30 min and label separation by ultrafiltration. Corrected retention is calculated by subtracting background retention (gained from assays using non-protein-bearing liposomes only) from the apparent iron retention in each assay. The results presented are the mean values of triplicate samples. The experiment was repeated a further three times; the results in each case were similar.

Protein content (µg)	Fe retention (pmol)
0	0
25	18
50	107
75	239
75 (heated)	66

Fig. 5. Effect of incorporation into liposomes on the ferri-exochelin-binding capacity of envelope proteins from iron-deficient *M. smegmatis*. Proteo-liposomes ($\blacktriangle$), liposomes containing no protein ($\blacklozenge$) and free envelope proteins ($\triangle$) were all labelled in a 1 ml reaction mixture [30 min; 37 °C; 50 nmol Fe(III) -exochelin, specific activity $1.09 \times 10^5 \text{ Bq nmol}^{-1}$] and then analysed by the elution of ^{55}Fe from the gel filtration column. The stability of the ^{55}Fe retention exhibited by the labelled proteo-liposomes was assessed by dialysing (16 h; 1000 vols, 4 °C) the sample before chromatography ($\triangle$).

peak II (Fig. 3) from the PEI-cellulose separations of the ferri-exochelin-29 kDa protein complex formed in CHAPS solution, one can quantify the enhancement of binding afforded by incorporation of the proteins into the liposomes: 0.7 nmol ferri-exochelin eluted with peak II when a 2 mg envelope protein sample was labelled, i.e. $0.35 \text{ nmol Fe (mg protein)}^{-1}$, but with the proteo-liposomes 3.5 nmol Fe was bound by 75 µg protein, i.e. $47 \text{ nmol Fe (mg protein)}^{-1}$. The binding activity of the envelope proteins was therefore enhanced 133-fold by their incorporation into proteo-liposomes.

Attempts to characterize ferri-siderophore-receptor complexes formed in liposomes. Having established that a ferri-exochelin-receptor complex could be formed in proteo-liposomes, attempts were made to extract the complex(es) with detergents in order to characterize their components and then compare these to the results achieved with the other approaches given above. Four detergent regimes were assessed for efficiency in releasing protein-bound ^{55}Fe from labelled proteo-liposomes: (i) 8 mM CHAPS, (ii) 8 mM CHAPS + 1 mM taurodeoxycholic acid, (iii) 8 mM CHAPS + 0.5 M NaCl and (iv) 0.5 % Triton X-100. In each case, the vesicles were digested for 16 h at 4 °C and then soluble and insoluble ^{55}Fe species were separated by centrifugation. ^{55}Fe radioactivity was measured in samples of each fraction and any diffusible ^{55}Fe removed from the soluble fraction by dialysis. Retained ^{55}Fe was considered to be of a high molecular mass (i.e. non-dialysable) and was probably a ferri-siderophore-receptor complex. The highest recovery of soluble, high-molecular-mass ^{55}Fe was achieved using Triton X-100 solubilization of the proteo-liposomes and, although recovery was far from ideal at 18.4 % of the original ^{55}Fe in the labelled proteo-liposome preparation, it was considered sufficient to allow the complex(es) to be characterized by electrophoresis.

As SDS-PAGE would lead to the denaturation of the complex and inadequate resolution had been achieved earlier on CHAPS-PAGE, Triton X-100-PAGE was used. However, autoradiography revealed that the soluble, high-molecular-mass ^{55}Fe run on Triton X-100-PAGE had electrophoresed at R_F 1, indicative of free ferri-exochelin. Thus the complex(es) applied to the PAGE system appeared to have dissociated to allow the ferri-siderophore to electrophorese freely.

DISCUSSION

Direct evidence for interaction of siderophores with envelope proteins is extremely sparse and identification of siderophore receptor proteins has rested mainly on genetic or immunological evidence (Roberts *et al.*, 1989; Schneider & Hantke, 1993; Meyer *et al.*, 1990; Heinrichs *et al.*, 1991; Wooldridge *et al.*, 1992; Henderson & Payne, 1994). An exception is the recent work of Zhou *et al.* (1993) and Zhou & van der Helm (1993), who have shown direct binding of ferri-enterobactin to the FecA ferric citrate receptor protein from *E. coli*. The principal difficulty that has faced workers is the absence of any satisfactory procedure for isolating an intact siderophore-receptor complex. The binding between the ligand and the protein must perforce be relatively weak and cannot depend on ionic or covalent bonds, as the two entities must dissociate to allow the transport of the ferri-siderophore to the inner membrane for uptake into the cytoplasm and removal of the iron. We can presume therefore that the binding of siderophore to its binding protein is created principally by a conformational change in the protein itself upon contact with the ferri-siderophore and is possibly stabilized, at best, by hydrogen bonding or even van der Waals' forces. Thus the conformation between the receptor and its ligand can only

be stable under a limited set of conditions. Consequently, evidence for the role of the multiplicity of different siderophore receptors in a variety of bacteria (see Guerinet, 1994) has relied either on deletion of individual genes with resultant loss of uptake function or upon antibodies raised against individual proteins blocking iron uptake. Previously in *M. smegmatis*, only the latter approach was feasible and indeed this strongly suggested a key role of the 29 kDa protein in ferri-exochelin uptake (Hall *et al.*, 1987). Until recently, genetic work with mycobacteria has lagged considerably behind studies with other bacteria. However, Fiss *et al.* (1994) have reported the isolation of several auxotrophic mutants of *M. smegmatis* that required exochelin for growth. Only one of these auxotrophs was characterized: it lacked the *N*-formyltransferase (part of the exochelin biosynthetic pathway), for which gene *fxuA* was designated. The loss of function in the other three mutants could not be characterized, although the affected genes, termed *fxuA*, *fxuB* and *fxuC*, shared amino acid sequence homology with, respectively, the iron permeases FepG, FepC and FepD from *E. coli*. These three proteins form a complex in the inner membrane of *E. coli* for the uptake of enterobactin (see Guerinet, 1994), and related proteins may be involved in the movement of exochelin though the cytoplasmic membrane of *M. smegmatis*.

The uptake of iron into any micro-organism is clearly complex, and mycobacteria, which show a multiplicity of uptake mechanisms, are no exception (Wheeler & Ratledge, 1994). The mechanism of ferri-exochelin uptake is an active, energy-requiring process (Stephenson & Ratledge, 1980). Two mechanisms can be envisaged for the energy transduction necessary for the dissociation of the ferri-exochelin-protein complex which is required to facilitate transport of the ferri-siderophore to the periplasm: either an analogue of the TonB protein of the *Enterobacteriaceae* may couple the proton-motive force to ferri-siderophore transport, or the hydrolysis of a nucleotide at the cytoplasmic face of the inner membrane could power the process. It was thought that this latter scenario could be modelled on the affinity column if all of the necessary components of the transduction machinery were retained on the column in an active state. Although displacement of the 29 kDa protein from the ferri-exochelin-Sephadex affinity column using 15 mM ATP in buffered CHAPS was achieved, the results were not consistent (L. G. Dover, unpublished). This elution of the 29 kDa protein was, however, the only one achieved without prior protein denaturation. The possible association of a 25 kDa protein with the 29 kDa putative receptor is suggested as it, too, was tightly bound to the affinity matrix but could be eluted separately from the 29 kDa protein. Possibly the smaller protein could be a second receptor for ferri-exochelin in a system similar to that described by Zhou *et al.* (1993) for *E. coli* enterobactin, which binds not only to its own receptor, FepA, but also to a ferric citrate protein receptor (FecA). These two proteins, however, are both approximately 80 kDa in size. For the record, *M. smegmatis* also has a ferric citrate transport system (Messenger & Ratledge, 1982). Altern-

atively, the 25 kDa protein could be to fulfil some 'helper' function for binding or transport of the exochelin akin to what is considered to occur in other bacteria (Guerinot, 1994).

The binding of ferri-exochelin by envelope protein(s) is greatly enhanced (133-fold) by the incorporation of the proteins into liposomes, suggesting that a hydrophobic environment is needed to maintain an active binding conformation. Although the protein-ferri-exochelin complex can be solubilized by Triton X-100, the association is lost during electrophoresis. If the association of ferri-exochelin to the receptor is fairly weak – perhaps by hydrogen bonding – and if the free NH_2 groups of exochelin (there are three) are not ionically bound to the protein, then the complex may easily become separated in an electric field. It would, in any case, be likely that the binding of ferri-exochelin to its receptor would be weak, as clearly once iron is removed for ingress into the cytoplasm, the desferri-exochelin must readily dissociate from the protein: if it were ionically bound it would be unable to do this.

The 29 kDa protein has been exceptionally difficult to substantiate as a ferri-exochelin binding protein: all our observations of its behaviour in the chromatography and PAGE systems described here have suggested it to be strongly lipophilic. It is readily denatured when not placed in detergents, and in SDS it is completely denatured, showing no ability to recombine with ferri-exochelin. Although proteins of a similar size have been found in *M. avium* and *M. leprae* (Sritharan & Ratledge, 1990) and a gene exhibiting significant homology to that encoding the putative iron-regulated 28 kDa antigen of *M. leprae* has been identified in *M. tuberculosis* (J. W. Dale, personal communication), *M. tuberculosis*, *M. avium* and *M. bovis* BCG do not produce an exochelin-type siderophore but instead elaborate a modified mycobactin – carboxymycobactin (Lane *et al.*, 1995) as the principal extracellular siderophore. These molecules are taken up by a non-active, facilitated diffusion type of mechanism (Stephenson & Ratledge, 1980) which is distinct from the exochelin-dependent system described here and which should not therefore involve an exochelin receptor protein. The relationship of the 29 kDa family of proteins between the pathogens and the non-pathogens remains to be resolved, although, as pointed out in the Introduction, Dale & Patki (1990) have found homology of the promoter region of a gene encoding a 28 kDa protein of *M. leprae* with the operator site of the Fur iron uptake regulatory protein of *E. coli*, suggesting an involvement of this 28 kDa protein in iron acquisition.

There is still much to be resolved about iron transport in mycobacteria. The ability of a BCG cosmid to restore synthesis of exochelin MS in *M. smegmatis* (Fiss *et al.*, 1994) has suggested that the exochelin transport system as described here may be found in all mycobacteria, but the peptido-siderophores, like exochelin MS, have not yet been found in the pathogenic mycobacteria (C. Ratledge & M. Ewing, unpublished) and earlier work (Stephenson & Ratledge, 1980) showed that $^{55}\text{Fe(III)}$ -exochelin MS

was not taken up by *M. bovis* BCG. The ubiquity of this 29 kDa exochelin receptor protein amongst all mycobacteria therefore seems unlikely; but this needs to be investigated by appropriate molecular techniques.

ACKNOWLEDGEMENTS

L. G. D. is indebted to the SERC (now BBSRC) for a research studentship and also to the MRC for support as a post-doctoral research assistant. We thank Mrs Maureen Ewing for her technical assistance.

REFERENCES

- Cavinato, A. G., Macleod, R. M. & Ahmed, M. S. (1988). A non-denaturing gel electrophoresis system for the purification of membrane proteins. *Prep Biochem* **18**, 205–215.
- Cherayil, B. J. & Young, R. A. (1988). A 28-kDa protein from *Mycobacterium leprae* is a target of the human antibody response in lepromatous leprosy. *J Immunol* **141**, 4370–4375.
- Dale, J. W. & Patki, A. (1990). Mycobacterial gene expression and regulation. In *Molecular Biology of the Mycobacteria*, pp. 173–198. Edited by J. McFadden, London: Academic Press.
- Dover, L. G. (1995). *Studies in the iron uptake mechanism of Mycobacterium smegmatis: identification of the iron uptake system*. PhD thesis, University of Hull.
- Fiss, E. H., Yu, S. & Jacobs, W. R. (1994). Identification of genes involved in the sequestration of iron in mycobacteria: the ferric exochelin biosynthetic and uptake pathways. *Mol Microbiol* **14**, 557–569.
- Guerinot, M. L. (1994). Microbial iron transport. *Annu Rev Microbiol* **48**, 743–772.
- Hall, R. M., Sritharan, M., Messenger, A. J. M. & Ratledge, C. (1987). Iron transport in *Mycobacterium smegmatis*: occurrence of iron-regulated envelope proteins as potential receptors for iron uptake. *J Gen Microbiol* **133**, 2107–2114.
- Heinrichs, D. E., Young, L. & Poole, K. (1991). Pyochelin-mediated iron transport in *Pseudomonas aeruginosa*: involvement of a high-molecular-mass outer membrane protein. *Infect Immun* **59**, 3680–3684.
- Henderson, D. P. & Payne, S. M. (1994). *Vibrio cholerae* iron transport systems: roles of heme and siderophore iron transport in virulence identification of a gene associated with multiple iron transport systems. *Infect Immun* **62**, 5120–5125.
- Lane, S. J., Marshall, P. S., Upton, R. J., Ratledge, C. & Ewing, M. (1995). Novel extracellular mycobactins, the carboxymycobactins from *Mycobacterium avium*. *Tetrahedron Lett* **36**, 4129–4132.
- Messenger, A. J. M. & Ratledge, C. (1982). Iron transport in *Mycobacterium smegmatis*: uptake of iron from ferric citrate. *J Bacteriol* **149**, 131–135.
- Meyer, J. M., Hohnadel, D., Kahn, A. & Cornelis, P. (1990). Pyoverdine-facilitated iron uptake in *Pseudomonas aeruginosa*: immunological characterization of the ferripyoverdine receptor. *Mol Microbiol* **4**, 1401–1405.
- Neilands, J. B., Kanopka, K., Schwyn, B., Coy, M., Francis, R. T., Paw, B. H. & Bagg, A. (1987). Comparative biochemistry of microbial iron assimilation. In *Iron Transport in Microbes, Plants and Animals*, pp. 3–33. Edited by G. Winkelmann, D. van der Helm & J. B. Neilands. Weinheim: VCH Publishers.
- Ratledge, C. & Hall, M. J. (1971). Influence of metal ions on the formation of mycobactin and salicylic acid in *Mycobacterium smegmatis* grown in static culture. *J Bacteriol* **108**, 314–319.

- Ratledge, C., Patel, P. V. & Mundy, J. (1982).** Iron transport in *Mycobacterium smegmatis*: the location of mycobactin by electron microscopy. *J Gen Microbiol* **128**, 1559–1565.
- Roberts, M., Wooldridge, K. G., Gavine, H., Kuswandi, S. I. & Williams, P. H. (1989).** Inhibition of biological activities of the aerobactin receptor protein in rough strains of *Escherichia coli* by polyclonal antiserum raised against native protein. *J Gen Microbiol* **135**, 2387–2398.
- Schneider, R. & Hantke, K. (1993).** Iron-hydroxamate uptake systems in *Bacillus subtilis*: identification of a lipoprotein as a part of a binding protein-dependent transport system. *Mol Microbiol* **8**, 111–121.
- Sharman, G. J., Williams, D. H., Ewing, D. F. & Ratledge, C. (1995).** Isolation, purification and structure of exochelin MS, the extra-cellular siderophore from *Mycobacterium smegmatis*. *Biochem J* **305**, 187–196.
- Sritharan, M. & Ratledge, C. (1990).** Iron regulated envelope proteins of mycobacteria grown *in vitro* and their occurrence in *Mycobacterium avium* and *Mycobacterium leprae* grown *in vivo*. *BioMetals* **2**, 203–208.
- Stephenson, M. C. & Ratledge, C. (1979).** Iron transport in *Mycobacterium smegmatis*: uptake of iron from ferriexochelin-exochelin. *J Gen Microbiol* **110**, 193–202.
- Stephenson, M. C. & Ratledge, C. (1980).** Specificity of exochelins for iron transport in three species of mycobacteria. *J Gen Microbiol* **116**, 521–523.
- Wheeler, P. R. & Ratledge, C. (1994).** Metabolism of *Mycobacterium tuberculosis*. In *Tuberculosis: Pathogenesis, Protection, and Control*, pp 353–385. Edited by B. R. Bloom. Washington, DC: American Society for Microbiology.
- Wooldridge, K. G., Morrissey, J. A. & Williams, P. H. (1992).** Transport of ferric-aerobactin into the periplasm and cytoplasm of *Escherichia coli* K12: role of envelope-associated proteins and effect of endogenous siderophores. *J Gen Microbiol* **138**, 597–603.
- Zhou, X. H. & van der Helm, D. (1993).** A novel purification of ferric citrate receptor (Fec A) from *Escherichia coli* UT 5600 and further characterization of its binding activity. *BioMetals* **6**, 37–44.
- Zhou, X. H., van der Helm, D., & Adjimani, J. (1993).** Purification of outer membrane iron transport receptors from *Escherichia coli* by fast protein liquid chromatography: Fep A and Fec A. *BioMetals* **6**, 25–35.
-

Received 18 September 1995; revised 19 January 1996; accepted 29 January 1996.