

Siderophore and haem iron use by *Tritrichomonas foetus*

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The ability of the parasitic flagellate *Tritrichomonas foetus* to use various iron sources for its physiological requirements was studied. The siderophores ferrioxamine B, ferrichrome, triacetylfusarinine, coprogen, enterobactin and pyoverdine sustained growth of the cells under iron-limited conditions, and siderophore iron was incorporated into the major iron protein of *T. foetus*, ferredoxin. The kinetics of siderophore uptake by the cells indicated that a non-saturable transport is involved, unlike the uptake of a ferrous salt. Siderophore uptake by the cells did not involve extracellular reductive dissociation of the ferric chelates, although *T. foetus* cells had some ferrireductase activity on ferric citrate. Fluorescent analogues of siderophores were used to show that the siderophores taken up by the cells were in small intracellular vesicles. The fluorescence emission maximum of pyoverdine in these intracellular vesicles shifted from 460 nm to 530 nm, indicating a very acidic environment. The results suggest that a wide range of chemically unrelated siderophores can be taken up non-specifically and efficiently used by *T. foetus*; the mechanism involved may be pinocytosis and removal of the iron from the siderophores in acidic intracellular vesicles. Haemin also sustained the growth of *T. foetus* cells under iron-limited conditions. The use of haemin iron by the cells probably involves haem oxygenase, since traces of biliverdin were found in the medium when haemin was the iron source. The iron uptake and ferrireductase activities of the cells do not seem to be regulated by the amounts of iron and copper in the growth medium.

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INTRODUCTION

The virulence of *Tritrichomonas foetus*, a parasitic flagellate that infests the urogenital tract of cattle, is greatly influenced by iron (Kulda *et al.*, 1999). The requirement of large amounts of nutritional iron is ascribed to the importance of [Fe-S] proteins for carbohydrate metabolism in trichomonads. This [Fe-S]-protein-dependent metabolism takes place in a specific organelle, the hydrogenosome, where pyruvate or malate is converted to CO₂, H₂ and acetate with concomitant substrate-level phosphorylation of ADP to ATP (reviewed by Müller, 2003). [Fe-S] clusters are assembled within the hydrogenosomes and incorporated into the apoproteins by a machinery similar to that operating in mitochondria (Sutak *et al.*, 2004a). The metabolic activity of *T. foetus* hydrogenosomes is markedly decreased when the supply of iron is restricted. The loss of ATP is

offset by the activation of the cytosolic glycolysis and the dominant end product of glucose breakdown becomes ethanol, probably due to the overproduction of pyruvate decarboxylase (Vanacova *et al.*, 2001; Sutak *et al.*, 2004b).

Little is known about iron uptake by trichomonads and nothing about its intracellular transport. There are independent mechanisms by which *T. foetus* takes up iron. Iron from low-molecular-mass complexes and from transferrin is taken up by one or more carrier-mediated transport systems, while receptor-mediated endocytosis is involved in the acquisition of lactoferrin-bound iron (Tachezy *et al.*, 1996, 1998). There is a cell ferrireductase activity, but its putative role in iron uptake is unknown (Tachezy *et al.*, 1998). Ferritin has not been found in trichomonads, and the intracellular storage of iron by these organisms is enigmatic. The major hydrogenosomal iron-containing protein is ferredoxin, as indicated by native gel electrophoresis of ⁵⁹Fe-labelled proteins of *T. foetus* cells (Suchan *et al.*, 2003).

Abbreviations: BCS, bathocuproin disulfonic acid; BPS, bathophenanthroline disulfonic acid.

The virulence of many pathogens requires specific iron-uptake systems, perhaps to counter iron-withholding host defences that operate in various environmental niches (Ratledge & Dover, 2000). Bacteria and fungi commonly produce siderophores to make iron available in an iron-limited environment (reviewed by Braun, 2001). Siderophores are small molecules that bind, solubilize and chelate extracellular ferric iron with a very high affinity. These ferrisiderophore complexes are subsequently taken up by the micro-organisms via specific transport systems. Some opportunistic organisms, such as the yeasts *Saccharomyces cerevisiae* and *Candida albicans*, cannot synthesize their own siderophores, but can use 'xenosiderophores' produced by other micro-organisms to fulfil their iron requirement (Lesuisse *et al.*, 1998; Ardon *et al.*, 2001). The ability of some organisms to use xenosiderophores could constitute a selective advantage of great value. Hydroxamate siderophores excreted by bacteria and actinomycetes are abundant in nature: their concentrations in bulk soils can reach 0.1 µM (Powell *et al.*, 1980). These concentrations can be even higher (5–10 µM) in rhizosphere soil or soil having high microbial activity (Crowley *et al.*, 1987). The concentration of siderophores in more specific ecological niches (the gastrointestinal or urogenital tracts, for example) is unknown and probably greatly depends on the circumstances, but there is no doubt that the opportunistic strategy of developing xenosiderophore uptake systems should provide a selective advantage for any pathogenic organism living in such specific environments.

Haem is also a source of iron that is frequently used by pathogenic micro-organisms (reviewed by Genco & Dixon, 2001), and haem-bound iron was recently shown to be a source of iron for *Trichomonas vaginalis* (Alderete *et al.*, 2004).

There have been no published reports of the synthesis of siderophores by protozoa, or the use of xenosiderophores by these organisms, to our knowledge. This study reinvestigates the mechanisms used by *T. foetus* to take up iron from various sources; we show that this organism can efficiently use siderophores and haemin as sources of iron.

METHODS

Organisms and cultivation. *T. foetus* strain KV-1 (Kulda *et al.*, 1984) was maintained in Trypticase-yeast extract-maltose (TYM) medium (Diamond, 1957) supplemented with 10 % heat-inactivated horse serum at pH 7.2.

Iron sources, iron uptake and reductase assays. The desferrisiderophores coprogen and enterobactin were purchased from EMC microcollection GmbH (Germany). Desferri-ferrichrome was purchased from Sigma. Ferrioxamine B refers to the commercially available mesylate derivative, Desferal (Novartis). Triacetylfusarinine was a gift from Dr H. Haas (Department of Molecular Biology, Medical University of Innsbruck, Austria). Pyoverdine was a gift from Dr J.-M. Meyer (Laboratoire de Microbiologie et de Génétique, CNRS/Université Louis-Pasteur, Strasbourg, France). Desferri-compounds were obtained as described by Wiebe & Winkelmann (1975). They were labelled with ⁵⁵Fe (50 mCi mg⁻¹; 1850 MBq mg⁻¹).

The fluorescent derivative of ferrichrome used was the glycine-based analogue B9 described by Berner *et al.* (1991). Total iron accumulation by *T. foetus* cells was measured by growing the cells overnight in TYM medium containing the ⁵⁵Fe-labelled iron source. Cells were washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 4.6 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) by centrifugation and the ⁵⁵Fe radioactivity measured by liquid scintillation. Rates of iron uptake were measured in micro-titration plates. *T. foetus* cells grown overnight under various conditions ('+Fe', 100 µM ferric citrate; '+Cu', 0.5 mM copper sulfate; '−Fe', 30 µM BPS; '−Cu', 200 µM bathocuproin disulfonic acid, BCS) were harvested by centrifugation, washed three times with PBS, and suspended in PBS at about 40 × 10⁶ cells ml⁻¹. The cells were then incubated at 37 °C in the wells of a microtitration plate (100 µl cell suspension per well) in the presence of various ⁵⁵Fe-labelled iron sources and for various times. The cells were harvested and washed (with PBS) on the filter of a cell harvester (Brandel) and their ⁵⁵Fe content measured. The ferrioreductase activity of the cells in PBS was measured spectrophotometrically by recording the increase in absorbance at 535 nm after adding 1 mM bathophenanthroline disulfonic acid (BPS) and 250 µM ferric citrate to the cell suspension kept at 37 °C.

Incorporation of ⁵⁵Fe from ferrioxamine B into ferredoxin.

Cells were incubated overnight with 25 nM [⁵⁵Fe]ferrioxamine B, harvested by centrifugation, washed three times in sucrose-Tris (ST) buffer (0.25 M sucrose, 10 mM Tris, 0.5 mM KCl, pH 7.2) and suspended in ST buffer supplemented with leupeptin (10 µg ml⁻¹) and tosyl-lysine chloromethyl ketone (1 mM). They were homogenized using a Potter homogenizer. Hydrogenosome-rich fractions were obtained by differential centrifugation (Cerkasov *et al.*, 1978), washed three times in ST buffer and separated on 15 % non-denaturing polyacrylamide gels at 4 °C. The gels were vacuum-dried and autoradiographed. The gels were rehydrated and the proteins transferred to nitrocellulose membranes. The bands of ferredoxin were visualized using a rabbit polyclonal anti-ferredoxin antiserum (provided by Patricia Johnson, UCLA, USA).

Microscopy. Cells were examined using a Leica IRE2 inverted microscope with a SP2 AOBs confocal laser scanning head. An argon laser was the source of excitation at 488 nm. Pyoverdine was excited with a two-photons Ti-Sa Laser set at 740 nm. The spectral capabilities of this microscope were used to select precise emission maxima in some experimental conditions, and thus detect any difference between the emission spectrum of intracellular pyoverdine and that of the siderophore in solution. Phase-contrast images are differential-interferential contrast images (Nomarski) recorded with the same microscope (488 nm beam through the sample, transmission detector).

Other. Biliverdin reductase was partially purified from rat liver as described by Kuty & Maines (1981).

RESULTS

T. foetus can use various siderophores as iron sources

We first compared the ability of *T. foetus* to accumulate iron from a ferric organic complex (ferric citrate) and from several siderophores (Fig. 1). Cells were grown overnight in medium containing 0.1 µM ferric citrate, ferrichrome, ferrioxamine B, enterobactin, triacetylfusarinine or pyoverdine. The total amounts of iron accumulated by the cells were not very different (Fig. 1), ranging from 0.6 % (ferrichrome) to 1.2 % (ferric citrate) of the total iron

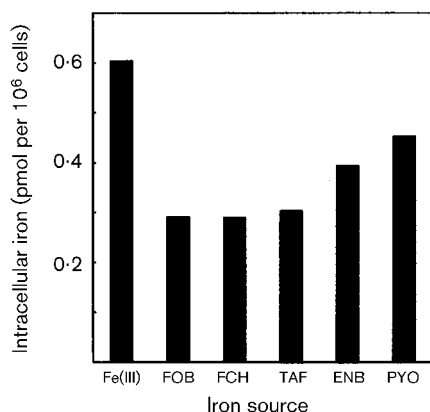


Fig. 1. ^{55}Fe accumulation from various iron sources by *T. foetus* cells. Cells were grown overnight in TYM medium containing $0.1 \mu\text{M}$ of the following ^{55}Fe -labelled iron sources: ferric citrate [Fe(III)], ferrioxamine B [FOB], ferrichrome [FCH], triacetylfusarinine [TAF], enterobactin [ENB], pyoverdine [PYO]. The cells were harvested, washed three times with PBS buffer and their ^{55}Fe radioactivity measured. Data from one representative experiment (out of two).

added to the medium (Fig. 1). This suggests that *T. foetus* can use iron from a wide variety of siderophores. We checked that the iron bound to one of these siderophores (ferrioxamine B) was indeed used by the cells by measuring the incorporation of iron from [^{55}Fe]ferrioxamine B into the major *T. foetus* iron protein, ferredoxin (Suchan *et al.*, 2003). Cells grown in the presence of a very low concentration of [^{55}Fe]ferrioxamine B (25 nM) efficiently incorporated the iron bound to the siderophore into ferredoxin (Fig. 2). Thus *T. foetus* can use siderophore iron, even when its concentration is subphysiological. The efficiency of this process may depend on the presence of specific siderophore receptors at the cell surface. We tried to evaluate the efficiency of siderophores in promoting cell growth by measuring the growth of cells cultivated in a large excess of desferri-siderophores (expected to chelate all of the medium iron as the ferri-siderophore complex) and cells cultivated with an excess of BPS (expected to chelate all of the medium iron as the $\text{Fe}^{2+}(\text{BPS})_3$ complex). Cell growth was completely inhibited by the excess BPS (Fig. 3), confirming that *T. foetus* requires iron (Tachezy *et al.*, 1996). In contrast, the cells grew in medium containing excess desferri-ferrioxamine B, desferri-ferrichrome or desferri-pyoverdine, but more slowly than control cells (Fig. 3).

Mechanism of siderophore iron use

Specific high-affinity iron-uptake systems are generally regulated in such a way that transport is induced by low-iron growth conditions. Iron transport is also copper dependent in several eukaryotic cells because copper is required for the redox processes involved in reductive iron uptake (i.e. reductive removal of iron from its ligands

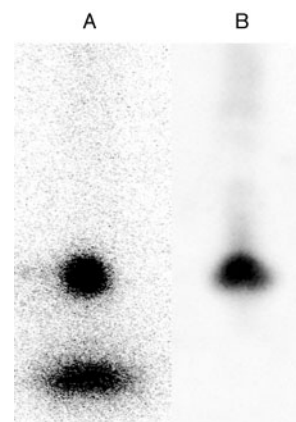


Fig. 2. Incorporation of ^{55}Fe from ferrioxamine B into ferredoxin. Cells were incubated overnight with 25 nM [^{55}Fe]ferrioxamine B and the hydrogenosome-rich fraction was isolated and analysed by native gel electrophoresis. The resulting phosphorimages were autoradiographed (A) and ferredoxin detected by Western blotting using polyclonal anti-ferredoxin antibodies (B).

prior to uptake of ferrous ions by the cells; for a review, see Kosman, 2003). We tested the effects of iron and copper concentrations in the medium on iron uptake and reduction by *T. foetus*. Cells were grown overnight in iron-rich ($100 \mu\text{M}$ ferric citrate), iron-poor ($30 \mu\text{M}$ BPS), copper-rich ($500 \mu\text{M}$ copper sulfate) and copper-poor ($200 \mu\text{M}$ BCS) medium, washed and suspended in PBS buffer. These different growth conditions did not affect the rate of iron uptake from either ferric citrate or a siderophore (ferrioxamine B) (Table 1). Similarly, there was no significant

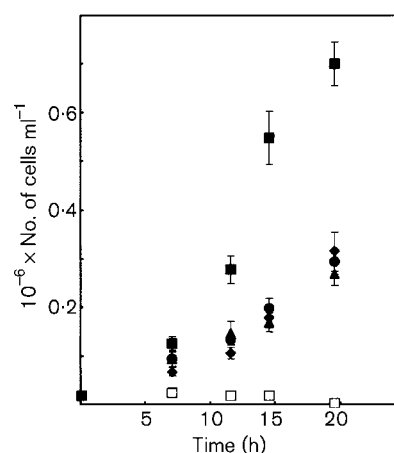


Fig. 3. Growth of *T. foetus* cells in the presence of different iron chelators. Cells were inoculated in TYM medium (■) or in TYM medium containing $200 \mu\text{M}$ BPS (□), or $100 \mu\text{M}$ desferri-ferrioxamine B, desferri-ferrichrome or desferri-pyoverdine (other symbols). Growth was followed by counting living cells under the microscope. Means \pm SE from three experiments.

Table 1. Effect of growth conditions on iron uptake and reduction by *T. foetus* cells

Cells were grown overnight in TYM medium supplemented with 30 μM BPS (iron-limited condition, '-Fe'), 100 μM ferric citrate (iron-rich condition, '+Fe'), 200 μM BCS (copper-limited condition, '-Cu') or 500 μM copper sulfate (copper-rich condition, '+Cu'). The cells were harvested, washed three times with PBS and suspended in PBS at about 40×10^6 cells ml^{-1} . Iron uptake was measured by incubating the cells for 10 min at 37 °C with either 1 μM ferric citrate [Fe(III)] or 1 μM ferrioxamine B [FOB]. The cells were then washed three times by centrifugation and incorporated ^{55}Fe was measured. Iron reduction was followed spectrophotometrically, by recording the increase in absorbance at 535 nm after adding 1 mM BPS and 250 μM ferric citrate to the cell suspension kept at 37 °C. Means \pm SE from three experiments are shown.

	Iron uptake and reduction rates (pmol min^{-1} per 10^6 cells)			
	-Fe	+Fe	-Cu	+Cu
Fe(III) uptake	0.48 ± 0.1	0.61 ± 0.07	0.65 ± 0.127	0.45 ± 0.04
FOB uptake	0.26 ± 0.04	0.31 ± 0.03	0.34 ± 0.02	0.28 ± 0.03
Fe(III) reduction	28.5 ± 2	33 ± 4	26.5 ± 1.5	24.5 ± 3.5
FOB reduction	0	0	0	0

difference between the ferrireductase activities of these cells (Table 1). Thus, iron uptake and ferrireductase activities were apparently not regulated by iron/copper availability in the growth medium. Moreover, cells were unable to reductively dissociate iron from the siderophore ferrioxamine B, whatever the growth conditions (Table 1). Thus, it does not seem probable that *T. foetus* cells take up siderophore iron by a reductive mechanism, as was proposed for ferric salts (Tachezy *et al.*, 1998).

We compared the kinetics of iron uptake from ferric citrate and various siderophores. The rates of iron uptake were

similar for all the iron sources tested (Fig. 4). Iron was taken up from siderophores that had very different structures (ferrioxamine B/ferrichrome, enterobactin, pyoverdine) at the same rate (Fig. 4). The prokaryote and eukaryote siderophore transport systems described to date are very specific and have high affinities for their substrates (for a review, see Neilands, 1995). *T. foetus* has been shown to contain a saturable transport system for an iron salt (Fe-NTA) (Tachezy *et al.*, 1998). We checked to see if the transport of siderophores was also saturable by comparing iron-uptake rates from increasing concentrations of ferrous ascorbate and of ferrioxamine B. The kinetic parameters

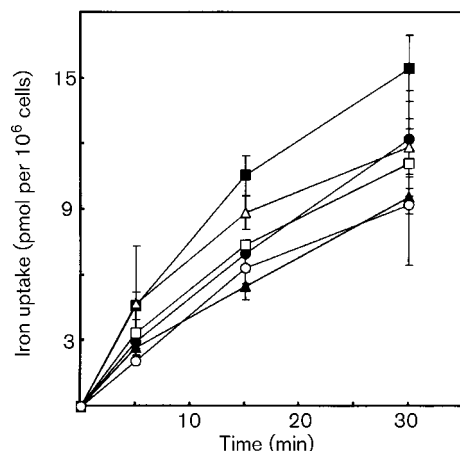


Fig. 4. Uptake kinetics of ^{55}Fe from various iron sources by *T. foetus* cells. Cells were grown overnight in TYM medium, washed three times with PBS, and incubated in PBS containing 1 μM of various iron sources at 37 °C. Cells were harvested at intervals, washed, and their ^{55}Fe contents determined by liquid scintillation. ■, Ferric citrate; ●, pyoverdine; △, enterobactin; □, ferrichrome; ▲, ferrioxamine B; ○, coprogen.

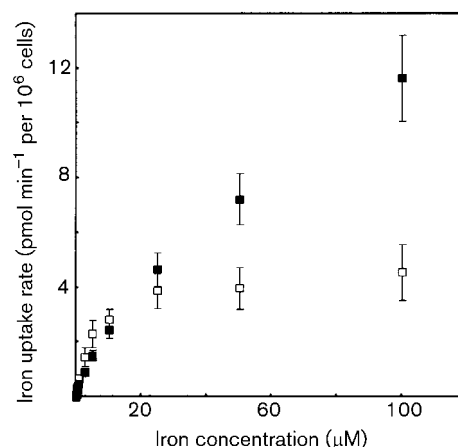


Fig. 5. Saturation of ferrous iron transport; unsaturation of siderophore transport. Cells were grown overnight in TYM medium, washed three times with PBS and incubated in PBS for 30 min at 37 °C with various concentrations (0.1–100 μM) of ^{55}Fe -labelled ferrous ascorbate (□) and ferrioxamine B (■). Cells were washed with the cell harvester and their radioactivity measured.

for the uptake of 'free' iron (ferrous ascorbate) and that of iron bound to a siderophore (ferrioxamine B) were very different (Fig. 5). The uptake of the ferrous salt was saturable, with an apparent K_m of about 5 μM (not shown), a value not very different from that found by Tachezy *et al.* (1998) for Fe-NTA. In contrast, the uptake of iron from ferrioxamine B showed no obvious saturation (Fig. 5). This confirms that siderophore uptake is not mediated by specific receptors at the cell surface. Fluorescent derivatives of siderophores were also used to show the presence of the siderophore inside cells. These imaging experiments used fluorescent derivatives of ferrioxamine B and ferrichrome and the naturally fluorescent siderophore pyoverdine (Meyer, 2000). Results obtained with the fluorescent derivative of ferrichrome and with pyoverdine are presented in Fig. 6. Results obtained with the fluorescent derivative of ferrioxamine B were the same as those obtained with ferrichrome (not shown). The maximum excitation of the fluorescent ferrichrome derivative was around 480 nm (Ardon *et al.*, 1998). Control cells (grown without any siderophore) excited

at this wavelength showed significant auto-fluorescence of small intracellular vesicles (Fig. 6A). The fluorescence was significantly greater when the cells were grown overnight with 10 μM fluorescent ferrichrome (Fig. 6B). The increased fluorescence was even clearer when the iron in the fluorescent complex was replaced by gallium (Fig. 6C). Ga^{3+} forms complexes with siderophore ligands that are isomorphous with the Fe^{3+} complexes but are not reducible (Emery, 1986). The fluorescence of the ferrichrome analogue is strongly quenched by iron (Ardon *et al.*, 1998), and thus the desferri-ferrichrome ligand fluoresces much more than the ferrichrome complex. In contrast, replacing iron by gallium increased the fluorescence of the ligand (data not shown). This explains why the fluorescence spots were more intense when the cells were grown with the gallium complex of fluorescent ferrichrome rather than with the iron complex (compare Fig. 6B and Fig. 6C). The fluorescence appeared as intense discrete spots on a background of low fluorescence in both cases. This suggests that the siderophore complexes were accumulated

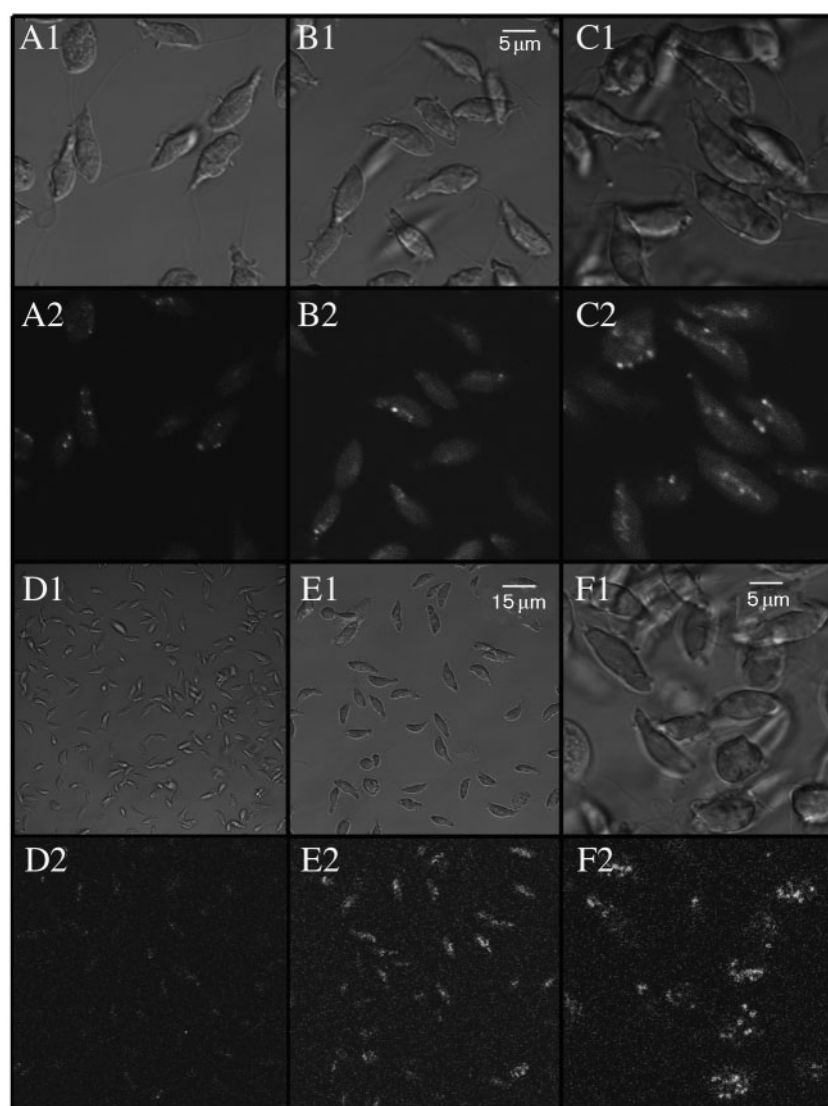


Fig. 6. Confocal microscopy showing fluorescent siderophores accumulated in intracellular vesicles. Cells were grown overnight in TYM medium containing (or not: control cells) 10 μM fluorescent siderophores, washed three times with PBS, and examined under the microscope. A–C: λ_{exc} 480 nm, λ_{em} 500–600 nm. A, control cells (no siderophore added); B, ferrichrome; C, gallium analogue of ferrichrome. D–F: λ_{exc} 380 nm, λ_{em} 530 nm. D, control cells (no siderophore added); E, F ferri-pyoverdine (two different scales). 1, phase-contrast pictures; 2, fluorescence pictures.

in intracellular vesicles, perhaps pinocytotic vesicles or lysosomes. We also studied the intracellular distribution of the naturally fluorescent siderophore pyoverdine. Desferri-pyoverdine fluoresces intensely around 460 nm when excited at 400 nm, while the ferric form of pyoverdine does not fluoresce (for a review, see Meyer, 2000). Cells grown overnight with 10 μ M ferri-pyoverdine did not fluoresce any more than control cells at these wavelengths (data not shown). However, emission and excitation scans revealed that cells grown in pyoverdine fluoresced much more than control cells, with a maximum at 530 nm when exciting at 380 nm (Fig. 6D, E, F). As fluorescence seemed to be localized in intracellular vesicles, we speculated that the pH within such vesicles could modify the fluorescent properties of desferri-pyoverdine. We measured the fluorescence spectra of desferri-pyoverdine at various pHs, and found that there was a shift in the maximum excitation wavelength from 400 to 380 nm and in the emission maximum from 460 to 530 nm below pH 3 (data not shown). Thus, our data are compatible with the hypothesis that siderophores are accumulated and dissociated in very acidic intracellular vesicles. Acidification strongly decreases the affinity of hydroxamate ligands for iron, and this could be a key parameter involved in removal of iron from internalized siderophores in *T. foetus* cells. The removal of iron from the siderophores inside the cells could involve reductive dissociation of the complex and/or hydrolysis of the siderophore itself. Both processes are likely to be favoured under acidic conditions.

Iron use from haemin

T. foetus cells readily used another iron source, haemin, in addition to iron salts/organic complexes and various siderophores. A high concentration (200 μ M) of the iron chelator BPS completely inhibited the growth of cells unless haemin was present (Fig. 7A). The iron in haemin is tightly bound to the porphyrin ring in both the ferric and the ferrous states (unlike siderophores). Iron cannot be released from haemin by reduction (Buchler, 1975). Its removal from haem requires the porphyrin ring to be opened, to form biliverdin. This reaction is catalysed by haem oxygenase, which is thus required for the assimilation of iron from haemin. Traces of biliverdin were found in culture supernatants of *T. foetus* cells grown with haemin as the sole iron source (Fig. 7B). The medium of cells grown with haemin showed a peak of absorbance around 670 nm, and this peak decreased as the absorbance around 480 nm increased following treatment of the medium supernatant with biliverdin reductase (Fig. 7B). These features are characteristic of the reduction of biliverdin to bilirubin (McDonagh & Palma, 1980). The use of haemin iron by *T. foetus* will be reported fully elsewhere.

DISCUSSION

T. foetus has an unusually high nutritional requirement for iron (Tachezy *et al.*, 1996) that is probably linked to the

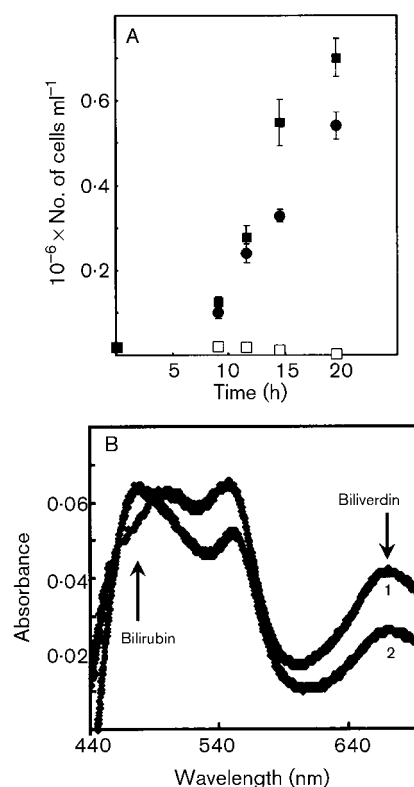


Fig. 7. Haem-iron use by *T. foetus*. A, Haemin restored the growth of the cells in iron-deprived medium. Cells were grown in TYM medium (■) or in TYM medium containing 200 μ M BPS (□) or 200 μ M BPS plus 50 μ M haemin (●). Growth was monitored by counting living cells. Means \pm SE from three experiments. B, Traces of biliverdin in culture containing haemin as the sole iron source. Cells were grown overnight in TYM medium containing 200 μ M BPS plus 50 μ M haemin, harvested, and the culture supernatant was analysed spectrophotometrically. Trace 1, culture supernatant (blank: same medium not inoculated with cells); trace 2, culture supernatant treated with biliverdin reductase (30 min at 37 °C plus 1 mM NADH and 1 mM NADPH).

importance of [Fe-S] proteins in mediating key steps of the trichomonad's energy metabolism. The present study shows that this organism also has an unusually great ability to use iron from very different iron sources, via mechanisms that are not necessarily specific, i.e. that do not involve inducible high-affinity, saturable uptake pathways. No report has been published, to our knowledge, of siderophore iron use by a protist. The use of siderophore iron by bacteria and fungi can reflect two different situations. The organism itself may synthesize and excrete (desferri)-siderophore(s) for which it has specific receptor(s). In that case, both siderophore synthesis and siderophore uptake pathways are highly specific and tightly regulated according to the iron requirement of the cell (reviewed by Neilands, 1995). Otherwise, 'opportunistic organisms' can use siderophores excreted by other organisms, although they

are unable to synthesize their own siderophore. These organisms have developed specific, iron-regulated pathways for uptake of 'xenosiderophores'. For example, *S. cerevisiae* and *C. albicans* do not seem to produce any siderophore, but they can produce specific uptake pathways for taking up exogenous siderophores (ferrichrome, ferrioxamine B, triacetylfusarinine and enterobactin for *S. cerevisiae*; ferrichrome for *C. albicans*; reviewed by Kosman, 2003). However, *S. cerevisiae* and *C. albicans* cells are unable to transport non-reductively siderophores for which they have no specific receptors/transporters (pyoverdine or coprogen, for example). Our present results show that *T. foetus* belongs to a third category of organisms that can use all siderophores via non-specific transport pathways. The lack of specific cell-surface receptors for siderophores is reflected by our observation that the cells can grow in a medium containing excess desferri-siderophores, but more slowly than control cells. When cells have specific siderophore receptors, addition of an excess desferri-siderophore to the growth medium either enhances or has no effect on cell growth, as shown in *S. cerevisiae* (Lesuisse *et al.*, 1998) and *C. albicans* (Lesuisse *et al.*, 2002). But there is no report of a desferri-siderophore inhibiting the growth of cells that have a specific receptor-mediated transport system for the corresponding siderophore. Thus, *T. foetus* cells can use siderophore iron, but probably not via specific siderophore receptors. Siderophore uptake is not saturable, and may occur by endocytosis/pinocytosis. In the endosome, iron could be removed from the siderophores by iron reduction and/or siderophore degradation. The low redox potential likely within the anaerobic cells of *T. foetus* should facilitate the reductive removal of iron from siderophores. Local acidification should also favour the removal of iron from siderophores. Our finding with pyoverdine as a fluorescent probe suggests that the internalized siderophores are indeed concentrated in very acidic intracellular vesicles where iron is removed. Siderophore iron, although usable by *T. foetus*, is less efficient at promoting cell growth than the 'free' iron present in the medium. As the rates of iron uptake from siderophores and iron salts/organic complexes are roughly similar, this could mean that the intracellular removal of iron from the siderophore is rate-limiting. Siderophore iron use by *T. foetus* may be physiologically relevant. The urogenital tract where the parasite lives is an ecological niche for various micro-organisms, including siderophore-excreting bacteria and fungi, under both normal and pathological conditions. The vaginal ecosystem is complex, and the ecology of the vaginal flora is readily influenced by numerous endogenous and exogenous factors, leading to changes in the overall microbial composition (Mardh, 1991). Further work would be required to determine whether the presence of siderophore-excreting bacteria and/or fungi in the urogenital tract promotes infection by *T. foetus*.

T. foetus has been shown to possess a saturable, energy-dependent pathway for the uptake of ferric salts (Tachezy

et al., 1998). It was suggested that low-molecular-mass ferric compounds could be taken up reductively, as in yeast (Lesuisse *et al.*, 1987), because iron reduction stimulates the uptake whereas adding a non-permeant ferrous chelator, BPS, inhibits it (Tachezy *et al.*, 1998). We now find that ferrous ascorbate is taken up faster by the cells than is ferric citrate via a saturable process, and that the rate of iron reduction by the cells is compatible with reductive uptake. However, the redox potential of the reducing system of *T. foetus* is not low enough to reduce siderophores, unlike that of *S. cerevisiae* (Lesuisse & Labbe, 1989). Hence it is unlikely that there is reductive uptake of siderophore iron. Other low-molecular-mass ferric compounds, with higher redox potentials, could be taken up reductively by the cells. Definitive evidence for the physiological involvement of a reduction step in iron uptake by *T. foetus* is difficult to provide, since there seems to be no relationship between the iron-reducing capacity of the cells and their iron status.

Haem is also a potential source of iron for *T. foetus*. This is probably very important physiologically, since *T. foetus* cells live in an ecological niche where haem-proteins and haem-containing micro-organisms are abundant. Specific erythrocyte binding to the surface of a related human parasite, *T. vaginalis*, has been reported (Lehker *et al.*, 1990) and haem iron use by this parasite has recently been shown (Alderete *et al.*, 2004).

Lactoferrin, transferrin and low-molecular-mass ferric compounds are also sources of iron for *T. foetus* (Tachezy *et al.*, 1996, 1998). Our findings show that hydroxamate, catecholate, pyoverdine-type siderophores and haemin must also be added to this list. Iron uptake from lactoferrin is saturable, with specific binding of the protein at the cell surface (Tachezy *et al.*, 1996), but the affinity of the process is rather low (K_D 3.6 μ M), whereas iron uptake from transferrin is not saturable, as shown here for siderophore uptake. Iron uptake from ferric nitriloacetate (Fe-NTA) is also a saturable process (Tachezy *et al.*, 1998), as is iron uptake from ferrous ascorbate (present work), but here again the affinity is not very high (K_m 2.7 μ M for Fe-NTA and about 5 μ M for Fe(II)-ascorbate). And none of these mechanisms of iron uptake is induced by iron-limited growth conditions. Finally, the optimal growth of *T. foetus* cells *in vitro* needs large amounts of iron (50–100 μ M) in the medium (Tachezy *et al.*, 1996), but only a small fraction of this iron is actually accumulated by cells grown overnight in such an iron-rich medium. In contrast, *S. cerevisiae* and *C. albicans* yeast cells have inducible iron-uptake systems with apparent affinities for their substrates (ferrous iron, siderophores or haemin) that are more than 10 times higher than those for the iron substrates used by *T. foetus* (Lesuisse *et al.*, 1998; Santos *et al.*, 2003). The parasite *Trypanosoma brucei* also has surface receptors that bind transferrin with affinity constants that are 10–100 times higher than the affinity constant for lactoferrin binding to *T. foetus* cells (Steverding *et al.*, 1995). Thus, there is an apparent paradox: *T. foetus* cells have high

requirements for iron but have no highly efficient inducible iron-uptake systems. But the cells can take up and use iron from a wide range of substrates: iron-binding proteins (lactoferrin, transferrin), siderophores, haemin, and low-molecular-mass ferric and ferrous compounds, which could compensate for the lack of high-affinity, inducible specific mechanisms.

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