***Staphylococcus aureus* but not *Staphylococcus epidermidis* can acquire iron from transferrin**

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Staphylococci grow and cause infection under the iron-restricted conditions found *in vivo*. They therefore must possess mechanisms to obtain iron for metabolism from this environment. To determine if staphylococci can extract iron bound to human transferrin, we labelled transferrin with ⁵⁵Fe and performed uptake assays on cells grown in iron-restricted and iron-plentiful conditions. Growing cultures of *Staphylococcus aureus* NCTC 8532 could take up radioactive iron during mid- to late-exponential phase of growth. This process was iron-regulated and did not require direct contact between the cell and the labelled transferrin. Siderophore production was detected during this phase, but reductase or protease activity was not. *S. epidermidis* ATCC 14990 could not access ⁵⁵Fe bound to transferrin, nor did this isolate produce siderophore, reductase or protease. This difference in the ability to acquire iron bound to transferrin may contribute to the increased virulence of *S. aureus* when compared to *S. epidermidis*.

Keywords: staphylococci, iron, transferrin, siderophore

INTRODUCTION

Iron is essential for the growth of virtually all living cells. Although intracellular iron is plentiful in host tissues, it is not readily available extracellularly as it is bound to specific iron-binding proteins, such as transferrin. Transferrin is found in serum and lymph and has an extremely high affinity for ferric iron. It is normally only 30% saturated, allowing it to scavenge iron and transport it around the body. For pathogenic bacteria to multiply in host tissues and cause infection, they must possess efficient mechanisms for the uptake of iron, which may include the uptake of iron bound to transferrin (for reviews see Williams & Griffiths, 1992; Wooldridge & Williams, 1993).

Many micro-organisms produce siderophores, compounds of low molecular mass with high affinities for iron, which compete with transferrin and other iron sources *in vivo* (Wooldridge & Williams, 1993). Organisms such as *Neisseria meningitidis*, *N. gonorrhoeae* and *Haemophilus influenzae* express specific transferrin receptors on their cell surfaces which bind and remove the iron (Williams & Griffiths, 1982). Some intracellular

pathogens such as *Listeria monocytogenes* produce ferric reductases, which reduce iron from the Fe³⁺ to the Fe²⁺ state and decrease its affinity for transferrin (Coward & Foster, 1985). Proteolytic cleavage of transferrin resulting in the disruption of iron-binding sites may also be important (Williams & Griffiths, 1992).

Staphylococci are important clinical pathogens. *S. aureus* causes a range of nosocomial and community-acquired pyogenic infections and toxin-related diseases. The coagulase-negative staphylococci (CoNS), including the major pathogen *Staphylococcus epidermidis*, are considered less pathogenic but are a frequent cause of infection in immunocompromised patients, particularly those with indwelling medical devices. Despite their medical importance, little is known of the ability of staphylococci to scavenge iron.

Schade (1963) showed that isolates of *Staphylococcus aureus* could grow in human serum *in vitro*. Growth was enhanced if excess iron was added, suggesting uptake of some transferrin-bound iron, but not enough to maintain maximal growth. *S. albus* isolates (CoNS) were not able to grow in serum but grew well if iron was added, indicating a poor iron-scavenging system.

In a simple plate assay, Marcelis *et al.* (1978) showed that the growth of *S. aureus* was not inhibited by the presence of transferrin while the growth of *S. epidermidis* was inhibited, albeit only by the highest concentration tested,

Abbreviations: CAS, chrome azurol S; CoNS, coagulase-negative staphylococci; SSD-0Fe, staphylococcal siderophore detection medium containing less than 0.05 µM iron; SSD-gc, SSD-0Fe prepared without glucose or Casamino acids.

approximately 46-fold greater than the concentration found in human serum. They concluded that *S. aureus* was able to access transferrin-bound iron, while *S. epidermidis* could not, and that this might be considered a virulence factor.

More recently, Brock *et al.* (1991) showed that *S. aureus* NCTC 8532 and a clinical isolate of *S. epidermidis* utilized only a small proportion of radioactive iron bound to transferrin, while taking up a larger proportion of radioactive iron released by the K562 erythroleukaemic cell line. In this case, transferrin was separated from the bacterial cells by a dialysis membrane, but it was not determined whether staphylococci could access iron bound to transferrin via a specific cell surface receptor such as that described for meningococci, gonococci and *Haemophilus* species. Recently, a 42 kDa transferrin-binding protein expressed on the surface of *S. aureus* and *S. epidermidis* and some other CoNS has been described (Modun *et al.*, 1994), although its role in the uptake of iron has not yet been determined.

S. aureus and some CoNS produce siderophores that react with the universal chrome azurol S (CAS) assay of Schwyn & Neilands (1987) (Meiwees *et al.*, 1990; Courcol *et al.*, 1991; Lindsay & Riley, 1994). Two siderophores, staphyloferrin A and staphyloferrin B, have been isolated and chemically characterized (Meiwees *et al.*, 1990; Drechsel *et al.*, 1993) although their relative importance in scavenging iron is unknown. Lindsay & Riley (1994) showed *S. aureus* isolates produced high levels of CAS activity which was not influenced by the precursors of staphyloferrin A and B, suggesting the CAS activity was due to the presence of uncharacterized siderophores. CoNS isolates produced significantly less CAS activity, although the production of staphyloferrin A and B was common. Some CoNS under iron-restricted conditions produced citrate, which may function like a siderophore. Citrate was not detected in *S. aureus* cultures.

The purpose of our study was to determine if staphylococci could take up ^{55}Fe bound to transferrin and, if so, whether this was achieved using: (i) a siderophore; (ii) direct cell contact between the transferrin and the cell surface; (iii) a reductase to reduce the affinity of transferrin for iron; or (iv) a protease capable of cleaving the transferrin molecule.

METHODS

Bacterial strains and growth media. *S. aureus* NCTC 8532 (ATCC 12600) and *S. epidermidis* ATCC 14990 were grown in SSD-0Fe medium (Lindsay & Riley, 1994). Briefly, SSD is a defined medium, apart from the use of Casamino acids as the nitrogen source. When treated with Chelex 100 the iron concentration is reduced to less than $0.05\ \mu\text{M}$ (SSD-0Fe). The medium is low in phosphates (2 mM) and devoid of citrate, making it compatible with the CAS assay for the detection of siderophores (Schwyn & Neilands, 1987). SSD-0Fe was supplemented with $0.5\ \mu\text{M}$ or $10\ \mu\text{M}$ $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (SSD-0.5Fe or SSD-10Fe) as required. *S. aureus* grows well in SSD-0Fe (iron-restricted) but *S. epidermidis* requires $0.5\ \mu\text{M}$ Fe for growth (iron-restricted) and $10\ \mu\text{M}$ Fe (iron-plentiful) for maximal growth (Lindsay & Riley, 1994).

Labelling of transferrin with ^{55}Fe . Human transferrin (10 mg; Sigma) was dissolved in 400 μl Tris/bicarbonate buffer (20 mM NaHCO_3 , 40 mM Tris, pH adjusted to 7.4 with HCl) and then dialysed against buffer overnight. A solution of 143 μM nitrilotriacetic acid and 143 μM $^{55}\text{FeCl}_3$ (Amersham; 1.04 MBq) in 600 μl buffer was equilibrated for 30 min. The two solutions were mixed, allowed to equilibrate for 30 min, and then dialysed against 200 ml buffer overnight.

To determine the concentration of unbound iron in the solution, the sample was centrifuged in a Centricon 10 filter at 5000 *g* for 15 min. The radioactivity of a sample of the filtrate compared to a sample of the uncentrifuged solution demonstrated that 0.184% of the radioactivity in the sample was unbound. Using radioactivity counts, it was estimated that 25% of the transferrin binding sites were occupied with ^{55}Fe . Labelled transferrin was run on PAGE gels and appeared as a 79 kDa band. No breakdown products or heterogeneity of the protein were observed (data not shown).

Uptake of ^{55}Fe bound to transferrin by non-growing cell suspensions (30 min). Several colonies from a blood agar plate were suspended in SSD-0Fe to an OD_{450} of 0.15, inoculated 1:100 into iron-restricted media (SSD-0Fe for *S. aureus* and SSD-0.5Fe for *S. epidermidis*) and incubated overnight with shaking at 37 °C. This inoculum was diluted 1:10 in either iron-restricted or iron-plentiful medium. Cultures (10 ml) were incubated in plastic 30 ml bottles with shaking at 37 °C until the cells reached mid-exponential phase (OD_{450} approximately 0.3). This took approximately 5 h for *S. aureus*, 6 h for *S. epidermidis* in iron-plentiful medium and 7 h for *S. epidermidis* in iron-restricted medium. Cultures were adjusted to a turbidity equivalent to 1×10^8 cells ml^{-1} , and 10 ml volumes were washed twice in SSD prepared without glucose or Casamino acids (SSD-gc), and resuspended in 0.8 ml SSD-gc. Cell suspensions were used in uptake studies within 20 min of preparation.

Labelled transferrin solution (5–10 μl) was mixed with 190–195 μl SSD-0Fe or supernatant 30 min prior to use. Supernatant was that from 24 h cultures of cells grown in iron-restricted or iron-plentiful conditions and siderophore activity of the supernatant was determined using the CAS assay as previously described (Lindsay & Riley, 1994). This assay detected chelating activity greater than $3\ \mu\text{M}$ desferrioxamine mesylate (Ciba-Geigy) equivalents. The transferrin mixture was added to the cell suspension and 100 μl samples taken at various time intervals were spotted onto cellulose nitrate membranes with a pore size of 0.22 μm (Sartorius) in a vacuum filter apparatus and washed twice with 2 ml 0.5% thioglycolic acid. The membranes had previously been blocked by soaking for 30 min in 1% (w/v) gelatin in T-TBS (20 mM Tris, 0.5 M NaCl, 0.05% Tween-80, pH 7.4) and then washed with SSD-gc containing 100 μM iron. The membranes were air dried, mixed with 5 ml of Filter-Count scintillant (Packard) and counted on a Packard Tri-Carb 1900CA liquid scintillation analyser. The amount of activity associated with the cells on the filter was compared to the amount of activity in a 100 μl sample, and expressed as a percentage. All experiments were performed at least three times.

Uptake of ^{55}Fe bound to transferrin by growing cells (8 h). Overnight cultures of *S. aureus* and *S. epidermidis* were prepared as described above and diluted 1:10 in either iron-restricted or iron-plentiful medium. In these experiments, *S. epidermidis* overnight cultures were diluted in SSD-0Fe rather than SSD-0.5Fe. Labelled transferrin was added directly to the plastic culture bottles (10 μl per 10 ml culture), or suspended in a dialysis membrane and added to the culture. This was to determine if direct contact between the staphylococcal cells and

transferrin was required for uptake. In some control experiments, pieces of dialysis membrane without transferrin were added to the growth cultures. Cultures were incubated with shaking at 37 °C. At 45 min intervals, 100 µl samples were spotted onto nitrocellulose membranes and their radioactivity measured as described above; pH was measured by spotting samples onto pH strips. Growth was determined by measuring OD₆₂₀ and chelating activity was measured by the CAS assay (Lindsay & Riley, 1994). The concentration of transferrin used was not detectable and did not interfere with the CAS assay measurements. Growth and chelating activity of cells in culture tubes without transferrin were also measured. All experiments were performed at least three times.

Reductase assay. Reductase activity of 8 h cultures was measured using the bathophenanthroline-disulfonic acid method of Cowart & Foster (1985) with some modifications. Transferrin was labelled as described above, except that 50 mg transferrin, and 0.8 mM nitrilotriacetic acid and FeCl₃·6H₂O, were used to make up the 1.5 ml of stock solution. Stock transferrin solution was used at a concentration of 33.5 µl ml⁻¹. The buffer used in the assay was 20 mM Tris/HCl, pH 7.4. The positive control was a clinical isolate of *Listeria monocytogenes* grown in brain heart infusion dialysate treated with CaCl₂ (Cowart *et al.*, 1980).

Cleavage of transferrin by staphylococcal proteases. ⁵⁵Fe-transferrin (2 µl) was mixed for 2 h at 37 °C with 22 µl supernatant from 8 h cultures of *S. aureus* and *S. epidermidis* grown in either iron-restricted or iron-plentiful medium. Controls of SSD-0Fe with the pH adjusted to 7.4 or 5.3 mixed with ⁵⁵Fe-transferrin were included. Samples were run on non-denaturing PAGE gels (mini-Protean, Bio-Rad), stained using the method of Neuhoff *et al.* (1988) without fixation, and compared to detect transferrin breakdown products.

RESULTS

S. aureus

In short-term (30 min) uptake experiments using washed exponential-phase *S. aureus* NCTC 8532 cells grown in iron-restricted medium and resuspended in fresh SSD-0Fe medium, an immediate association of the ⁵⁵Fe-transferrin with the cells was seen (Fig. 1). There was no increase in the amount of cell-associated radioactivity with time. To determine if siderophore was required for uptake to be detected, transferrin was mixed with supernatant from a 24 h culture of *S. aureus* grown in SSD-0Fe. Uptake of ⁵⁵Fe by cells occurred (Fig. 1). This supernatant had CAS activity equivalent to 7.0 ± 2.0 µM desferrioxamine equivalents and a pH of 5.4. However, uptake did not occur if the pH of the supernatant was adjusted to 7.4 (CAS activity equivalent to 6.7 ± 1.6 µM desferrioxamine). When supernatant from an iron-plentiful culture was used (pH 5.3, no CAS activity) uptake was detected, but when the pH was adjusted to 7.4 (no CAS activity), no uptake occurred (data not shown). *S. aureus* cells grown in iron-plentiful conditions showed a similar response to the iron-restricted cells (data not shown). Since we could not demonstrate pH-independent uptake of ⁵⁵Fe bound to transferrin over 30 min, we looked for uptake by growing cells over 8 h.

Growth of *S. aureus* was not affected by the small amount of labelled transferrin added. However, cell yield was

Fig. 1. Uptake of ⁵⁵Fe bound to transferrin by exponential-phase cells of *S. aureus* NCTC 8532 over 30 min with: SSD-0Fe added (■); supernatant from a 24 h iron-restricted culture of *S. aureus* (pH 5.4, 7.0 µM desferrioxamine equivalents) added (▲); or supernatant from a 24 h iron-restricted culture of *S. aureus* with pH adjusted (pH 7.4, 6.7 µM desferrioxamine equivalents) added (△). Data shown are the mean and standard deviation of three experiments.

increased slightly by the presence of dialysis membrane, so dialysis membrane with or without transferrin was added to all *S. aureus* cultures in the series of 8 h growth experiments. In these experiments, as with the 30 min experiments, radioactivity was associated with the cells immediately. The level of ⁵⁵Fe associated with the cells remained constant until mid- to late-exponential phase, when the cell-associated ⁵⁵Fe began to increase (Fig. 2a). During this time, the pH decreased from 8.4 to 6.8. If the experiment continued beyond 8 h, the pH fell very quickly, and caused iron to dissociate from transferrin. Siderophore was detectable at low levels during the lag phase (presumably this was carried over from pre-cultures), but production increased immediately prior to when the increase in ⁵⁵Fe uptake was observed.

When *S. aureus* was grown in iron-restricted medium with the labelled transferrin inside the dialysis membrane, growth was not affected (Fig. 2b). The amount of radioactivity associated with the cells was much lower initially, approximately 1%. This increased slowly until mid-exponential phase, where the maximum increase occurred, although it did not reach the same level as when the transferrin had direct contact with the cells. Siderophore production was unaffected by the lack of contact with transferrin.

When *S. aureus* cells were grown in iron-plentiful medium with direct access to transferrin, growth was relatively unaffected (Fig. 2c). Radioactivity associated with the cells was high initially, with a slight increase towards the

Fig. 2. For legend see facing page.

end of the experiment, but not as high as when the cells were grown in iron-restricted conditions (Fig. 2a). No siderophore was detected.

When *S. aureus* was grown in iron-plentiful medium with transferrin inside the dialysis membrane (Fig. 2d), growth was similar to the previous experiments. Radioactivity associated with the cells was low initially, and stayed low with a slight increase towards the end of the experimental period, but not as much as when the cells had direct contact with the transferrin (Fig. 2c) or when they were grown in iron-restricted conditions (Fig. 2b). No siderophore was detected.

Reductase activity was not detected in the supernatant from 8 h *S. aureus* cultures. No breakdown products of transferrin could be seen on non-denaturing PAGE gels, indicating that the supernatant did not contain protease activity directed toward transferrin.

S. epidermidis

Uptake experiments conducted over 30 min with *S. epidermidis* ATCC 14990 produced results very similar to those seen with *S. aureus* NCTC 8532 in Fig. 1. Uptake was not seen in experiments where labelled transferrin was mixed with SSD-0Fe, although initial cell-associated radioactivity was seen. Supernatants with a pH of 5.6 caused an increase in uptake, but if the pH was adjusted to 7.4 this uptake did not occur (data not shown).

S. epidermidis grew poorly in SSD-0Fe over 8 h and growth was not improved when labelled transferrin was added in concentrations containing enough iron to stimulate growth, nor when cultures were grown for up to 24 h. Dialysis membrane did not affect growth. During growth experiments there was some initial radioactivity associated with the cells, similar to the 30 min experiments. This activity declined and remained constant at approximately 6%, while pH did not fall below 8.0. No siderophore was detected. If the transferrin was separated from the cells by a dialysis membrane, growth was not affected, but the amount of cell-associated ^{55}Fe was lower and remained constant at approximately 2%.

When *S. epidermidis* was grown in iron-plentiful medium, growth was much stronger. A high level of radioactivity was associated with the cells (11% within 45 min), which slowly increased to 14% during the course of the experiment. No siderophore was detected. If the transferrin was separated from the cells by a dialysis membrane, the amount of radioactivity associated with the cells remained at less than 2%.

Reductase activity and protease activity directed toward transferrin were not detected in *S. epidermidis* supernatant.

DISCUSSION

When Schade (1963) showed that *S. aureus* could grow in serum *in vitro*, this was evidence that *S. aureus* was able to use iron bound to transferrin as a nutritional iron source. Iron-supplemented serum enabled *S. aureus* to grow better, indicating that the amount of iron taken up from the transferrin was not enough to achieve maximal growth. The results of our experiments support this observation. Uptake of ^{55}Fe bound to transferrin by *S. aureus* occurred, but was delayed and could not be detected in 30 min experiments.

Uptake by 8 h cultures of growing *S. aureus* cells occurred during the mid- to late-exponential phase. Uptake was regulated by iron since cells grown in iron-plentiful conditions showed much lower levels of cell-associated radioactivity. Uptake did not require direct cell contact since uptake occurred when the transferrin was separated from the cells by a dialysis membrane. This suggests that specific receptors or a transferrin-binding protein, such as that described by Modun *et al.* (1994), were not responsible for the uptake seen, and that it was mediated via a component of supernatant that was able to pass through a dialysis membrane. CAS activity (siderophore) was produced by *S. aureus* cells immediately prior to uptake of ^{55}Fe . Exponential-phase supernatant did not have detectable levels of reducing ability or protease activity. It is therefore likely that the component of supernatant responsible for the uptake of ^{55}Fe bound to transferrin was siderophore. The structure of the siderophore(s) produced by *S. aureus* in this medium is presently unknown, but there is evidence to suggest (Lindsay & Riley, 1994) that it is not staphyloferrin A described by Meiwes *et al.* (1990), staphyloferrin B described by Dreschel *et al.* (1993), or citrate.

Uptake of ^{55}Fe bound to transferrin occurred slowly and only in growing cells after many hours incubation. As the number of cells in the culture increased (Fig. 2a, b), the amount of ^{55}Fe per cell did not increase. Therefore, the uptake detected is not typical of an active process, and is relatively inefficient. This agrees with the observations of Schade (1963) and Brock & Ng (1983), who showed that *S. aureus* cells did not grow at an efficient rate in serum since growth could be enhanced by adding iron or desferrioxamine. The use of metabolic inhibitors such as KCN to demonstrate active transport is inappropriate when the test cells are in a growing culture. Attempts to fractionate cells were unsuccessful, and we were unable to demonstrate association of ^{55}Fe with a cytoplasmic fraction.

Schade (1963) also showed that isolates of *S. albus* (CoNS) were unable to grow in serum that had not been supplemented with iron. Again, our observations support

Fig. 2. Growth (■; measured as OD_{620}), uptake of ^{55}Fe bound to transferrin (□, expressed as percentage of available radioactivity associated with cells), siderophore production (▲; expressed in μM desferrioxamine equivalents), and pH (△) of cultures of *S. aureus* NCTC 8532 over 8 h when grown in: (a) iron-restricted SSD-0Fe with ^{55}Fe -transferrin; (b) iron-restricted medium with ^{55}Fe -transferrin inside a dialysis membrane; (c) iron-plentiful medium with ^{55}Fe -transferrin; and (d) iron-plentiful medium with ^{55}Fe -transferrin inside a dialysis membrane. Data shown are the mean of at least three experiments. Standard deviations are indicated for percentage ^{55}Fe uptake.

these results. *S. epidermidis* was unable to grow in the presence of ^{55}Fe -transferrin. Uptake of ^{55}Fe bound to transferrin was not seen in 30 min experiments nor by growing cells over 8 h, suggesting that *S. epidermidis* cannot use transferrin as an iron source. Siderophore activity, reducing ability or protease activity was not detected in *S. epidermidis* supernatant.

In 30 min or 8 h experiments with *S. aureus* or *S. epidermidis*, ^{55}Fe was associated with cells immediately upon contact. This was not regulated by iron concentration and required direct contact between the cells and transferrin. This activity may be due to transferrin binding to the cell surface, although it is unlikely that this binding is associated with ^{55}Fe uptake since *S. epidermidis* exhibits this binding but cannot extract enough ^{55}Fe to stimulate growth. Binding of transferrin to the cell surface could be due to non-specific forces, or binding may be specific and due to a transferrin-binding protein such as that described by Modun *et al.* (1994). However, the expression of the protein detected by Modun *et al.* (1994) was regulated by iron in *S. epidermidis*. This was not seen in our experiments.

The effect of pH on uptake of iron bound to transferrin was highlighted in the 30 min experiments. The affinity of transferrin for iron falls when pH is lower than 6. The supernatants with pH values of 5.4 appeared to enhance uptake of ^{55}Fe bound to transferrin by *S. aureus* and *S. epidermidis* cells in 30 min experiments. However, this is likely to be due to the reduced affinity of transferrin for iron at low pH, since the effect was abolished if the pH was raised. Therefore it is very important to monitor pH in experiments such as these. Growth experiments for extended periods may allow the pH to drop to levels where it can affect transferrin binding of iron. The experiments of Marcelis *et al.* (1978) in particular may have been influenced by unchecked pH, as could those of Schade (1963), since the pH of spent growth medium was not reported.

These experiments were performed in artificial media *in vitro* and the ability of staphylococci to take up iron bound to transferrin *in vivo* is unknown. Our results are consistent with Schade's observations when staphylococci were grown in serum *in vitro*. In the body, transferrin is not the only potential source of bacterial iron. Lactoferrin, an iron-binding protein similar to transferrin, is found in milk, tears and mucus, and also in polymorphonuclear leucocytes, where it is released during inflammation. A human lactoferrin-binding protein produced by *S. aureus* has recently been described (Naidu *et al.*, 1991, 1992), although it has not been established whether this binding is involved in the uptake of iron bound to lactoferrin. The method used in the present study was not successful for measuring uptake of ^{55}Fe bound to lactoferrin, as the protein bound to the cellulose nitrate membranes and a suitable blocking agent could not be found (J. A. Lindsay, unpublished observations). Since haemolysins are produced by many staphylococci, haemoglobin and haem are potential sources of nutritional iron *in vivo* (Francis *et al.*, 1985). Brock *et al.* (1991) have suggested that cell-derived iron may be more readily available to staphylococci than

iron bound to transferrin. They did not detect significant levels of iron uptake from transferrin. This may have been due to a shorter incubation time (4.5 h) than the one used in the present study. Differing growth media and levels of iron restriction may also have been important.

Much work is still required to determine which mechanisms for the uptake of iron are possessed by staphylococci and which are activated during infection. Evidence that *S. aureus* is better equipped to handle iron-restriction than *S. epidermidis* is accumulating, including factors such as the ability to survive and grow in serum and in SSD-0Fe, the production of siderophores and iron-regulated proteins in SSD-0Fe, and now the ability to take up iron bound to transferrin in SSD-0Fe. These factors may contribute to the increased pathogenicity of *S. aureus* when compared to *S. epidermidis* and the other CoNS.

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