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Dependence of *Staphylococcus epidermidis* on non-transferrin-bound iron for growth

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Abstract

The ability of *Staphylococcus epidermidis* strains to grow in the presence of human transferrin and varying amounts of ferric iron was studied. At initial bacterial densities up to 10^4 cfu ml^{-1}, none of the three strains grew when transferrin iron saturation was below the full saturation point, whereas the bacteria grew consistently when transferrin was fully iron-saturated and there was non-transferrin-bound iron in the medium. Precultivation of the bacteria under iron-restricted conditions to induce siderophore production did not abolish the growth dependence on non-transferrin-bound iron. At initial bacterial densities of 10^6 cfu ml^{-1}, the bacteria proliferated consistently also in the presence of partially saturated transferrin. The results indicate that at low bacterial densities, *S. epidermidis* cannot utilise transferrin-bound iron for growth and that its proliferation is dependent on non-transferrin-bound iron.

Keywords: *Staphylococcus epidermidis*; Iron; Transferrin; Non-transferrin-bound iron

1. Introduction

Staphylococcus epidermidis is a major causative agent of septic infections in neutropenic patients receiving high-dose chemotherapy and other immunocompromised patients [1,2]. High-dose chemotherapy is associated with high serum iron concentrations, which exceed the iron-binding capacity of the major iron carrier protein, transferrin, in the host. This results in the appearance of non-transferrin-bound iron in the circulation of the patients [3–5]. As iron is an essential growth factor for bacteria, it has been suggested that hyperferremia, together with the lack of neutrophilic granulocytes, predisposes these patients to infections with opportunistic bacteria [4,6,7].

Under normal conditions, transferrin in plasma and extracellular fluids of the host is only partially saturated with iron. Due to its very high affinity for ferric iron, transferrin keeps the level of free ionic iron too low to sustain bacterial growth [6]. Pathogenic bacteria, such as *Neisseria meningitidis* and *Haemophilus influenzae*, have evolved mechanisms to acquire iron from transferrin [8,9]. These bacteria express cell surface transferrin receptors and obtain iron from the receptor-bound transferrin. Another mechanism of iron assimilation employed by many Gram-negative species involves the synthesis of low-molecular mass iron chelators, termed siderophores. Siderophores are secreted in response to iron deprivation and once complexed with iron, they are transported back into the cell via a specific receptor on the outer membrane [8,9]. Siderophores differ in their affinity to iron and ability to remove iron from transferrin in vitro. A further effective mechanism of bacterial iron acquisition in a plasma milieu is from heme compounds released from damaged cells, particularly erythrocytes [8–10].

Several studies have addressed iron uptake by staphylococci from human transferrin. Schade [11] demonstrated that *Staphylococcus aureus* strains grew in normal human serum in vitro whereas several coagulase-negative staphylococci strains were not able to grow in serum without iron supplementation. This suggested that the *S. aureus* strains could acquire iron from partially saturated transferrin whereas the other staphylococci could not. Iron uptake studies using human transferrin labelled with iron isotope showed that *S. aureus* acquired iron from transferrin whereas *S. epidermidis* did not [12]. The iron acquisition did not require bacterial cell surface contact suggest-

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ing a siderophore-mediated mechanism. On the other hand, *S. aureus* and several coagulase-negative staphylococci including *S. epidermidis* possess a 42-kDa cell wall transferrin-binding protein, which was identified as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase [13]. Staphylococci expressing this transferrin receptor were able to grow in the presence of iron-saturated human transferrin and remove iron from transferrin in vitro [14]. Staphylococci also produce at least three siderophores, of which staphyloferrin A was shown to remove iron from transferrin in vitro [14].

Recognition of the possible pathogenetic role of hyperferremia in patients receiving high-dose chemotherapy has led to the evaluation of the potential therapeutic value of iron chelation therapy in these patients [15,16]. Considering the potential value of iron chelation therapy in the prevention of septic infections caused by *S. epidermidis*, it is important to know whether bacteria at densities occurring in septicemia can utilise transferrin-bound iron for growth. Therefore, we have in the present study investigated the ability of *S. epidermidis* to grow in the presence of human transferrin and different amounts of ferric iron.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *S. epidermidis* ATCC 12228 strain and two strains (16779 and 19435) of *S. epidermidis* isolated from neutropenic patients with a hematological malignancy and septicemia were kindly provided by Dr. Pentti Kuusela and Dr. Aulikki Sivonen (Clinical Microbiology, Helsinki University Central Hospital, Helsinki, Finland). The strains were identified with standard laboratory methods including fermentation of mannitol and trehalose and the urease and DNAse tests. RPMI 1640 (R-7509, Sigma) supplemented 50 mM HEPES buffer, pH 7.4 and 2 mM L-glutamine was depleted from iron by incubating twice with 30 g l^{-1} of Chelex-100 (Bio-Rad Laboratories, Hercules, CA, USA) overnight at 4°C. After removal of the resin, the medium was supplemented with $CaCl_2$ (10 μM) and $MgCl_2$ (100 μM) and the solution was sterile-filtered. To obtain an inoculum for the growth assays, the bacteria were cultured in the iron-depleted RPMI with or without iron supplementation and diluted to different cell densities with 0.9% NaCl. 50 μl was used to inoculate 250 μl of RPMI containing 2.5 g l^{-1} of apotransferrin and different amounts of ferric nitrilotriacetic acid (FeNTA). The growth was monitored by turbidity measurements every 30 min in microtitration plates for 96 h at 37°C with periodic shaking in a Bioscreen C Microbiology Reader (Labsystems, Finland).

The siderophore index of the bacterial cultures was determined with the Chrome Azurol S (CAS) liquid assay of Payne [17]. Briefly, 0.1 ml of the culture supernatant was mixed with 0.1 ml of CAS assay solution and 2 μl of 5-sulfosalicylic acid. The optical density (OD) at 630 nm was measured for loss of blue colour resulting from the siderophore production. The siderophore index was calculated as a percentage of decrease in the OD using the growth medium before inoculation as a reference.

2.2. Transferrin and iron preparations

Human apotransferrin with a purity over 98% and iron saturation less than 0.5% was prepared from human plasma as described in detail elsewhere (von Bonsdorff et al., submitted for publication). The protein concentration was determined by OD at 280 nm using the extinction coefficient of 11.1 for apotransferrin [18]. A solution of FeNTA was prepared from an iron atomic absorption standard solution (in 1% HCl, Sigma-Aldrich) and 5 mM nitrilotriacetic acid (NTA) dissolved in 0.1 M Tris–HCl buffer, pH 8.0. The final solution contained 3 mM Fe^{3+} and 4.2 mM NTA, pH 7.4. Apotransferrin with a final concentration of 2.5 g l^{-1} in the iron-depleted RPMI medium was saturated to different saturation values by adding FeNTA and incubating overnight at 4°C without agitation.

2.3. Determination of transferrin saturation and non-transferrin-bound iron

Calculated transferrin saturation was deduced from the molar ratio of ferric iron to transferrin. The titration of transferrin saturation was carried out by measuring the OD at 450 nm of the samples containing increasing levels of FeNTA. Proportions of transferrin iron forms (apo, monoferric and diferric transferrins) were analysed by urea polyacrylamide gel electrophoresis (6% acrylamide gels with 6 M urea) as described before [19]. Samples containing 15 μg of transferrin were separated in 10×10-cm gels and visualised by Coomassie brilliant blue staining. Non-transferrin-bound iron was determined by a colourimetric method as described before [20] except that no NTA was added to the samples. Briefly, 1 ml of each transferrin-containing growth medium was centrifuged in a Centricon YM-30 filter (Millipore). Iron in the ultrafiltrates was measured with bathophenantroline at 535 nm.

3. Results

To obtain bacteria with and without siderophore induction, the staphylococci were cultivated in iron-depleted and iron-replete RPMI without transferrin. The strains grew slowly in the iron-depleted RPMI medium, which contained 0.1 μM iron as determined by atom absorption spectrophotometry. Increase of the ferric ion concentration up to 1–2 μM effectively promoted bacterial growth whereas further increment in the iron level had only little

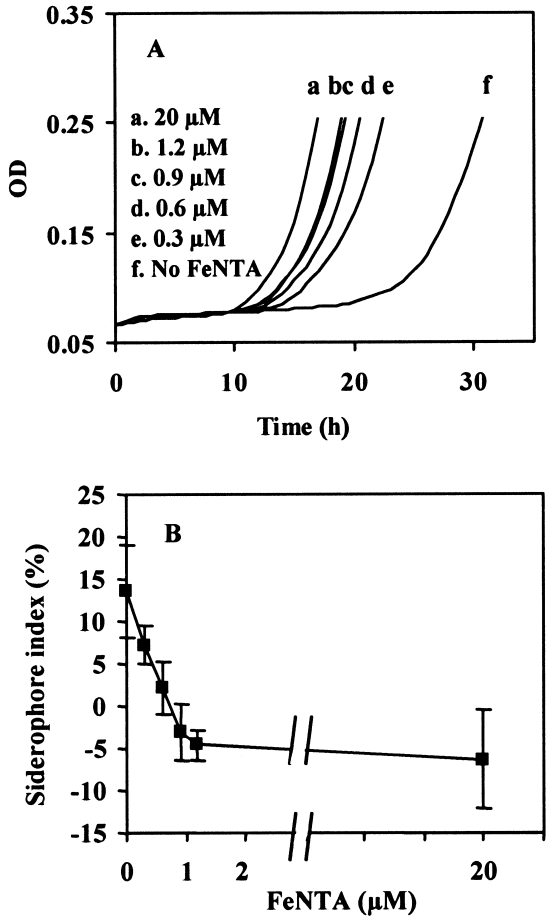

Fig. 1. Siderophore induction by iron depletion. (A) The growth was measured as OD for the bacteria grown in the medium supplemented with different concentrations of FeNTA. (B) The siderophore index was determined for each culture supernatant after 48 h of growth. Values in (B) are mean of five independent experiments ± S.D.

effect (Fig. 1A). Siderophore activity was studied in the culture supernatants after 48 h when all cultures had entered into the stationary phase. There was a strong inverse relationship between the siderophore index and iron concentration in the growth medium at iron concentration below about 1 μM (Fig. 1B). Bacterial cultures grown without added FeNTA, which showed maximal siderophore index, and with 20 μM FeNTA, which showed the lowest index, were used for inoculation of the growth assays in the presence of transferrin.

The ability of *S. epidermidis* to utilise transferrin-bound iron for growth was studied in the iron-depleted RPMI containing transferrin, which had been adjusted to different calculated saturation values by stepwise addition of FeNTA. When initial bacterial densities of 10^2–10^4 cfu ml^{-1} were studied, no detectable growth took place in the media that contained transferrin with a calculated saturation below 90%. This was indicated by monitoring the turbidity of the growth medium for 96 h in the Bioscreen C analyser (Fig. 2). Determining viable counts indicated slight growth during the first 24 h but no viable cells after 96 h. In contrast, bacterial growth consistently took place when the calculated transferrin saturation was over 90% (Fig. 2). After the critical transferrin saturation level had been reached, further addition of FeNTA did not increase the growth rate. All three *S. epidermidis* strains showed the same dependence on full transferrin saturation for growth. No difference was observed between bacteria with high and low siderophore activity (Fig. 2A,B). This indicated that the induction of siderophore production did not promote bacterial growth in the presence of partially saturated transferrin.

To confirm the actual transferrin saturation in the growth assays, the OD of the transferrin-containing medium was measured at 450 nm, which is directly proportional to the transferrin saturation level. The OD increased linearly as a function of added FeNTA up to the calculated saturation of about 90%, above which the OD reached a plateau indicating full transferrin iron saturation (Fig. 3A). The level of non-transferrin-bound iron was below the limit of detection of the assay in the samples containing transferrin with a saturation level below 90%, whereas the samples with a calculated transferrin satura-

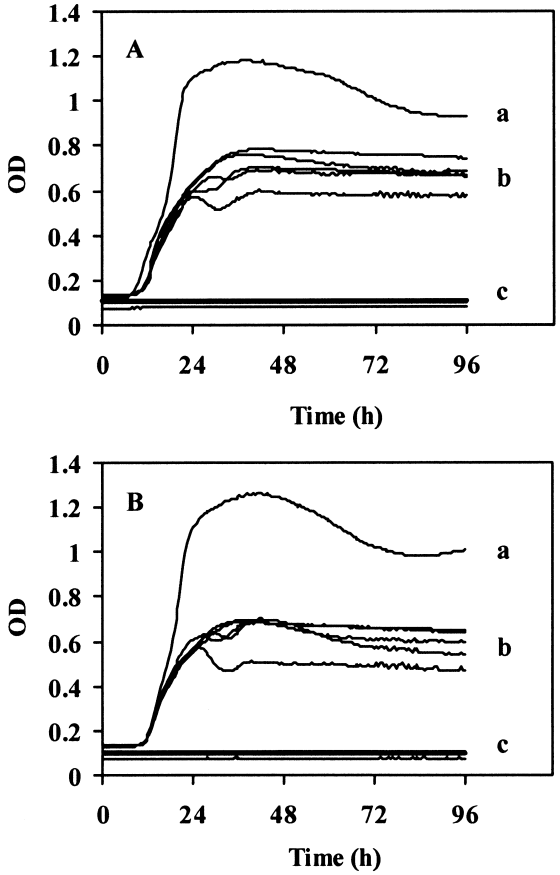

Fig. 2. Growth of non-induced (A) and induced (B) *S. epidermidis* in the presence of transferrin saturated to a varying degree with iron. Growth curves measured as OD are shown for the soy broth (a) and for RPMI containing 2.5 g l^{-1} transferrin with iron saturation >90% (b) or ≤90% (c). An initial bacterial density of 4×10^3 cfu ml^{-1} was used.

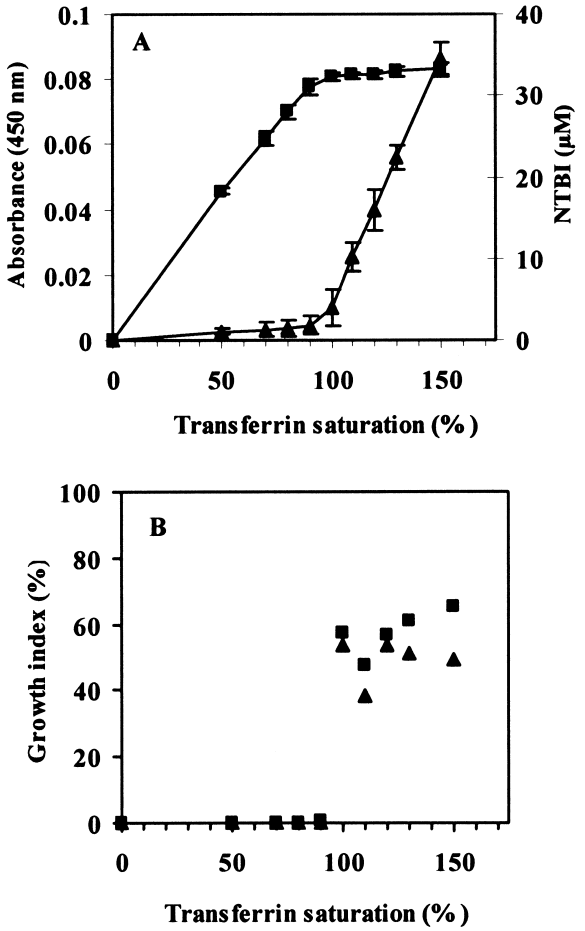

Fig. 3. Dependence of *S. epidermidis* on non-transferrin-bound iron for growth. (A) The iron saturation of transferrin in the test media was confirmed by measuring the OD at 450 nm (■) and by determining non-transferrin-bound iron (NTBI) (▲). (B) Parallel cultures from one experiment with non-induced (■) and induced (▲) bacteria. A growth index was defined as a ratio of the areas limited by the growth curves for the test medium and for the soy broth. An initial bacterial density of 4×10^3 cfu ml^{-1} was used. Values in (A) are mean of four independent experiments ± S.D.

tion greater than 90% contained detectable non-transferrin-bound iron, the level of which increased apparently linearly with increasing FeNTA concentrations (Fig. 3A). At the initial density of about 10^4 cfu ml^{-1}, the bacteria grew only in the media which contained fully saturated transferrin and detectable non-transferrin-bound iron (Fig. 3B).

Table 1
The effect of initial bacterial count on the growth of *S. epidermidis* in the presence of partially saturated transferrin

Initial cell count (cfu ml^{-1})	Transferrin saturation (%)					
	0	50	70	80	90	100
10^3	−	−	−	−	+	++
10^4	−	−	−	−	+	++
10^5	+	+	+	+	+	++
10^6	++	++	++	++	++	++

The results are from four different experiments with both induced and non-induced bacteria. Growth in none of the experiments (−), in part of the experiments (+), or in all experiments (++).

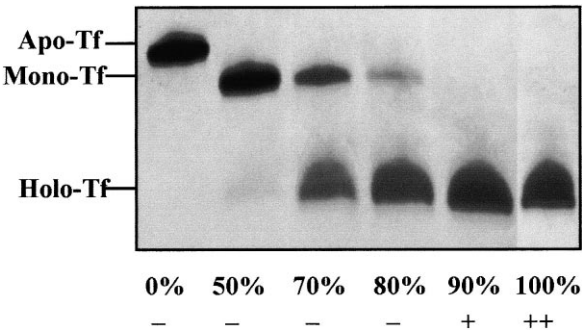

Fig. 4. Iron forms of transferrin and bacterial growth. Transferrin iron forms were analysed in urea gel electrophoresis. Staphylococci were cultured at initial bacterial densities of 10^4 cfu ml^{-1}. The calculated transferrin saturation values and bacterial growth in four different experiments are indicated below the figure: no growth (−), growth in part of the experiments (+) or growth in all experiments (++).

The full transferrin saturation point was further assessed by studying the distribution of transferrin iron forms in the growth media by urea gel electrophoresis (Fig. 4). The proportion of diferric transferrin increased in the media with increasing calculated transferrin iron saturation, and the media with calculated saturation greater or equal to 90% contained only diferric transferrin. When compared with bacterial growth, it could be concluded that bacteria did not grow in the media below the full transferrin saturation point, even if most of the transferrin was in the diferric form (Fig. 4). When the initial bacterial density in the growth assay was increased to 10^5 cfu ml^{-1}, bacteria grew in some experiments even in the presence of partially saturated transferrin (Table 1). At initial densities of 10^6 cfu ml^{-1} and higher, the bacteria grew consistently irrespective of the transferrin saturation level.

4. Discussion

The results of the present study indicated that at bacterial densities up to about 10^4 cfu ml^{-1}, the growth of *S. epidermidis* in the presence of human transferrin was critically dependent on full transferrin saturation and the presence of non-transferrin-bound iron. The growth inhibitory effect of partially saturated transferrin was not abolished when staphylococci were precultivated under iron-depleted conditions, which induced siderophore production in the bacteria. However, the growth inhibition by transferrin was overcome when initial bacterial densities of 10^6 cfu ml^{-1} or higher were used.

The dependence of the growth of *S. epidermidis* on full transferrin saturation and the presence of non-transferrin-bound iron was indicated by the following lines of evidence. Firstly, titrimetric determination of the saturation point of transferrin in the culture medium indicated that

transferrin was fully saturated in the samples in which the staphylococci grew, whereas no growth was observed in the samples containing partially saturated transferrin. Secondly, all samples that supported bacterial growth contained non-transferrin-bound iron, whereas no growth was observed in the samples without detectable non-transferrin-bound iron. Finally, the samples in which bacteria did not grow contained at least traces of monoferric transferrin in urea gel electrophoresis, whereas the samples that supported growth contained only diferric transferrin. The different assays indicated that the growth inhibitory activity of transferrin was maintained even very close to the full saturation point.

The very high affinity constant of transferrin for ferric iron implies that the level of non-transferrin-bound iron remains very low until the saturation of transferrin becomes close to 100% saturation [6]. In the present study, non-transferrin-bound iron increased to a micromolar level at an iron concentration corresponding to a calculated transferrin saturation of about 90%. Taking into account a certain inaccuracy in the determination of the molar amount of transferrin and ferric iron in the system, this was reasonably close to 100%.

It has been shown in two earlier studies that *S. epidermidis* grows in the presence of iron-saturated human transferrin [12,14]. This was also observed in the present study. On the other hand, the inability of the staphylococci to grow in the presence of partially saturated transferrin even close to the full saturation point indicated that at densities up to 10^4 cfu ml^{-1} the bacteria could not utilise transferrin-bound iron for growth. According to our experience (unpublished results), transferrin preparations saturated in the presence of excessive iron typically contain redox-active iron even after extensive buffer exchange, indicating that the transferrin preparations contain residual iron not bound by the high affinity binding sites of transferrin [21]. Residual non-transferrin-bound iron may influence bacterial growth observed in the presence of iron-saturated holotransferrin preparations in the earlier studies.

It has previously been demonstrated that *S. epidermidis* at a density of 10^8 cfu ml^{-1} removed iron from transferrin [14]. This is consistent with the finding of the present study that the staphylococci proliferated in the presence of partially saturated transferrin when bacterial densities of 10^6 cfu ml^{-1} or higher were used. The inability of the bacteria to grow at low initial densities in the presence of partially saturated transferrin suggests that iron displacement from transferrin requires high bacterial density. It has been suggested that diphosphoglycerate, whose formation is catalysed by the transferrin receptor glyceraldehyde-3-phosphate dehydrogenase, may facilitate iron removal from the receptor-bound transferrin [13]. Diphosphoglycerate and other organic phosphates were previously shown to displace iron from transferrin at millimolar concentrations [22]. If iron displacement from transferrin would depend on extracellular organic phosphates, this could explain why relatively high densities of bacteria were required to initiate proliferation in the presence of partially saturated transferrin. However, more studies are required to clarify the mechanisms by which *S. epidermidis* at high cell densities can acquire iron from transferrin.

In conclusion, the results of the present study suggest that the presence of non-transferrin-bound iron in the circulation may predispose patients receiving high-dose chemotherapy to septic infections by *S. epidermidis*. Therefore, administration of apotransferrin or other iron chelators, which would bind non-transferrin-bound iron in the patients, deserves further evaluation.

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