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Staphylococci Express a Receptor for Human Transferrin: Identification of a 42-Kilodalton Cell Wall Transferrin-Binding Protein

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Staphylococcus aureus **and the coagulase-negative staphylococci are commonly responsible for peritonitis in renal patients undergoing continuous ambulatory peritoneal dialysis. To simulate growth conditions in vivo, staphylococci isolated from peritoneal infections were cultured in used human peritoneal dialysate (HPD). Immunoblotting experiments using cell wall preparations from these staphylococci revealed the presence of the host iron-binding glycoprotein transferrin bound to *S. aureus*, *S. epidermidis*, *S. capitis*, *S. haemolyticus*, and *S. hominis* but not to *S. warneri* or *S. saprophyticus*. Similar results were obtained by incubating broth-grown staphylococci with human transferrin, although, in contrast to *S. aureus*, the coagulase-negative staphylococci bound more transferrin after growth in iron-restricted broth. To determine whether the staphylococci express a saturable specific receptor for human transferrin, the interaction of human ^{125}I-transferrin with the staphylococci was examined. Both *S. aureus* and *S. epidermidis* bound the radiolabelled iron-saturated ligand in a time- and concentration-dependent manner. From competition binding assays, the affinity (K_d) and number of receptors were estimated for *S. epidermidis* (K_d, 0.27 μM; 4,200 receptors per cell) and *S. aureus* (K_d, 0.28 μM; 4,200 receptors per cell). *S. epidermidis* but not *S. aureus* receptor activity was partially iron regulated. Human apotransferrin and iron-saturated transferrin and rabbit and rat transferrins competed equally well for the staphylococcal receptor. Bovine and porcine transferrins and ovotransferrin as well as human and bovine lactoferrins were much less effective at competing with human transferrin. Treatment of whole staphylococci with proteases abolished transferrin binding, indicating the involvement of cell surface protein. Western blots (immunoblots) of cell wall preparations probed with human transferrin revealed the presence of a 42-kDa transferrin-binding protein common to both *S. aureus* and *S. epidermidis*. On Western strip blots, the binding of human transferrin to this protein was blocked by labelled human transferrin but not by albumin, immunoglobulin G, or bovine transferrin or ovotransferrin. To assess the conservation of the 42-kDa transferrin-binding protein, cell wall proteins of *S. epidermidis*, *S. haemolyticus*, *S. capitis*, *S. hominis*, *S. warneri*, and *S. saprophyticus* were Western blotted and probed with human transferrin. Only *S. warneri* and *S. saprophyticus* lacked the 42-kDa wall protein, consistent with their inability to bind transferrin. These data show that the staphylococci express a specific receptor for human transferrin based at least in part on a common 42-kDa cell wall protein.**

The transferrins constitute a group of iron-binding glycoproteins found in blood (serotransferrin or transferrin), secretions such as milk (lactotransferrin or lactoferrin), and egg white (ovotransferrin or conalbumin). Their discovery is generally associated with the work of Schade and Caroline (33), who noted that the growth inhibitory properties of raw egg white could be overcome by the addition of iron but no other metal cation. The active ingredient was later discovered to be ovotransferrin, and subsequently, a protein with similar properties was isolated from human plasma (34). The transferrins have since been the subject of intensive investigation (for reviews, see references 1, 7, 13, 16, and 49). They are monomeric glycoproteins ranging in molecular mass from 76 to 81 kDa and consist of two similar but not identical domains, each of which contains a single iron-binding site. Iron binding is reversible and requires the presence of an anion, usually bicarbonate, and the transferrins bind two atoms of ferric iron per molecule of protein. The main physiological role of transferrin is to transport iron from sites of absorption and storage to sites of utilization. The binding of iron-saturated transferrin to the mammalian cell surface transferrin receptor leads to the endocytosis of the receptor-transferrin complex, release of the protein-bound iron within the endosome, and the recycling of both the receptor and apotransferrin.

In extracellular body fluids, transferrin and lactoferrin, which are usually only partially saturated with iron (30%), maintain the level of free ionic iron at about 10^{-18} M, a level far too low to sustain the growth of most microorganisms (5). Thus, the iron-withholding properties of the transferrins are also considered to contribute to host defenses by rendering unavailable an essential microbial nutrient (12, 49). However, pathogens multiply successfully in body fluids and cause infection in the absence of exogenously supplied iron because they possess efficient iron-scavenging mechanisms capable of removing iron bound to transferrins. The best understood of these high-affinity iron transport systems are those based on siderophores, low-molecular-mass iron chelators which bind iron with high affinity and specificity (11, 12, 51). However, certain gram-negative pathogens, notably *Haemophilus influenzae* and *Neisseria meningitidis*, use siderophore-independent receptor-mediated iron uptake systems which involve a direct

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interaction between the bacterial cell surface and the iron-binding glycoprotein (49). These systems are further distinguished from siderophore-mediated mechanisms by their high degree of specificity for the iron-binding glycoprotein of the natural host; humans, for example, are the only natural reservoir for *H. influenzae*, which shows considerable specificity for human transferrin (25, 26).

In contrast to our knowledge of the iron uptake systems of gram-negative bacteria, there is comparatively little information concerning the mechanisms by which gram-positive pathogens acquire iron during growth in extracellular body fluids. Both *Staphylococcus aureus* and the coagulase-negative staphylococci have been reported to produce siderophores (15, 21). Of these, a carboxylate-type siderophore termed staphyloferrin A has been isolated and chemically characterized (15, 21). The ability of staphyloferrin to compete for transferrin-bound iron was not, however, reported. Furthermore, *S. aureus* appears capable of binding both human and bovine lactoferrins, while bovine mastititis isolates of coagulase-negative staphylococci can also bind bovine lactoferrin (27, 28).

S. epidermidis and, to a lesser extent, *S. aureus* are commonly responsible for peritonitis in renal patients undergoing continuous ambulatory peritoneal dialysis (CAPD) (39). In the context of CAPD peritonitis, the pathogenesis of staphylococcal infection is likely to be related, at least in part, to the ability of the organism to multiply within the dialyzed peritoneum. Although staphylococci cannot grow in commercial peritoneal dialysis solutions, these fluids are modified during dialysis and become enriched by a plasma ultrafiltrate. This modified human peritoneal dialysate (HPD) contains several human serum proteins, including albumin, transferrin, and immunoglobulins, and can support staphylococcal growth (19, 44, 48). Thus, in common with other extracellular tissue fluids, HPD represents a severely iron-restricted environment for any infecting pathogen.

Previously, we have shown that the growth of *S. epidermidis* in HPD results in a cell envelope phenotype markedly different from that of staphylococci grown in a conventional laboratory medium (38, 48). Compared with proteins in nutrient broth-grown cells, many *S. epidermidis* cell wall proteins were repressed in HPD, although three major proteins of 42, 48, and 54 kDa predominated. In addition, growth in HPD resulted in the induction of two immunodominant, iron-regulated cytoplasmic membrane proteins of 32 and 36 kDa (38, 48). These iron-regulated proteins appear to be common to many *S. epidermidis* isolates and are also expressed by *S. epidermidis* grown in vitro in iron-restricted nutrient broth and in vivo in a chamber implanted intraperitoneally in rats (24). The 32- but not the 36-kDa iron-repressible protein is also shared by *S. aureus* (38). During the course of these studies, immunoblotting experiments indicated that both *S. epidermidis* and *S. aureus* bound host proteins after growth in HPD. This was perhaps not unexpected given that gram-positive bacteria such as the staphylococci and streptococci are known to bind plasma proteins such as albumin, immunoglobins, fibrinogen, and fibronectin. For *S. aureus*, cell wall plasma protein receptors such as the immunoglobulin-binding protein (protein A) (35) and the fibronectin-binding proteins (17, 31) have been well studied. However, staphylococcal cell walls contain numerous other proteins, the functions of most of which have yet to be elucidated. In the present article, we demonstrate that both coagulase-positive and coagulase-negative staphylococci express a receptor for the human iron-binding plasma glycoprotein transferrin, which is based at least in part on a 42-kDa cell wall transferrin-binding protein.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Coagulase-negative staphylococci (*S. epidermidis* 2, 31, 59, 112, 138, and 901, *S. capitis*, *S. haemolyticus*, *S. warneri*, *S. hominis*, and *S. saprophyticus*) and *S. aureus* N100 were isolated from HPD collected from CAPD patients with peritonitis. Staphylococci were grown statically in nutrient broth or pooled noninfected HPD as described before at 37°C in air enriched with 5% carbon dioxide to mimic physiological conditions in the peritoneal cavity (46, 48). Iron-restricted conditions were achieved in nutrient broth as described before by the addition of 800 μM ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA) (24).

Preparation of cell wall and cytoplasmic membrane fractions. Cell wall and cytoplasmic membrane proteins were prepared as described by Cheung and Fischetti (6) for *S. aureus*, adapted for coagulase-negative staphylococci as described previously (38, 47). Briefly, staphylococci were harvested from 100 ml of growth medium, washed twice in phosphate-buffered saline (PBS; 120 mM NaCl, 10 mM sodium phosphate [pH 7.4]), suspended in 0.6 ml of digestion buffer (30% [wt/vol] raffinose, 1 mg of benzamidine per ml, 0.5 mg of phenylmethylsulfonyl fluoride per ml in 10 mM Tris-hydrochloride [pH 7.4]) containing 100 μg of lysostaphin, and incubated for 60 min at 37°C (38). Protoplasts were removed by centrifugation (11,600 × *g* for 3 min), and the supernatant containing the cell wall proteins was stored frozen at −20°C prior to electrophoresis. The protoplast pellet was suspended in distilled water and sonicated at 4°C for two 30-s periods. After lysis, cytoplasmic membranes were collected by centrifugation (100,000 × *g* for 30 min).

Preparation of ^{125}I-labelled human transferrin. Two hundred microliters of a 100-μg/ml solution of Iodo-Gen (Pierce Europe B.V., BA oud Beijerland, The Netherlands) in dichloromethane was added to a 4-ml test tube, and the dichloromethane was allowed to evaporate by rotating the test tube in a water bath at 37°C. Three hundred micrograms of iron-saturated human transferrin (Sigma) and approximately 6 MBq of carrier-free sodium ^{125}I (Amersham International plc, Amersham, United Kingdom) were added to each Iodo-Gen-coated tube in 300 μl of PBS. The mixture was incubated with agitation at room temperature for 15 min, and the unincorporated ^{125}I was removed by passing the ^{125}I-transferrin down a Sephadex G-25 column (Pharmacia) preequilibrated with PBS containing 0.25% (wt/vol) transferrin.

Transferrin binding assays. Preliminary experiments to determine whether staphylococci bound human transferrin after growth in nutrient broth with or without EDDA were carried out by resuspending the bacterial cells (10^9 cells per ml) in PBS containing 3.75 mM human transferrin. After a 2-h incubation at 4°C, staphylococci were washed at least three times in PBS, and cell walls were then isolated as described above prior to immunoblotting and probing with anti-human transferrin as described below.

For determination of the kinetics of ^{125}I-transferrin binding, 10^8 CFU of *S. epidermidis* or *S. aureus*, in a final assay volume of 1 ml, grown in nutrient broth with or without EDDA, was incubated with iron-saturated ^{125}I-transferrin (5.1 nM in PBS) for periods of 5, 10, 20, 30, 60, and 90 min at 37°C in a water bath. Bacteria were pelleted and washed three times in PBS and transferred to a fresh microcentrifuge tube, and the amount of cell-associated ^{125}I-transferrin was determined with an LKB 1282 Compugamma counter (Pharmacia LKB, Uppsala, Sweden). Specific binding was defined as the difference between the amounts of ^{125}I-transferrin bound in the absence

and presence of a 100-fold excess of the unlabelled ligand. Receptor number and affinity (K_d) were calculated from competitive binding experiments in which the binding of a single concentration of radioligand was competed for by increasing concentrations of the same unlabelled ligand by using iron-saturated human transferrin as radioligand and competitor as described by DeBlasi et al. (8). By using the curve-fitting program Graph Pad Inplot (ISI Inc.), the curves obtained were fitted to the following four-parameter logistic equation:

$$y = A + \frac{B - A}{1 + (10^C/10^x)^D}$$

where A is the bottom plateau of the curve, B is the top plateau, x is the value in the middle of the curve, and D is Hill coefficient or slope factor. In these experiments, staphylococci were incubated for 30 min with 4.7 nM ^{125}I-transferrin in the presence of a range of concentrations of unlabelled iron-saturated human transferrin (0 to 3 μM). For competitive binding experiments with human apotransferrin or iron-saturated transferrin, bovine, porcine, rat, and rabbit transferrins, ovotransferrin, and human and bovine lactoferrins (Sigma), the concentration of competing ligand used was 700 nM.

Transferrin binding was also examined by using a nonisotopic dot blot enzyme assay based on the method described by Morton and Williams (26). Briefly, 5 μl of staphylococcal cell wall preparations (5 μg) in PBS was spotted on a nitrocellulose membrane and allowed to dry at room temperature for 10 min. Nonspecific binding sites were blocked with 0.3% (vol/vol) Tween 20 in Tris-buffered saline (50 mM Tris-hydrochloride containing 120 mM NaCl [pH 7.4]) and probed with a human transferrin-horseradish peroxidase (HRP) conjugate (2.5 μg/ml; Jackson Immunoresearch). Dot blots were developed in a solution of 4-chloro-1-naphthol (25 μg/ml; Sigma) in Tris-buffered saline containing 0.01% (vol/vol) hydrogen peroxide. Following development, dots were quantified by using a Shimadzu CS-9000 scanning densitometer as described previously (50).

Proteolytic and heat treatment of staphylococci. Bacteria (1 ml containing 10^9 cells per ml) were incubated at 37°C for 2 h with the following enzymes: (i) trypsin (2,500 U/ml in Tris-HCl [pH 8.0]), (ii) *S. aureus* V8 protease (110 U/ml in 50 mM Tris-HCl [pH 7.8]), (iii) proteinase K (10 U/ml in 50 mM Tris-HCl [pH 7.5]), (iv) pepsin (1,000 U/ml in sodium acetate buffer [pH 4.5]), and (v) pronase E (0.5 U/ml in 30 mM Tris-HCl [pH 6.2]). Proteolysis with trypsin, protease V8, and proteinase K was stopped by the addition of benzamidine (500 μg/ml) and phenylmethylsulfonyl fluoride (500 μg/ml). Digestion with pronase E was stopped by the addition of iodoacetamide (0.5 M) (28), and the pH was raised to 7.4 to stop the reaction with pepsin. As a control, cells were also incubated at 37°C with buffer instead of protease. For some experiments, staphylococci were also incubated in a water bath for 1 h at the following temperatures: 37, 65, 80, and 100°C. Enzyme-treated, heat-treated, and control cells were washed in PBS prior to the preparation of cell walls as described above before examination for transferrin-binding ability on dot blots and for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting) experiments.

SDS-PAGE and Western blotting. Cell wall proteins were separated on SDS–12.5% polyacrylamide gels as described before (38). Samples were solubilized in sample buffer in either the presence or absence of 2-mercaptoethanol at 37°C for 30 min or at 100°C for 10 min. Approximately 2 μg of protein was loaded per lane, and molecular size standards (Sigma) were run concurrently. Gels were stained with Coomassie brilliant blue.

For Western blotting, proteins were electrophoretically transferred to nitrocellulose and blocked with 1% (wt/vol) skim milk. Human transferrin bound to staphylococcal cell surfaces was identified after SDS-PAGE by incubating the nitrocellulose in a rabbit antiserum to human transferrin (diluted 1:400; Dakopatts, Copenhagen, Denmark). The molecular mass and position of transferrin were confirmed by running a human transferrin control and by counterstaining the blots with Ponceau S (Sigma; 0.5% [wt/vol] in 1% glacial acetic acid). To identify a putative transferrin-binding protein(s), Western blots were probed with human transferrin-HRP (2.5 μg/ml). Competitive binding assays were also carried out on Western strip blots as follows. Cell wall protein blots were incubated with a mixture of 0.18 nM of the human transferrin-HRP conjugate and either a range of concentrations of human transferrin (0 to 11.25 μM) or 11.25 μM bovine transferrin, ovotransferrin, human albumin, or human immunoglobulin G. Secondary detection of bound antibodies was achieved following incubation with protein A-HRP conjugate (Sigma; 10 μg/ml). Reactive bands on Western blots were visualized either with a 25-μg/ml solution of 4-chloro-1-naphthol containing 0.01% (vol/vol) hydrogen peroxide or by using an enhanced chemiluminescent substrate kit (ECL; Amersham International).

RESULTS

Binding of human transferrin to staphylococcal cell walls. Following growth of staphylococci in HPD, cell wall preparations were isolated from 11 coagulase-negative staphylococcal strains and an *S. aureus* strain isolated from CAPD patients with peritonitis. Immunoblotting experiments with anti-human transferrin revealed the presence of an 80-kDa band associated with the cell wall preparations derived from *S. epidermidis*, *S. aureus*, *S. capitis*, *S. haemolyticus*, and *S. hominis* but not *S. warneri* or *S. saprophyticus*. This band was confirmed as transferrin by running purified human transferrin alongside the cell wall preparations (data not shown).

To determine whether the staphylococci bound human transferrin during growth in conventional laboratory media, the same staphylococcal strains were grown either in nutrient broth or in nutrient broth made iron restricted by the addition of EDDA and then incubated for 2 h at 4°C with human transferrin. Figure 1 shows an immunoblot of the cell wall preparations derived from these staphylococcal cells probed with anti-human transferrin. As with the HPD-grown staphylococci, transferrin bound to *S. epidermidis*, *S. aureus*, *S. capitis*, *S. haemolyticus*, and *S. hominis* but not to *S. warneri* or *S. saprophyticus* was observed. Furthermore, more transferrin appeared to be bound to *S. epidermidis* after growth in nutrient broth containing EDDA than in nutrient broth alone (Fig. 1, compare lanes 11 and 12). Addition of excess iron sulfate to media containing EDDA reduced the level of transferrin bound to that observed for cells grown in nutrient broth alone, providing additional confirmation that iron availability influenced transferrin binding (data not shown). For *S. aureus*, similar levels of transferrin appeared to be bound irrespective of the growth medium iron availability (Fig. 1, compare lanes 13 and 14). As with *S. epidermidis*, the presence of EDDA in the growth medium appeared to increase the amount of human transferrin bound to *S. hominis*, *S. capitis*, and *S. haemolyticus* (Fig. 1, compare lanes 3 and 4, 7 and 8, and 9 and 10).

To examine further the transferrin-binding ability of the staphylococci, dot blots of the cell walls of *S. epidermidis* and *S.*

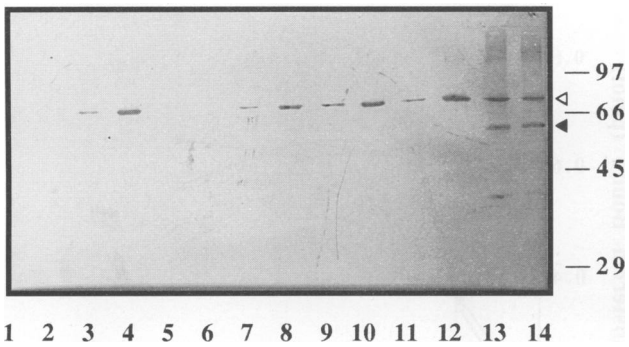

FIG. 1. Immunoblot of the cell wall proteins prepared from staphylococci grown in nutrient broth in the absence (lanes 1, 3, 5, 7, 9, 11, and 13) or presence (lanes 2, 4, 6, 8, 10, 12, and 14) of EDDA. After being harvested, bacterial cells were incubated in PBS containing human transferrin (3.75 mM) for 2 h at 4°C and washed thoroughly. Cell walls were then isolated and probed with antisera raised against human transferrin. Lanes: 1 and 2, *S. saprophyticus*; 3 and 4, *S. hominis*; 5 and 6, *S. warneri*; 7 and 8, *S. capitis*; 9 and 10, *S. haemolyticus*; 11 and 12, *S. epidermidis*; 13 and 14, *S. aureus*. The positions of molecular mass markers (in kilodaltons) are indicated on the right. The open arrowhead shows the positions of human transferrin (80 kDa). The lower band (closed arrowhead) observed in lanes 13 and 14 is protein A.

aureus grown in nutrient broth or in nutrient broth plus EDDA were probed with human transferrin-HRP conjugate. Both organisms were found to be capable of binding transferrin, and scanning densitometry of the dot enzyme assays indicated that for *S. epidermidis*, transferrin binding was regulated to some extent by the availability of iron in the growth medium since binding to cell walls derived from staphylococci grown in iron-restricted medium was increased by approximately 50% (Fig. 2). Iron restriction did not, however, influence the expression of transferrin binding by *S. aureus*. These data are

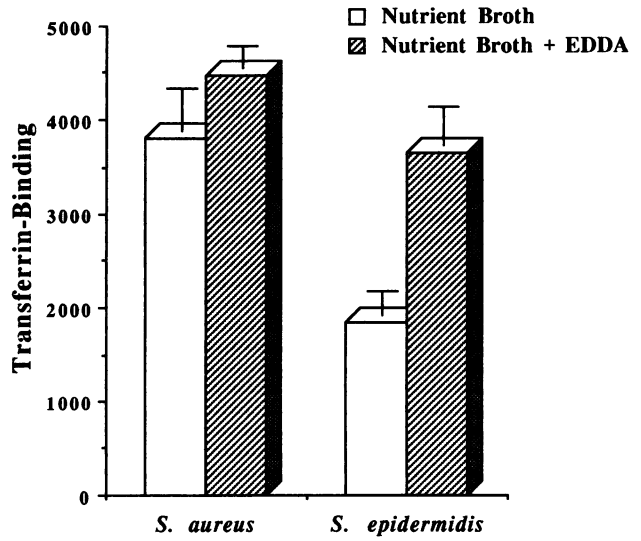

FIG. 2. Influence of EDDA on the expression of transferrin binding by *S. aureus* and *S. epidermidis*. After overnight growth in nutrient broth with or without EDDA, cell walls were prepared and spotted onto nitrocellulose and probed with a transferrin-HRP conjugate. After color development, dots were quantified by scanning densitometry. Values (arbitrary units) are means ± standard deviations.

consistent with those obtained following the growth of *S. aureus* and *S. epidermidis* in broth and incubation with human transferrin.

Transferrin receptor saturability, affinity, and copy number. To determine whether the staphylococci express a specific receptor for human transferrin, the interaction of whole staphylococcal cells with ^{125}I-transferrin was examined. Both *S. epidermidis* and *S. aureus* bound ^{125}I-transferrin in a time-dependent (Fig. 3) and saturable (displaceable) manner (Fig. 4), with saturation being reached after 30 min of incubation with the labelled ligand. In agreement with the dot blot and Western blot data presented above, *S. epidermidis* grown in nutrient broth bound less transferrin than *S. epidermidis* grown in broth plus EDDA (Fig. 3A), while iron availability had no apparent effect on the saturability of transferrin binding to *S. aureus* (Fig. 3B).

The specificity of transferrin binding by *S. epidermidis* and *S. aureus* was established by using the method described by DeBlasi et al. (8) and is demonstrated in Fig. 4. The receptor number and affinity (K_d) were calculated for both *S. epidermidis* and *S. aureus* from binding experiments using human transferrin as both radioligand and competitor (Fig. 4). *S. epidermidis* cells grown in iron-restricted nutrient broth expressed approximately 4,200 receptors per cell, with an affinity (K_d) of 0.27 μM. For *S. aureus*, a receptor number of 4,200 per cell was calculated, with a K_d of 0.28 μM.

Characterization of a cell wall transferrin-binding protein. Following treatment of whole cells of *S. epidermidis* and *S. aureus* with the proteolytic enzyme pronase E, cell walls were extracted and examined by dot enzyme assay for transferrin binding. Binding was abolished, indicating the involvement of a cell wall protein(s) (data not shown). To identify the putative transferrin-binding protein(s), cell wall preparations of *S. epidermidis* and *S. aureus* were solubilized in SDS-PAGE sample buffer at 37°C prior to electrophoresis. After SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose and probed with the human transferrin-HRP conjugate. Figure 5 reveals the presence, in *S. epidermidis*, of a 42-kDa transferrin-binding protein induced in the cell walls of bacteria grown in iron-restricted broth. A transferrin-binding protein of approximately 42 kDa was also observed in the *S. aureus* cell wall, although its expression did not appear to be affected by the availability of iron in the growth medium (Fig. 5). While the relative migration of the 42-kDa transferrin-binding protein was unaffected by solubilization in SDS-PAGE sample buffer at 37 or 100°C, the transferrin-binding activity of this protein was sensitive to temperature. For *S. epidermidis* and *S. aureus*, transferrin binding was abolished at solubilization temperatures higher than 37 and 65°C, respectively. Furthermore, the 42-kDa protein of both organisms was not a disulfide-linked subunit of a larger protein since its migration on SDS-polyacrylamide gels was unaffected by the presence of 2-mercaptoethanol in the sample buffer (data not shown). Western blots of cell wall preparations derived from *S. capitis*, *S. haemolyticus*, and *S. hominis* but not from *S. warneri* or *S. saprophyticus* also revealed the presence of an iron-regulated 42-kDa transferrin-binding protein (data not shown).

Influence of iron saturation on transferrin binding. In competition binding studies using iron-saturated ^{125}I-transferrin, unlabelled iron-saturated transferrin blocked the binding of the radiolabelled ligand. To determine whether transferrin iron saturation influenced receptor binding, studies were also carried out with the ^{125}I-labelled apotransferrin or iron-saturated transferrin in competition with each respective form of the unlabelled ligand. Iron saturation of transferrin had no effect on receptor binding (data not shown).

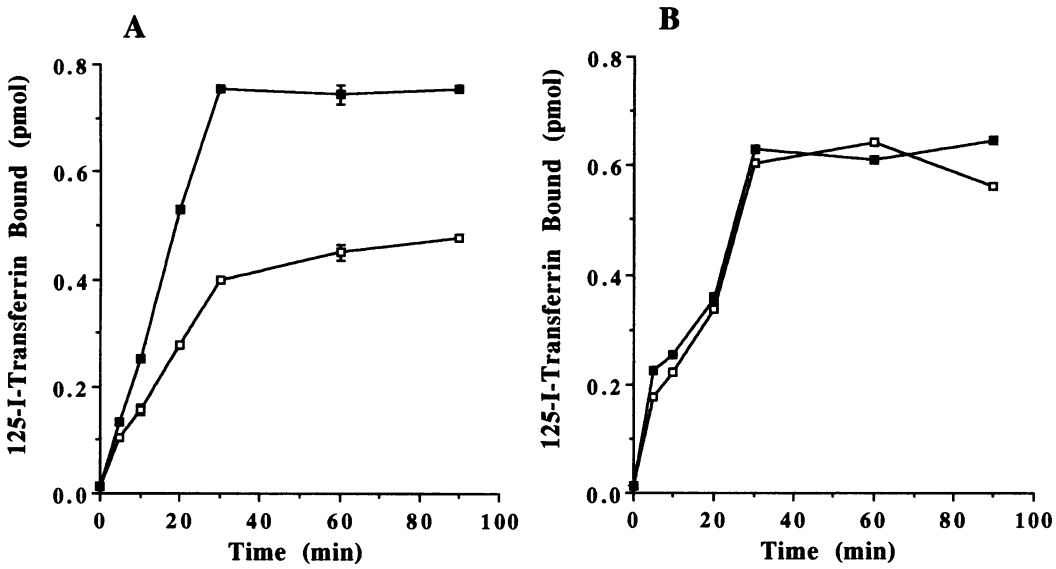

FIG. 3. Time course of ^{125}I-transferrin binding to *S. epidermidis* (A) and *S. aureus* (B). After overnight growth in nutrient broth (□) or in nutrient broth supplemented with EDDA (■), staphylococci were harvested, washed, resuspended in PBS (10^8 cells per ml) containing 5.1 nM ^{125}I-labelled iron-saturated human transferrin, and incubated at 37°C. At intervals of 5, 10, 20, 30, 60, and 90 min, bacteria were pelleted and the amount of cell-associated ^{125}I-transferrin was determined. Data presented are the means of three independent experiments ± standard deviations.

Transferrin species specificity. To examine further the specificity of binding, we evaluated the ability of other mammalian transferrins (rat, rabbit, porcine, and bovine transferrins, ovotransferrin, and human and bovine lactoferrins) and serum proteins (albumin and immunoglobulin G) to inhibit the binding of human transferrin to whole staphylococcal cells (Fig. 6). Rat and rabbit transferrins were almost as effective as human transferrin, while porcine and bovine transferrins and ovotransferrin competed much less effectively. Some reduction in binding was also observed with human and bovine lactoferrins (Fig. 6). To determine whether these data correlated with the binding of transferrin to the 42-kDa protein, competition Western strip blot assays were carried out with a concentration of human transferrin (11.25 μM) established to be well in excess of that required to block the binding of the HRP-transferrin conjugate. Figure 7 shows that the binding of transferrin-HRP to the 42-kDa transferrin-binding protein of *S. epidermidis* was inhibited by human transferrin but not by bovine transferrin or ovotransferrin or by the human serum proteins albumin and immunoglobulin G. Similar results were obtained for *S. aureus* (data not shown).

Expression of the 42-kDa transferrin receptor by staphylococci grown in HPD. Since we had originally observed that staphylococci grown in peritoneal dialysate bound human transferrin, HPD-grown staphylococci were examined for expression of the 42-kDa transferrin-binding protein. Figure 8A demonstrates that each of five *S. epidermidis* isolates and one *S. aureus* isolate from CAPD patients with peritonitis expressed a 42-kDa transferrin-binding protein after growth in HPD. Similar results indicating the presence of a conserved transferrin-binding protein were obtained for *S. haemolyticus*, *S. capitis*, and *S. hominis* (Fig. 8B). *S. saprophyticus* and *S. warneri*, which did not bind transferrin after growth in HPD, lacked the 42-kDa transferrin-binding protein (Fig. 8B). In addition, one or two bands below that of the 42-kDa transferrin-binding protein were sometimes apparent in the HPD-grown (but not broth-grown) staphylococci (e.g., Fig. 8A, lanes 1 and 2, and

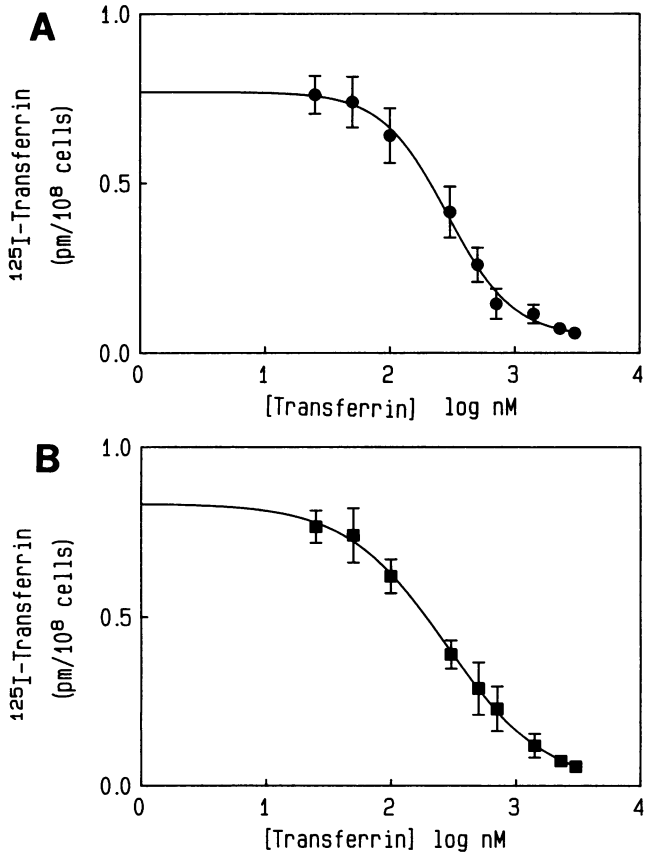

FIG. 4. Competition binding assay using iron-saturated human transferrin as radiolabelled ligand and competitor. (A) *S. epidermidis*; (B) *S. aureus*. Results depict the means and standard deviations of three experiments. The curves were fitted by using the curve-fitting program Graph Pad Inplot (ISI Inc.).

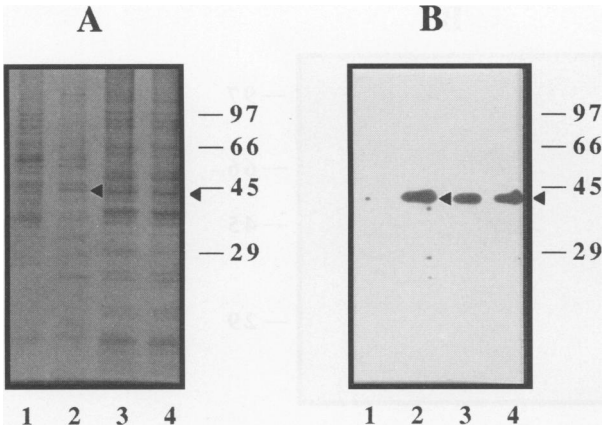

FIG. 5. (A) SDS-PAGE of the cell wall proteins of *S. epidermidis* (lanes 1 and 2) and *S. aureus* (lane 3 and 4) grown in nutrient broth without (lanes 1 and 3) or with (lanes 2 and 4) EDDA. (B) Western blot of the samples described for panel A probed with a human transferrin-HRP conjugate. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the gels. The closed arrowheads indicate the position of the 42-kDa transferrin-binding protein.

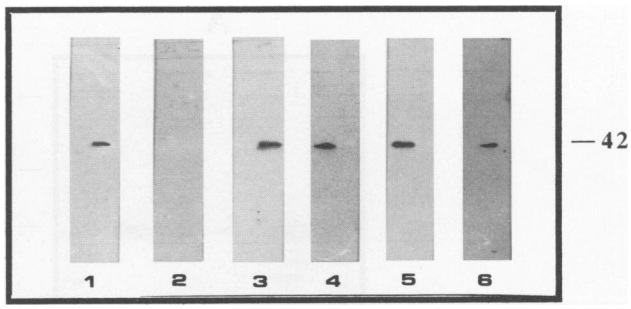

FIG. 7. Western strip blot competition assay showing the transferrin specificity of the 42-kDa *S. epidermidis* transferrin-binding protein. Western strip blots of cell wall proteins were incubated with human transferrin-HRP conjugate (0.18 nM; lane 1) or a mixture of the HRP conjugate (0.18 nM) and 11.25 μM human transferrin (lane 2), bovine transferrin (lane 3), ovotransferrin (lane 4), human albumin (lane 5), or human immunoglobulin G (lane 6). The position of the 42-kDa transferrin-binding protein is indicated on the right.

Fig. 8B, lanes 2 and 4) and are probably breakdown products of the 42-kDa protein.

DISCUSSION

Information on the nature of the molecular interactions occurring between staphylococcal cell envelope components and the CAPD host environment should aid our understanding of the pathogenesis of CAPD peritonitis. To mimic conditions within the dialyzed peritoneum, we routinely grow staphylococci in HPD in air enriched with 5% carbon dioxide, gaseous conditions which closely resemble those found physiologically (46). Growth in such an environment profoundly influences staphylococcal surface physiochemistry, adherence to catheter polymers, and cell envelope protein profile (38, 45–48). With regard to the latter, we wished to determine whether any of the novel cell wall proteins observed were host rather than bacterially derived since HPD contains numerous plasma proteins (19, 48). Immunoblotting studies with antisera to human transferrin indicated that this iron-binding glycoprotein could be detected bound to the cell walls of *S. aureus*, *S. epidermidis*, *S. capitis*, *S. haemolyticus*, and *S. hominis* after growth in HPD even though the cells had been thoroughly washed in PBS after harvesting. However, no transferrin was detected bound to *S. warneri* or *S. saprophyticus*, suggesting that the observed transferrin binding to the other staphylococcal strains was unlikely to be a nonspecific phenomenon. Furthermore, these results could be reproduced by growing the same staphylococcal strains in conventional laboratory media and then incubating them with human transferrin or by probing staphylococcal cell walls with a human transferrin-HRP conjugate. Interestingly, for *S. epidermidis*, *S. capitis*, *S. haemolyticus*, and *S. hominis* but not *S. aureus*, more transferrin appeared to be associated with the cell well after growth in nutrient broth made iron restricted by the addition of the synthetic iron chelator EDDA, suggesting that the expression of the surface component involved in transferrin binding was regulated to some extent by the availability of iron in the growth medium. Given that staphylococci require iron for growth, such data also imply that the binding of transferrin may constitute the initial step in the acquisition of glycoprotein-bound iron. For gram-negative pathogens such *H. influenzae* (26) and *N. meningitidis* (37, 43), this has been shown to be the case in that the acquisition of iron from transferrin is dependent on direct contact between a bacterial cell surface receptor and the host iron-binding glycoprotein.

To demonstrate that the transferrin-binding staphylococci express a specific receptor for human transferrin, we sought to determine whether the major prerequisites for a biological receptor could be fulfilled, namely, ligand specificity and concentration-dependent saturability. Transferrin binding to

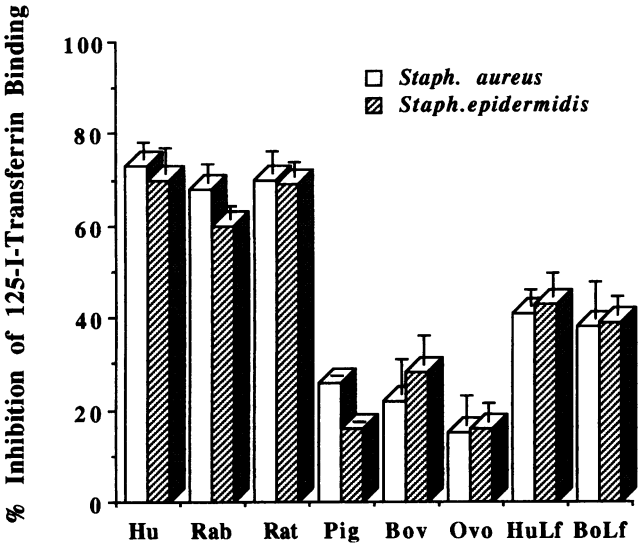

FIG. 6. Whole-cell competition binding assay showing the transferrin species specificity of the staphylococcal transferrin receptor. Staphylococci (10^8 cells per ml) were incubated with ^{125}I-apotransferrin (4 nM) in the presence of one of the following unlabelled apoproteins (700 nM): human (Hu), rabbit (Rab), rat, pig, and bovine (Bov) transferrins; ovotransferrin; and human (HuLf) and bovine (BoLf) lactoferrins. After 30 min at 37°C, bacteria were pelleted and the amount of cell-associated ^{125}I-transferrin was determined. Data presented are the means of three independent experiments ± standard deviations.

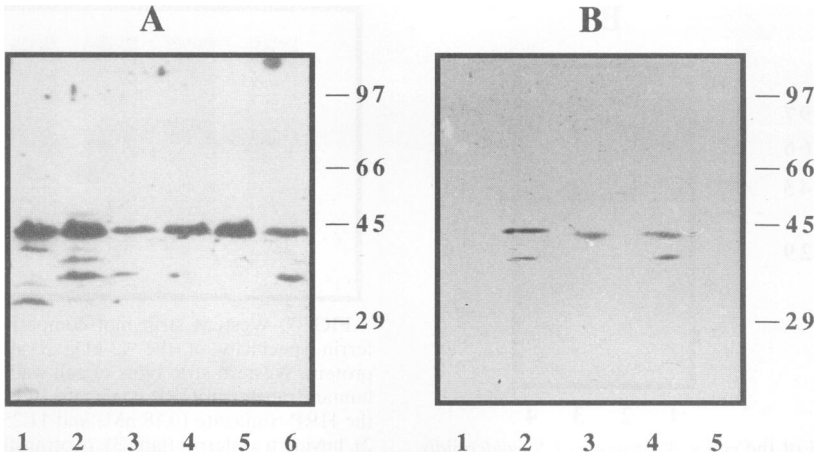

FIG. 8. Western blots of the cell wall proteins of CAPD peritonitis isolates of *S. aureus* and coagulase-negative staphylococci grown in HPD and probed with a human transferrin-HRP conjugate. (A) *S. aureus* (lane 1) and *S. epidermidis* (lanes 2 to 6); (B) *S. saprophyticus* (lane 1), *S. haemolyticus* (lane 2), *S. hominis* (lane 3), *S. capitis* (lane 4), and *S. warneri* (lane 5). The positions of molecular mass markers (in kilodaltons) are indicated to the right of the blots.

both *S. epidermidis* and *S. aureus* was time and concentration dependent (demonstrated by the ability of the unlabelled ligand to compete for binding with ^{125}I-transferrin), and from competition binding assays, the existence of a staphylococcal transferrin receptor was established. Although the affinities of the staphylococcal receptors for transferrin are much lower (*S. aureus* K_d, 0.28 μM; *S. epidermidis* K_d, 0.27 μM) than those of mammalian cells (e.g., the rabbit reticulocyte diferric transferrin receptor has a K_d of 11 nM) (52), they are similar to that described by Tsai et al. (43) for the transferrin receptor of *N. meningitidis* (K_d, 0.7 μM). In addition, the staphylococci did not distinguish between human apotransferrin and the iron-saturated form. This finding is similar to that described for other prokaryotes but contrasts with that for the eukaryotic transferrin receptors, which have far higher affinities for the iron-loaded form of the protein (41, 52). In this context, given that bacteria do not appear to endocytose transferrin (20, 37), it is possible that a lower-affinity receptor might facilitate more rapid turnover and iron release from transferrin at the bacterial cell surface or, since serum transferrin is only 30% iron saturated, enable the microorganism to sort through the available iron for iron-bearing molecules.

Having established the existence of receptors for transferrin on the staphylococci, we wished to identify the bacterial cell wall component(s) involved. Treatment of whole staphylococci with the proteolytic enzyme pronase E abolished transferrin binding, indicating the probable involvement of a protein(s) located at the cell surface. Previously, we have shown that bacterial transferrin-binding proteins can be renatured to bind transferrin after SDS-PAGE and Western blotting provided that the samples were solubilized in SDS-PAGE sample buffer at 37°C rather than 100°C (14, 26, 40). By adopting this procedure, a 42-kDa cell wall protein common to both *S. aureus* and *S. epidermidis* could be renatured to bind human transferrin. Furthermore, omission of 2-mercaptoethanol from the sample buffer did not influence the migration of the protein on SDS-polyacrylamide gels, indicating that it was not a disulfide bridge-linked subunit of a larger receptor protein. Thus, the transferrin receptor consists of at least a 42-kDa protein in both *S. aureus* and *S. epidermidis*. *S. haemolyticus*, *S. capitis*, and *S. hominis* also possess a 42-kDa transferrin-binding protein which is absent from *S. saprophyticus* and *S. warneri*, consistent with the transferrin binding data. Further work is required to determine how closely these proteins are related. Interestingly, the *S. epidermidis* and *S. aureus* proteins exhibited a differential susceptibility to proteases in that although both were susceptible to pronase E, only the *S. epidermidis* protein was cleaved by trypsin and protease K (23).

The staphylococcal transferrin receptor differs from the mammalian transferrin receptor, a 180-kDa transmembrane protein consisting of two identical disulfide bridge-linked subunits of 90 kDa (49). It also differs from the transferrin receptors of *Haemophilus* and *Neisseria* spp., which consist of two distinct transferrin-binding proteins, termed TBP1 and TBP2, which range in molecular mass from 68 to 105 kDa depending on the strain (49). Interestingly, for both organisms, TBP2 but not TBP1 can be renatured to bind transferrin after SDS-PAGE and Western blotting (40). The staphylococcal transferrin-binding protein described here also appears to be unrelated to the *S. aureus* human lactoferrin-binding protein described by Naidu et al. (27) as an approximately 450-kDa protein. Under reducing conditions, this lactoferrin receptor was resolved on SDS-polyacrylamide gels into two components of 67 and 62 kDa, both of which bound a lactoferrin-peroxidase conjugate after Western blotting (27).

A variety of mammalian cells expressing transferrin receptors are capable of both binding and acquiring iron from the transferrins of other species. Thorstensen and Romslo (42) noted that for isolated rat hepatocytes, binding of transferrin and uptake of iron are essentially the same for both rat and human transferrins. Others have shown that rabbit, pig, and horse transferrins exhibited the same patterns of binding to the human MOLT-3 cell receptors as human transferrin, while binding of mouse and dog transferrins was weaker and that of bovine transferrin was negative (2). In contrast, a common feature which has emerged from studies of the transferrin-binding proteins of gram-negative bacteria is their remarkable specificity for the transferrin of their natural host (25, 26, 36, 49). For example, humans are the only known natural reservoir for *H. influenzae*, which shows a marked preference for human transferrin and is unable to bind or remove iron from porcine, rabbit, mouse, or dog transferrin (26). In contrast, the porcine pathogen *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* is able to bind and acquire iron from porcine but not human

transferrin (9, 25, 26). In competition binding assays, the staphylococcal receptor also demonstrated some transferrin species specificity in that neither bovine or porcine transferrin nor ovotransferrin effectively blocked the binding of human transferrin. This is perhaps surprising given the high degree of amino acid sequence conservation among the mammalian transferrins (1, 3). When human transferrin and ovotransferrin are compared, some 348 of 699 amino acid residues occupy identical positions (49.8% homology). The binding of transferrin to the staphylococcal receptor was, however, inhibited by rat and rabbit transferrins and, to a lesser extent, by human lactoferrin, which shares some 59% amino acid sequence homology with human transferrin (3). Interestingly, human transferrin was reported not to block binding of ^{125}I-labelled human or bovine lactoferrin to the lactoferrin receptors of either *S. aureus* or *S. epidermidis*, although moderate inhibition of lactoferrin binding was observed with *S. hyicus* and *S. hominis* (28). In addition, neither human nor bovine staphylococcal isolates showed any preference for the lactoferrins of their respective hosts, further distinguishing the lactoferrin receptor from the staphylococcal human transferrin receptor described in this article. Although staphylococci do not generally exhibit a restricted mammalian host specificity, further work is required to determine whether nonhuman isolates such as *S. hyicus*, which is usually isolated from the skins of pigs and cattle (29), exhibit any alternative transferrin species specificity.

The gram-negative bacterial transferrin receptors described to date are regulated by growth medium iron availability (49). For the fresh CAPD peritonitis-associated isolates of *S. aureus* examined in this study, the transferrin receptor was constitutively expressed after growth in both iron-plentiful and iron-restricted nutrient broth. Holland et al. (14) observed constitutive expression of the transferrin receptor in fresh clinical isolates of *H. influenzae* type b, which became iron regulated only after prolonged subculture in vitro on iron-rich laboratory media. Although we have not as yet carried out similar experiments with the CAPD peritonitis isolates of *S. aureus* described here, we have observed iron-regulated expression of the transferrin receptor in other non-CAPD isolates of *S. aureus*. Further work is thus required to determine whether this constitutive phenotype is unique to CAPD isolates and whether it confers a survival advantage within the dialyzed peritoneum. The staphylococcal transferrin receptor protein is expressed by staphylococci grown under conditions which mimic those encountered in vivo, i.e., in HPD and also in cells recovered without subculture from a rat peritoneal chamber implant (23). The presence of antibodies to the transferrin receptor in HPD provides further evidence that the receptor is expressed during episodes of peritonitis in CAPD patients (23). Work is under way to determine whether such antibodies can compete with transferrin for the transferrin receptor and perhaps interfere with staphylococcal iron acquisition.

Further work is also required to elucidate the contribution of the transferrin receptor to the acquisition of protein-bound iron. Apart from *Bordetella pertussis* and *Bordetella bronchiseptica* (10, 22, 30), none of the gram-negative bacteria currently known to express transferrin-binding proteins synthesize siderophores (49). Siderophore production has, however, been described in both *S. aureus* and the coagulase-negative staphylococci (15, 21). It is, therefore, possible either that the staphylococci possess at least two independent iron uptake systems or that there is cooperation between transferrin receptor and siderophore to maximize the efficiency of transferrin iron acquisition. In *Bordetella* spp., both systems appear to operate, although direct binding of transferrin provides for far more efficient iron uptake than siderophore activity alone (22, 30). Interestingly, neither *S. aureus* nor *S. epidermidis* appears to acquire iron efficiently from transferrin when separated from the iron-binding protein by a dialysis membrane (4). Given that the staphylococci grow in serum (32) and HPD (38, 48), both of which contain transferrin, as well as in laboratory media supplemented with transferrin (18), these data suggest that efficient iron uptake requires direct cell surface contact. Work is currently under way to investigate the role of the 42-kDa transferrin receptor protein in the uptake of glycoprotein-bound iron.

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