The *Staphylococcus aureus* and *Staphylococcus epidermidis* transferrin-binding proteins are expressed *in vivo* during infection

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Staphylococci express a 42 kDa cell-wall-associated protein which functions as a receptor for the mammalian iron-binding glycoprotein transferrin. To determine whether this transferrin-binding protein (TBP) is expressed during infection, *Staphylococcus aureus* and *Staphylococcus epidermidis* were grown *in vivo* in chambers implanted intraperitoneally in rats. SDS-PAGE and Western blotting of cell wall proteins prepared from staphylococci recovered directly from the chambers revealed the presence of both the TBP and bacterial-surface-associated rat transferrin. To obtain evidence for the *in vivo* expression of the staphylococcal TBPs in humans, sera and human peritoneal dialysate (HPD) from non-infected patients undergoing continuous ambulatory peritoneal dialysis (CAPD) and sera from healthy human volunteers were screened for anti-TBP antibodies. Western immunoblots revealed that three out of ten samples from the latter group, seven out of ten HPD samples and ten of ten CAPD patient serum samples contained antibodies to the TBP of both *S. aureus* and *S. epidermidis*. To gain further insights into the appearance of TBP antibodies, HPD samples were collected over time from CAPD patients whose HPD samples taken immediately after catheter insertion lacked anti-TBP antibodies. In two of these patients, each of whom experienced an episode of peritonitis due to *S. epidermidis* or *Staphylococcus hominis*, antibodies to the TBP appeared in the HPD collected immediately post-infection. To determine whether such TBP antibodies were capable of blocking interactions between transferrin and its staphylococcal receptor, HPD immunoglobulin fractions were purified using protein A-Sepharose beads. In competition assays, these immunoglobulins blocked the binding of 125I-labelled transferrin both to whole bacteria and to the isolated 42 kDa TBPs of *S. aureus* and *S. epidermidis*. These provide evidence to show that staphylococcal TBPs are expressed *in vivo* during infection.

**Keywords:** transferrin, transferrin-binding protein, *Staphylococcus aureus*, *Staphylococcus epidermidis*, continuous ambulatory peritoneal dialysis

### INTRODUCTION

*Staphylococcus aureus* is a primary human pathogen frequently responsible for a broad spectrum of pyogenic and toxin-mediated infections (Mollby *et al.*, 1994). In contrast, the pathogenic potential of coagulase-negative staphylococci, such as *Staphylococcus epidermidis*, has only been recognized relatively recently and they have emerged as important opportunistic pathogens, especially in infections associated with indwelling medical devices (Mollby *et al.*, 1994; Pfaffer & Herwaldt, 1988). For example, *S. epidermidis* is a major cause of peritonitis in patients undergoing treatment for end-stage renal failure by continuous ambulatory peritoneal dialysis (CAPD) (Spencer, 1988; Brown *et al.*, 1991; Williams *et al.*, 1995). In CAPD, sterile dialysis fluid (a hypertonic solution containing dextrose) is introduced into the peritoneal cavity via an abdominal catheter.

**Abbreviations:** CAPD, continuous ambulatory peritoneal dialysis; HPD, human peritoneal dialysate; HRP, horseradish peroxidase; TBP, transferrin-binding protein.
After a suitable dwell time (4–8 h), 'spent dialysate', i.e. the commercial dialysate now enriched by a plasma ultrafiltrate (containing low levels of serum proteins such as albumin, transferrin, immunoglobulins and complement components), is drained and replaced with fresh fluid. A major complication of this technique is that most patients experience one to two episodes of peritonitis per year, although multiple recurrent episodes are also a common feature (Spencer, 1988; Brown et al., 1991). Peritonitis not only threatens patient survival but also compromises the use of the peritoneal membrane as a dialysis membrane, repeated attacks being responsible for considerable morbidity and increased treatment costs. In CAPD peritonitis, bacteria gain entry either intra- or extraluminally via the CAPD catheter, and although microbial contamination does not always lead to infection, peritonitis can develop readily despite a vigorous host cellular response (Williams et al., 1995). Thus, on entry and during the development of peritonitis, the infecting bacterium encounters biomaterial, cellular and fluid-phase environments with which to interact.

Previously we have shown that both S. epidermidis and S. aureus, grown in spent human peritoneal dialysate (HPD) as a simulated CAPD fluid phase, exhibit markedly different properties to those of staphyloccoci grown in conventional laboratory media (Williams et al., 1988; Smith et al., 1991; Wilcox et al., 1990; Modun et al., 1994). In particular, we noted the appearance of a number of iron-repressible cell-wall- and cytoplasmic-membrane-associated proteins in both S. epidermidis and S. aureus (Williams et al., 1988; Smith et al., 1991; Modun et al., 1994). Amongst these antigens, the 32 and 36 kDa proteins proved to be the immunodominant S. epidermidis cell envelope proteins (Smith et al., 1991; Modun et al., 1992). These iron-repressible proteins were also found to be expressed during the growth of S. epidermidis in the peritoneal cavities of both rats (Modun et al., 1992) and pigs (McDermid et al., 1993). Although the function(s) of these proteins has not yet been elucidated, they may constitute components of high-affinity iron-scavenging mechanisms. Both the 32 and 36 kDa proteins are components of ATP-binding cassette transporter systems (A. Cockayne, P. Hill, J. Morrissey & P. Williams, unpublished data) which may conceivably contribute to the uptake of staphylococcal siderophores [low-molecular-mass iron(III) chelators] such as staphyloferin A and staphyloferin B (Meiwes et al., 1990; Konetschny-Rapp et al., 1991; Drechsel et al., 1993), the chemically uncharacterized siderophore 'aureochelin' (Courcol et al., 1997) or α-keto acids (Heuck et al., 1993).

Alternatively, these iron-repressible 32 and 36 kDa proteins may be involved in the uptake of iron from transferrin. This mammalian iron-binding glycoprotein is present in HPD and transferrin can be detected bound to the cell walls of both S. epidermidis and S. aureus grown in HPD (Modun et al., 1994). Human pathogens such as Haemophilus influenzae and Neisseria meningitidis acquire iron from transferrin via a siderophore-independent process which involves a direct interaction between a bacterial surface receptor and transferrin (Williams & Griffiths, 1992). The presence of transferrin bound to HPD-grown staphylococci therefore suggested that these Gram-positive pathogens may possess an analogous receptor. Using 125I-labelled human transferrin, Modun et al. (1994) showed that both S. epidermidis and S. aureus bound the radio-labelled ligand in a time- and concentration-dependent manner, thereby confirming the existence of a specific transferrin receptor. In addition, the staphylococcal receptor exhibited a significant transferrin species preference by binding human, rat and rabbit transferrins but not pig, bovine or ovo-transferrins, despite their very similar amino acid sequences (Modun et al., 1994). Ligand-blotting techniques established that the 42 kDa cell wall protein identified as one of the three major HPD-induced S. epidermidis wall proteins (Smith et al., 1991) is in fact a transferrin-binding protein (TBP) (Modun et al., 1994). Other coagulase-negative staphyloccoci, including Staphylococcus hominis, Staphylococcus haemolyticus and Staphylococcus capitis, also possess a 42 kDa TBP (Modun et al., 1994). Recently we have demonstrated that the staphylococcal transferrin receptor is involved in the direct acquisition of iron from transferrin (B. Modun, C. Ioannou, R. Evans & P. Williams, unpublished data). It is therefore possible that iron released from the TBP-bound transferrin is possibly internalized via either the 32 or 36 kDa iron-repressible cytoplasmic-membrane-associated protein.

The ability to compete successfully for transferrin-bound iron is likely to be an important prerequisite for bacterial multiplication in vivo. In addition, bacterial transferrin receptors have attracted considerable interest as candidate vaccine antigens (Williams & Griffiths, 1992; Gray-Owen & Schryvers, 1996). This is because they recognize the same human serum protein and are therefore likely to share highly conserved cell-surface-exposed epitopes. In the present paper, we sought to determine (i) whether staphyloccoci recovered without subculture from a rat intraperitoneal chamber model were coated with surface-bound transferrin and (ii) whether they expressed the 42 kDa TBP. We also investigated whether infected CAPD patients produced antibodies to this TBP and whether such antibodies were capable of blocking the ligand–receptor interaction.

METHODS

Bacterial strains and growth conditions. S. epidermidis strains 138 and 901, S. aureus strain W and S. hominis strain 441 were isolated from HPD obtained from infected CAPD patients at the Renal Unit, City Hospital, Nottingham, and have been described previously (Modun et al., 1994). Staphyloccoci were grown in vitro statically at 37 °C for 18 h in (i) nutrient broth or (ii) nutrient broth rendered iron-restricted by addition of the non-utilizable iron chelator ethylenediamine-di-α-hydroxyphenylacetic acid (800 μM) (Modun et al., 1992) or (iii) pooled HPD in a gaseous atmosphere enriched with 5% CO₂ (Wilcox et al., 1990).
Intraperitoneal rat chamber model for *in vivo* growth of staphylococci. *S. epidermidis* strain 138 and *S. aureus* strain W were grown in intraperitoneal chambers implanted in Wistar SBW rats essentially as described by Pike et al. (1991) and Modun et al. (1992). The titanium chambers incorporate two 0.2 μm pore-size membrane filters and possess an external sampling port which permits repeated sampling of the contents without killing the animal. Chambers (one per rat; two rats per strain were used) were inoculated with 0.5 ml bacterial suspension containing 10^6 cfu ml⁻¹ of either *S. epidermidis* strain 138 or *S. aureus* strain W which had been grown for 18 h in nutrient broth, washed and resuspended in PBS (120 mM NaCl, 10 mM sodium phosphate, pH 7.4). Staphylococci grown *in vivo* (10^6 cfu ml⁻¹ ml⁻²) could be recovered without subculture after 48 h (Pike et al., 1991).

Preparation of staphylococcal cell wall proteins. Cell wall and cytoplasmic membrane proteins were prepared essentially as described previously by Smith et al. (1991). Briefly, staphylococci were harvested, washed twice in PBS, resuspended in 0.6 ml digestion buffer (30% [w/v] raffinose, benzamidine (1 mg ml⁻¹), PMSF (0.5 mg ml⁻¹), in 10 mM Tris/HCl, pH 7.4), containing 100 μg lysozyme) and incubated for 60 min at 37°C (Smith et al., 1991). Protoplasts were removed by centrifugation (11,600 g for 3 min) and the supernatant fraction containing the cell wall proteins was stored frozen at -20°C prior to electrophoresis.

Human sera and spent peritoneal dialysates. Patients with end-stage renal failure being treated by CAPD for the first time at the Renal Unit, City Hospital, Nottingham, were recruited. Samples of serum and HPD were collected as soon as practicable following insertion of the peritoneal catheter and then at 3–4 month intervals. For patients who subsequently developed staphylococcal peritonitis, serum and HPD fluid were also collected at the time of infection and at 3 weeks post-infection. Serum samples from ten healthy human volunteers were kindly provided by Dr W. Irving (Department of Microbiology, University Hospital, Nottingham).

SDS-PAGE and Western blotting. Staphylococcal cell wall proteins were solubilized by incubation in sample buffer without 2-mercaptoethanol at 37°C for 30 min and loaded onto a 12.5% SDS-polyacrylamide gel. After electrophoresis, proteins were either stained with Coomassie Brilliant Blue or probed with a human transferrin-horseradish peroxidase control (Sigma) and by counterstaining the blots with Ponceau S (Sigma; 0.5%). Bands on Western blots were visualized either with a 25 pg ml⁻¹ solution of 4-chloro-1-naphthol containing 0.01% (v/v) hydrogen peroxide or by using an enhanced chemiluminescent substrate kit (ECL; Amersham International).

Protein A-Sepharose purification of human immunoglobulins. To 1 ml serum or 2 ml infected HPD, 50 μl protein A immobilized on Sepharose beads (Sigma) was added. The mixture was incubated for 2 h at room temperature and then the beads were collected and washed three times in TBS. Bound immunoglobulins were eluted using glycine (0.1 M, pH 2.5), and the solution obtained was neutralized with Tris/HCl (1 M, pH 8.0). A sample of immunoglobulin purified with protein A-Sepharose was subjected to SDS-PAGE and Western blotting as described above but probed with protein A-peroxidase to confirm the presence of immunoglobulins (primarily IgG).

Preparation of 125I-labelled human transferrin. Two hundred microlitres of a 100 μg ml⁻¹ solution of iodogen (Pierce Europe) in dichloromethane was added to a 4 ml test tube and the dichloromethane was allowed to evaporate by rotating in a water bath at 37°C. To each iodogen-coated tube, 300 μg iron-saturated human transferrin (Sigma) and approximately 6 MBq carrier-free 125I (in NaOH) (Amersham) were added in 300 μl PBS. The mixture was incubated with agitation at room temperature for 15 min and the unincorporated 125I was removed by passing the 125I-labelled transferrin down a Sephadex G-25 column (Pharmacia) pre-equilibrated with PBS containing 0.25% (w/v) human transferrin.

Competitive binding assays. Whole staphylococcal cells were incubated with a mixture of purified immunoglobulins and 125I-labelled human transferrin in PBS for 30 min at 37°C. Bacteria were washed three times in PBS and the associated radioactivity was measured using a Compu gamma counter (Pharmacia-LKB). Competitive binding assays were also carried out on Western strip blots essentially as described previously (Modun et al., 1994). Briefly, strips of nitrocellulose containing the 42 kDa staphylococcal TBP were incubated with the human transferrin–HRP conjugate in the presence or absence of a range of dilutions of the human HPD IgG purified using protein A-Sepharose. Subsequently the strip blots were washed and developed using an enhanced chemiluminescence kit as described above.

**RESULTS AND DISCUSSION**

**Staphylococci grown *in vivo* bind rat transferrin and express the 42 kDa TBP**

Although both the *S. aureus* and *S. epidermidis* transferrin receptors exhibit significant transferrin species specificity, we have previously shown that rat transferrin will effectively compete with human transferrin (Modun et al., 1994). Given this information, we were able to make use of the rat intraperitoneal chamber model to determine whether staphylococci growing *in vivo* bind transferrin. Cell wall proteins isolated from staphylococci recovered without subculture from the intraperitoneal chambers were subjected to Western blotting and probed with anti-human transferrin (which cross-reacts with rat transferrin). When compared with cells grown in nutrient broth, staphylococci grown *in vivo* possessed surface-bound rat transferrin (data not shown). To confirm that both staphylococcal species express the 42 kDa TBP after growth *in vivo*, blots were also probed with a human transferrin–HRP conjugate. Both *S. epidermidis* and *S. aureus* clearly expressed the TBP during intraperitoneal growth (Fig. 1).

These findings are in accordance with our previous observations (Modun et al., 1994) that staphylococci grown *in vitro* in pooled HPD express the 42 kDa TBP and are coated with surface-associated transferrin. Since
expression of the *S. epidermidis* TBP in vitro is dependent on the concentration of iron available, these data suggest that the peritoneal cavity constitutes a severely iron-restricted environment. Similar conclusions have been made with respect to *H. influenzae*. When recovered without subculture from the intraperitoneal cavities of infant rats, this Gram-negative pathogen expresses its transferrin receptor (Holland *et al.*, 1992). Given that these TBPs are involved in iron-scavenging (Williams & Griffiths, 1992), their expression in vivo is likely to confer a significant survival advantage in the severely iron-restricted environment of the peritoneum.

**Infected CAPD patients produce antibodies to the staphylococcal TBP**

To obtain further evidence for the in vivo expression of the staphylococcal TBPs, we probed Western blots of the cell wall proteins of staphylococci grown in HPD with serum and HPD from CAPD patients and serum from healthy volunteers. With regard to the latter, Fig. 2 shows that of the ten serum samples, only three contained antibodies which recognized the TBP from both staphylococcal species. Given that human skin and other body sites are colonized by *S. epidermidis* and *S. aureus*, it is perhaps surprising that only three of the ten samples contained anti-TBP antibodies. Others have previously noted the presence of naturally occurring antibodies to iron-regulated bacterial antigens, in particular the iron-regulated *Escherichia coli* outer-membrane proteins (Griffiths *et al.*, 1985) and the TBPs of *H. influenzae* (Holland *et al.*, 1996), a bacterium which colonizes the upper respiratory tract of up to 80% of children and adults (Moxon & Wilson, 1991). It is possible that the lack of reactivity to the staphylococcal TBP reflects the major staphylococcal colonization site, i.e. the skin (Kloos & Musselwhite, 1975; Möllby *et al.*, 1994), a body site from which transferrin is likely to be absent (Bezkorovainy, 1987).

In contrast to the data obtained with sera from healthy volunteers, all ten serum samples from CAPD patients collected immediately after insertion of catheters contained antibodies which, on Western blots, recognized the TBPs of both *S. epidermidis* (Fig. 3a) and *S. aureus* (data not shown). However, not all samples of HPD fluid obtained immediately after catheter insertion from the same ten patients contained antibodies to the staphylococcal TBPs. Fig. 3(b) shows that the HPD fluid from three out of ten of these CAPD patients lacked antibodies to the *S. epidermidis* TBP (see lanes 2, 7 and 8). Similar results were obtained with the *S. aureus* TBP (data not shown). Thus, the same serum and HPD samples which contain antibodies to the *S. epidermidis* TBP also recognized the *S. aureus* TBP. This suggests that the two 42 kDa proteins are likely to be highly antigenically conserved. Despite the human antibody responses observed, we have been unable to raise antibodies to the staphylococcal TBPs in rabbits, rats or mice (B. Modun, A. Cockayne & P. Williams, unpublished data). We have therefore been unable to explore further the antigenic relationships between the *S. epidermidis* and *S. aureus* TBPs. Furthermore, the presence of antibodies to the TBPs in all of the CAPD patient serum samples was surprising given the results obtained with sera from healthy volunteers. However, this may reflect either the relatively small number of samples examined or the previous medical history of the CAPD patients in relation to prior intravenous- or haemodialysis-catheter-related staphylococcal infections.

To obtain further insights into the appearance of antibodies to the staphylococcal TBPs, we collected

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**Fig. 1.** SDS-PAGE (a, c, e) and Western blot (b, d, f) of the cell wall proteins of *S. epidermidis* (a, b, c, d) and *S. aureus* (e, f) grown in nutrient broth (a, b), HPD (lane 1 of c, d, e, f) and in vivo in the rat chamber model (lane 2 of c, d, e, f). Western blots (b, d, f) were probed with a human transferrin-peroxidase conjugate. Molecular mass markers (in kDa) are shown in lane 3 of (e). The position of the TBP in each panel is marked with an arrowhead.
Fig. 2. Western blots of the cell wall proteins of *S. epidermidis* grown in HPD and probed with serum obtained from ten healthy volunteers (lanes 1–10). The position of the 42 kDa TBP is marked with an arrowhead. The positions of the molecular mass markers are shown on the left-hand side.

Fig. 3. Western blots of the cell wall proteins of *S. epidermidis* probed with (a) serum samples from 10 different CAPD patients and (b) HPD fluid obtained after insertion of a catheter. All samples in (a) contain antibodies to the TBP. The position of the 42 kDa TBP is marked with an arrowhead on each strip in (b). The positions of the molecular mass markers are shown on the left-hand side.
HPD samples over time from CAPD patients whose dialysate samples collected immediately after insertion of catheters lacked anti-TBP antibodies. Fig. 4(a) shows the results obtained for a patient who experienced two episodes of *S. epidermidis* peritonitis. Although antibodies to the TBP were lacking in the post catheter insertion HPD sample, a dialysate sample taken at the onset of peritonitis clearly contained anti-TBP antibodies (Fig. 4a, compare lanes 1 and 2). Similar results were obtained with CAPD patients lacking HPD antibodies to the TBP and who experienced infections due to *S. hominis* (Fig. 4b, lanes 1 and 2). In a CAPD patient who experienced an episode of peritonitis due to *Staphylococcus warneri*, no anti-TBP antibodies were observed in either the post catheter insertion dialysate or HPD collected at the onset of infection or at 20 d post-infection (data not shown). This result, although a useful control, is perhaps not surprising, given that *S. warneri* does not express a TBP (Modun et al., 1994).

These data provide further evidence that the staphylococcal TBPs are expressed during human infection and by implication suggest that the staphylococci acquire iron from transferrin *in vivo* via their TBPs. In addition, the appearance of antibodies to the TBP in HPD fluid at the onset of peritonitis may reflect prior colonization of the CAPD catheter well before the onset of peritonitis.
Human antibodies block the binding of transferrin to staphylococcal TBPs

It is not known whether antibodies to the staphylococcal TBP are protective or whether they contribute to the resolution of peritonitis in CAPD patients. Since antibodies to the TBP were present in (i) the post catheter insertion dialysate of some CAPD patients and (ii) the HPD fluid of others but only following an episode of peritonitis, we sought to determine whether such antibodies could block the binding of transferrin to the staphylococcal cell surface and to the 42 kDa TBP. To explore this possibility, we first purified the antibodies as described in Methods to avoid competition with transferrin present in the HPD fluid. Competition binding assays were then performed with radiolabelled human transferrin. Fig. 5(a) summarizes the data obtained and demonstrates that the binding of transferrin to whole staphylococcal cells was blocked by an immunoglobulin fraction derived from HPD containing TBP antibodies but not from an HPD or serum lacking such antibodies. Since the 42 kDa TBP retains its transferrin-binding capacity after SDS-PAGE and Western blotting, we also performed Western strip blot competition assays. Fig. 5(b) shows that the anti-TBP antibodies effectively block binding of a human transferrin conjugate to the 42 kDa TBP of S. epidermidis. Similar results were obtained with S. aureus (data not shown). The presence of blocking antibodies was confirmed by the subsequent incubation of the strip blot with protein A-peroxidase (Fig. 5b, lane 4). As controls, the blocking ability of affinity-purified antibodies from an HPD sample and from a serum sample previously shown to lack antibodies to the TBP was examined. No inhibition of transferrin binding was observed (Fig. 5b, lane 3). We were unable to obtain sufficient human TBP antibodies to determine whether they could block staphylococcal growth in an iron-restricted medium containing transferrin. However, the preliminary data presented suggest that the staphylococcal TBP may be well worthy of consideration as a novel candidate vaccine antigen, especially since the protein is shared by both coagulase-negative staphylococci and S. aureus and appears to be antigenically conserved.

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