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Cell Envelope Proteins of *Staphylococcus epidermidis* Grown In Vivo in a Peritoneal Chamber Implant

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***Staphylococcus epidermidis* was grown in vivo in chambers implanted intraperitoneally in rats. The cell wall and cytoplasmic membrane protein profiles of the in vivo-grown organisms were compared with those of *S. epidermidis* grown in vitro in nutrient broth (NB), in iron-restricted NB, or in pooled human peritoneal dialysate (HPD). Compared with growth in broth and in common with growth in HPD, growth in vivo in chambers resulted in the repression of many *S. epidermidis* wall proteins, with proteins of 27, 42, 54, and 70 kDa predominating. Growth in vivo also resulted in the induction of two iron-repressible cytoplasmic membrane proteins of 32 and 36 kDa, which were also present in staphylococci grown in HPD and in iron-restricted NB. Immunoblotting experiments revealed that in sera taken 21 days after inoculation of the intraperitoneal chambers, the predominant antibody response to cell envelope proteins was directed against the 32- and 36-kDa iron-repressible membrane proteins.**

Coagulase-negative staphylococci are most commonly encountered as skin commensals but in recent years have emerged as significant pathogens in medical device-related infections (7). *Staphylococcus epidermidis*, for example, is the predominant cause of peritonitis in patients undergoing treatment for renal failure by continuous ambulatory peritoneal dialysis (CAPD) (1). In CAPD peritonitis, the staphylococci appear to gain entry intra- or extraluminally via the CAPD catheter and rapidly establish themselves as an adherent biofilm on the catheter and/or peritoneal membrane surface. Under these conditions, infection develops rapidly despite a readily demonstrable host cellular immune response (10). The virulence determinants of *S. epidermidis* have not been well characterized, although in the context of CAPD peritonitis, pathogenesis is likely to be related to the ability to multiply in vivo within the peritoneum (11, 16) and to adhere to catheter polymer and peritoneal tissue surfaces (10). Although *S. epidermidis* cannot grow in commercial peritoneal dialysis solutions, these fluids are modified during dialysis and become enriched by a plasma ultrafiltrate. This modified human dialysate, human peritoneal dialysate (HPD), can support staphylococcal growth (11, 16).

The growth environment is known to exert a considerable effect on the structure of bacterial cell envelopes and the expression of virulence determinants, and there are many reports indicating considerable differences between in vivo- and in vitro-grown organisms (2, 3, 15). In particular, the extremely low availability of iron in mammalian body fluids (including HPD [16]) constitutes a major environmental signal for infecting pathogens (2, 3, 15). This lack of readily available iron is due to the presence of transferrins, which are high-affinity iron-binding glycoproteins. Pathogenic bacteria compete for this transferrin-bound iron by derepressing high-affinity iron-sequestering mechanisms usually based on low-molecular-mass iron chelators (siderophores) and their corresponding cell envelope protein receptors (15). Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* have been isolated without subculture from both human and animal infections and shown to express iron-regulated outer membrane proteins that are indicative that growth in vivo occurs under conditions of iron restriction (4, 8, 12).

Since information on the nature of the molecular interactions between staphylococcal cell envelope components and the CAPD host would be of value, and in an attempt to mimic in vivo conditions, we grew *S. epidermidis* in pooled HPD (9, 16). Cell wall and cytoplasmic membrane protein profiles of staphylococci cultured in either nutrient broth or HPD were then examined. HPD-grown *S. epidermidis* lacked many of the proteins present in the cell wall of broth-grown cells; however, two iron-repressible cytoplasmic membrane proteins (IRMPs) of 32 and 36 kDa were induced (9, 16). The molecular masses and antigenic homology of these IRMPs were highly conserved among clinical isolates of *S. epidermidis* (9). In addition, both *S. aureus* and other coagulase-negative species expressed similar proteins of between 32 and 36 kDa which were antigenically related to the 32-kDa *S. epidermidis* IRMP (9, 14). The aim of the present study was therefore to examine (i) the cell envelope protein profiles of *S. epidermidis* recovered without subculture after in vivo growth in an experimental animal model and (ii) the antibody response mounted against these in vivo-grown bacteria. In particular, we wished to determine whether the IRMPs were expressed during growth in vivo.

S. epidermidis 901 was isolated from the dialysis fluid of a patient on CAPD with peritonitis (16). In vitro, this strain was grown statically in (i) nutrient broth (NB), (ii) an iron-restricted NB (achieved by the addition of 800 μM ethylenediamine di-*o*-hydroxyphenylacetic acid [EDDA] [Sigma]; this concentration of EDDA was determined empirically to be the minimum required for induction of the IRMPs [see Fig. 2]), and (iii) sterile pooled antibiotic-free

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HPD at 37°C in an atmosphere of 95% air and 5% carbon dioxide (13). Strain 901 was also grown in vivo in a diffusion implant chamber described by Pike et al. (6) and implanted intraperitoneally in Wistar SBW rats. The chamber, which was constructed of titanium (instead of polytetrafluoroethylene as described in reference 6), incorporated two 0.4-μm-pore-size membrane filters and possessed an external sampling port which permitted multiple sampling of the inoculum without killing the rat. Chambers (one per rat; four rats in total were used) were inoculated with 0.5 ml of *S. epidermidis* 901 (5×10^3 CFU/ml) (grown in NB) in phosphate-buffered saline (pH 7.4), and 10^9 CFU/ml could be recovered after 48 h (6).

Cell wall and cytoplasmic membrane proteins were prepared as described previously (9) from staphylococci cultured in vivo and in vitro from experiments which were repeated on at least three separate occasions. Briefly, staphylococci (10^9 cells) were resuspended in 0.6 ml of digestion buffer (30% [wt/vol] raffinose, 1 mg of benzamidine per ml, and 0.5 mg of phenylmethylsulfonyl fluoride per ml in Tris-buffered saline [pH 7.4] containing 100 μg of lysostaphin) and incubated at 37°C for 60 min. Protoplasts were removed by centrifugation, and the supernatant containing the cell wall proteins was stored at −20°C until required. Cytoplasmic membranes were collected by centrifugation after sonication and lysis of the protoplast suspensions. Cell wall and membrane preparations were heated at 100°C in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 2-mercaptoethanol and loaded on SDS–12.5% polyacrylamide gels. After electrophoresis, gels were either fixed and stained with Coomassie blue or electrophoretically transferred to nitrocellulose membranes. The membranes were then probed with either (i) monospecific polyclonal antisera raised as described before (9) against the purified 32- and 36-kDa IRMPs (diluted 1:400), (ii) pooled preimmune rat sera (diluted 1:50) collected before inoculation of the chamber implant, or (iii) pooled rat sera (diluted 1:50) collected 21 days after inoculation of the chamber with *S. epidermidis*. Bound antibodies were detected with a protein A-peroxidase conjugate (Sigma), and reactive bands were visualized with a 25-μg/ml solution of 4-chloronaphthol containing 0.01% (vol/vol) hydrogen peroxide.

Cell wall and cytoplasmic membrane proteins were prepared from *S. epidermidis* 901 grown in either NB, NB plus EDDA, or HPD or after recovery without subculture from the peritoneal chamber and subjected to SDS-PAGE. Figure 1 shows that there was a marked reduction in the total number of cell wall-associated proteins in HPD- and in vivo-grown bacteria compared with staphylococci cultured in NB. The protein profile of in vivo-grown cells closely resembled that of *S. epidermidis* cultured in HPD, with major common proteins of 42, 54, and 70 kDa predominating, although a 27-kDa protein present in the in vivo-grown bacteria appeared to be repressed in the HPD-grown cells (compare lanes 3 and 4 of Fig. 1). The addition of the iron chelator EDDA to NB to induce iron-restricted conditions did not result in such major changes in the cell wall protein profiles of the NB-grown staphylococci, although a 39-kDa protein in the cell walls of NB-grown staphylococci was repressed not only in NB plus EDDA but also in HPD- and in vivo-grown cells (compare lane 1 with lanes 2 to 4 in Fig. 1).

The cytoplasmic membrane protein profiles of *S. epidermidis* grown under the different nutritional conditions also showed marked variations (Fig. 2A). In particular, proteins

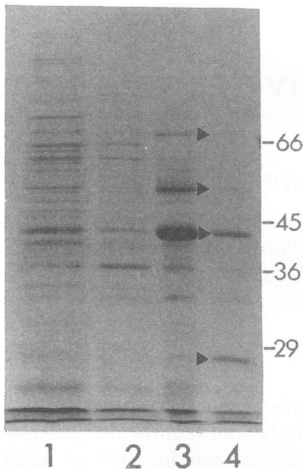

FIG. 1. SDS-PAGE of the cell wall proteins of *S. epidermidis* after growth in NB (lane 1), in NB plus EDDA (lane 2), in HPD (lane 3), and in vivo (lane 4). Arrowheads (lane 4) mark the positions of the 27-, 42-, 54-, and 70-kDa proteins (bottom to top). The molecular masses of marker proteins are indicated on the right in kilodaltons.

of 41 and 45 kDa were strongly expressed in HPD- and in vivo-grown bacteria but not in broth-grown cells. In addition, an 88-kDa protein present in NB-plus-EDDA-grown and in vivo-grown *S. epidermidis* appeared to be repressed in NB-grown and HPD-grown cells. Growth in vivo in the implanted chamber also resulted in the expression of the 32- and 36-kDa IRMPs, which were previously reported (9, 16) to be induced after growth in HPD (Fig. 2A). This finding was confirmed by probing the proteins derived from in vivo-grown staphylococci with a mixture of two monospecific antibodies raised against each IRMP (Fig. 2B).

We have previously shown that the IRMPs of *S. epidermidis* and other coagulase-negative staphylococcal species

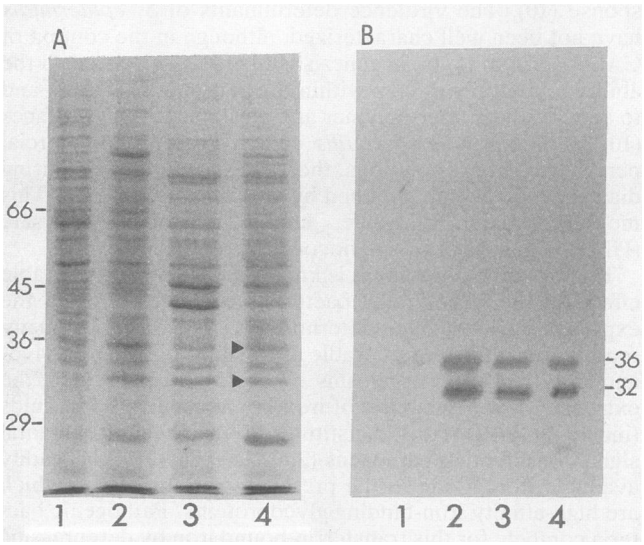

FIG. 2. (A) SDS-PAGE of the cytoplasmic membrane proteins of *S. epidermidis* after growth in NB (lanes 1), in NB plus EDDA (lanes 2), in HPD (lanes 3), and in vivo (lanes 4). Arrowheads mark the positions of the IRMPs in lane 4. The molecular masses of markers are indicated on the left in kilodaltons. (B) Immunoblot of the same samples probed with a mixture (1:1) of two monospecific antibodies raised in rabbits against the purified 32- and 36-kDa IRMPs.

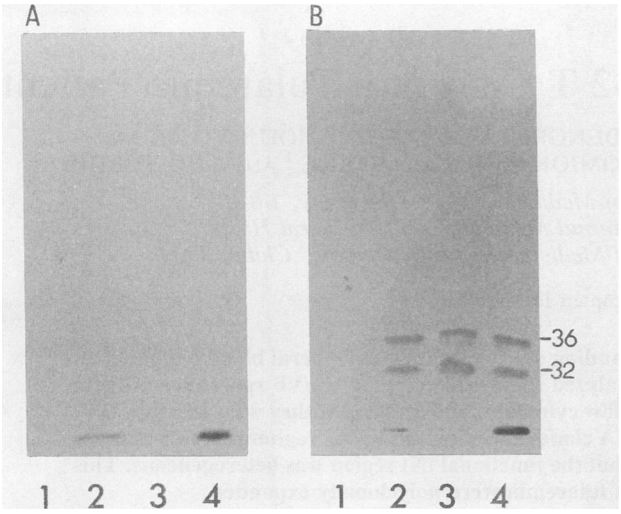

FIG. 3. Immunoblots showing reaction of antibodies with cytoplasmic membrane proteins in preimmune rat sera (A) and rat sera obtained 21 days after inoculation of the intraperitoneally implanted chambers with *S. epidermidis* (B). Cytoplasmic membranes were prepared from staphylococci grown in NB (lanes 1), in NB plus EDDA (lanes 2), in HPD (lanes 3), and in vivo (lanes 4). The molecular masses of the IRMPs are indicated on the right in kilodaltons.

are the immunodominant cell envelope protein antigens and that normal human sera as well as sera and HPD fluid from patients on CAPD contained antibodies directed against these proteins (9, 14, 16). To examine the humoral immune response mounted by the rats against *S. epidermidis* growing within the implanted peritoneal chambers, immunoblots of both cell wall and cytoplasmic membrane proteins were probed with preimmune rat sera taken before inoculation of the chamber or sera obtained 21 days after inoculation. Figure 3, which shows a Western blot (immunoblot) of the cytoplasmic membrane preparations, reveals a strong antibody response in the 21-day postinfection sera to the 32- and 36-kDa IRMPs only. Apart from the 27-kDa antigen noted in the in vivo-grown staphylococcal cell walls (Fig. 1), no antibody reaction with cell wall proteins in the preimmune sera or 21-day sera was observed, although the low level of IRMPs contaminating the cell wall fraction (9) reacted weakly (data not shown).

In conclusion, the present study clearly demonstrates that (i) *S. epidermidis* multiplying in vivo within the rat peritoneum grows under iron-restricted conditions and (ii) the IRMPs are the immunodominant cell envelope protein antigens. Further work is under way to determine whether the IRMPs are components of a high-affinity iron transport system which is essential for growth in vivo. The relatively poor immunogenicity of *S. epidermidis* cell wall proteins previously noted in other studies (5, 9, 14) also appears to be borne out by the lack of an antibody response to these proteins observed in the present study. This phenomenon, in conjunction with the observed reduction in the total number of cell wall proteins, could conceivably contribute to the avoidance of host defense mechanisms within the dialyzed peritoneum. In addition, the similarity between the cell envelope protein profiles of in vivo chamber-grown and in vitro HPD-grown staphylococci strengthens previous suggestions that the latter growth environment represents an excellent in vitro culture medium for mimicking conditions within the dialyzed peritoneum.

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