Effect of Growth Conditions on Expression and Antigenicity of Staphylococcus epidermidis RP62A Cell Envelope Proteins

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Received 8 December 1992/Accepted 8 February 1993

Staphylococcus epidermidis RP62A (ATCC 35984) was grown in tryptic soy broth (TSB), iron-depleted TSB (TSB–Fe), iron-reconstituted TSB–Fe (TSB+Fe), a chemically defined medium, and fetal calf serum (FCS) and on silastic disks in chambers that were sutured to the pig peritoneal wall. Bacterial cell wall proteins were extracted by digestion with recombinant lysostaphin, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and detected by silver staining. Cell wall proteins from TSB-, chemically defined medium-, or FCS-grown cells had a complex profile of greater than 25 protein bands spanning the full molecular mass range. By contrast, a digest obtained from in vivo-grown cells had only five major proteins of 40 kDa or greater. Proteins of 130 and 106 kDa were present in the cell envelopes of TSB–Fe and in vivo-grown cells but not in those grown in TSB or TSB+Fe. A 43-kDa protein expressed by in vitro-grown cells and 52- and 96-kDa proteins expressed by in vivo-grown cells reacted with antisera from pigs with the chamber implants and from catheterized, paracatheter-inoculated pigs but not with hyperimmune sera from pigs immunized with TSB-grown cells. The data indicate that S. epidermidis, growing under in vivo conditions, expresses antigens distinct from those that are grown in vitro.

Staphylococcus epidermidis, a member of the permanent cutaneous microflora of humans, is emerging as a major pathogen associated with chronic infections of implanted foreign devices. Although the precise mechanism of infection is unclear, the organism appears to be exquisitely suited for adherence to biomaterial surfaces, perhaps mediated by the production of an exopolysaccharide adhesin (27) or by surface protein (16). Indeed, a correlation between slime production and virulence of S. epidermidis strains has been made (32). Furthermore, direct observation of a variety of biomaterials has invariably demonstrated surface-associated slime-enclosed microcolonies. It has been suggested that a biofilm mode of growth may be, in part, responsible for the chronicity of these infections by protecting the cells from the action of antibiotics or engulfment by leukocytes.

With the exception of slime, there have been few reports of other S. epidermidis virulence factors. Patrick et al. (23) examined cell surface proteins of various S. epidermidis clinical isolates in order to define potential virulence factors and/or proteins that might be useful in serodiagnostic analysis. Under constant conditions, they were able to identify common antigenic proteins. However, Smith et al. (25) grew S. epidermidis isolates, from continuous ambulatory peritoneal dialysis patients, in nutrient broth or spent human peritoneal dialysate (HPD) and found differences in cell envelope protein expression. They have also reported that growing S. epidermidis in 5% carbon dioxide alters its cell surface chemistry (10).

It is now firmly established that pathogenic bacteria respond to external stimuli, altering their phenotype to better adapt to different microenvironments (13). Survival of the bacterium in the host depends on the interaction between cell surfaces (15), and their characteristics are subject to phenotypic change (2, 5, 12, 25). Since the pathogenesis of catheter-associated infection involves adherent bacterial growth, it is important to determine cell surface protein and antigen composition of the biofilm cells under in vivo conditions. In order to do this we used catheterized pigs or intraperitoneal (i.p.) devices infected with an archetypal catheter-associated S. epidermidis strain, RP62A. The data indicate that antisera developed against the bacteria in these pigs have different antigenic specificities than conventionally raised antistaphylococcal antiserum.

MATERIALS AND METHODS

Organism and growth conditions. S. epidermidis RP62A (ATCC 35984) was used. It was isolated from the blood of a patient with intravascular catheter-related sepsis (8). The organism was revived and stored at −70°C on tryptic soy agar slants (Difco Laboratories, Detroit, Mich.). When required for use, the slants were thawed and the organism was streaked on tryptic soy agar plates. Isolated colonies were identified by a combination of a biotype (ApiStaph API Laboratory Products Ltd., St. Laurent, Quebec, Canada) and an antibiogram pattern.

For in vitro growth, single colonies were transferred to a 100-ml volume of tryptic soy broth (TSB; Difco), iron-depleted TSB (TSB–Fe), iron-reconstituted TSB–Fe (TSB+Fe), chemically defined medium (CDM) (17), or 20% (vol/vol) fetal calf serum (FCS; Sigma Chemical Co., St. Louis, Mo.). TSB–Fe was made by treating double-strength TSB with Chelex-100 cation-exchange resin (Bio-Rad) as described by Domingue and coworkers (11, 12). TSB–Fe ([Fe] < 1 μM) and TSB+Fe were reconstituted with vitamin and mineral mixtures in order to support growth (12). The concentration of iron in TSB+Fe medium was 20 μM.

In vivo growth. Bacterial cells were grown in vivo on silastic (Dow Corning Corp., Midland, Mich.) implant de-
vices, as described by Ward et al. (28), which were sterilized with ethylene oxide and seeded by immersing them in a S. epidermidis culture in TSB medium overnight. They were washed by dipping them in sterile phosphate-buffered saline (PBS) and transferred individually to gas-sterilized acrylic implant chambers equipped with 0.45-μm-pore-size filters on both ends to prevent the migration of phagocytes into the chamber (21, 28). The chambers were filled with ca. 3 ml of PBS to facilitate growth. A mid-line incision was made in two anesthetized pigs and four precolonized chambers were sewn to the peritoneal wall of each pig and left in situ for 21 days. The pigs were then euthanized by a barbiturate overdose, and the devices were aseptically removed from the chambers and immediately placed in 50 ml of sterile PBS. Bacteria were removed from the disks by vortexing vigorously for 30 s and then by 5 min of ultrasonication. The cells were enumerated by plate count and identified by biotype and antibiogram.

Animals. Domestic, female, weaner pigs weighing 10 to 15 kg were used. Care, housing, and treatment of animals were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals (Canadian Council on Animal Care, Ottawa, Ontario, Canada).

Modified whole-cell lysates. Cell wall proteins were extracted by the method of Cheung and Fischetti (5) with slight modifications. The cells were harvested at late logarithmic phase by centrifugation (12,000 × g, 10 min, 4°C), washed three times in ice-cold PBS (pH 7.4), and resuspended to an optical density at 540 nm of 1.0 (10^6 CFU/ml). The volume was noted, and the cells were resuspended in 0.98 ml of phosphate-buffered digestion buffer containing 30% (wt/vol) sucrose in 0.05 M Tris (pH 8.0; Sigma) with 0.145 M NaCl. A 0.01-ml volume of a 100-μl/m suspension of DNase (Sigma) was added. The enzymatic reaction was initiated by adding 0.01 ml of a 1-mg/ml suspension of recombinant lysozyme (Applied Microbiology Inc., New York, N.Y.). Protease activity was inhibited by 1 mg of phenylmethylsulfonyl fluoride (Sigma) per ml and 1 mg of benzamidine (Sigma) per ml. The mixture was incubated at 37°C for 2 h, and the reaction was stopped by centrifuging out the protoplasts at 8,000 × g for 10 min. The supernatant was stored at −70°C until required. In some cases, the bacterial membranes were isolated by breaking the protoplasts by ultrasonication with two bursts of 30 s. Membranes were harvested at 100,000 × g for 30 min (25).

Immune sera. Antistaphylococcal antiserum were raised by intramuscularly (i.m.) immunizing two pigs with 10^9 CFU of live, TSB-grown S. epidermidis. The cells were washed three times in PBS and emulsified in Freund complete adjuvant (Difco) before injection. A booster shot was given 14 days later. Antisera were collected from these pigs at 28 days postimmunization and from the pigs with the i.p. chambers on the day of euthanasia.

Peritoneal dialysis catheter-associated infection was modelled by surgically installing catheters made by adhering Dacron velour to a 5-cm portion of a 60-cm piece of silastic (Dow Corning) tubing (interior diameter, 0.265 cm; outer diameter, 0.488 cm) 15 cm from the i.p. (proximal) end. The catheter was implanted by a paramedian incision through the peritoneal wall. The proximal end was inserted into the peritoneal space ventrally and cranially towards the liver. The Dacron cuff was sutured to the muscle and parietal peritoneum. A subcutaneous tunnel was formed dorsally by blunt dissection through which the catheter was passed. It was then exteriorized by a stab incision and sewn to the subcutaneous tissue by a purse string. Live, TSB-grown S. epidermidis (10^9 CFU) was inoculated between the exit site tissue and the catheter (pericatheterly), mimicking an exit site infection. At 21 days postsurgery, sera from six pigs were sampled. In all cases antiserum was pooled, and the titre was quantified by an enzyme-linked immunosorbent assay with formalin-killed whole cells as antigen.

SDS-PAGE. The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) method of Laemmli (19) was used with 12 or 9.0% (vol/vol) acrylamide gels. The samples were heated at 37°C for 15 min prior to loading. Molecular mass standards from 94 to 14 kDa or 20 to 29 kDa were from Pharmacia (Uppsala, Sweden) and Sigma, respectively. The gels were stained with the Bio-Rad (Richmond, Calif.) silver stain kit. Protein concentrations were measured by the method of Bradford (4). Membrane samples were boiled in 0.5 M NaOH to solublize the proteins. Standard curves were prepared with bovine serum albumin (Sigma) and the appropriate concentration of NaOH.

Western immunoblotting. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) from polyacrylamide gels by the method of Towbin as modified by Matsudaia (22). The immobilized staphylococcal proteins were incubated with porcine antiserum diluted 1:50 in Tris-buffered saline (pH 7.4) with 0.5% (wt/vol) Tween 20 (TBS-Tween). Antistaphylococcal antibodies were detected by a 1:1,000 dilution in TBS-Tween of goat-raised, phosphatase-labeled, affinity-purified, anti-pig immunoglobulin G antibody (Kirkegaard and Perry, Gaithersburg, Md.). Bands were detected by incubation in a substrate mixture consisting of 2 mg of nitroblue tetrazolium (Sigma) in 20 ml of Tris (pH 8.8) with 4 mM MgCl2 and 1 mg of 5-bromo-4-chloro-3-indoly phosphate (Sigma) dissolved in dimethyl sulfoxide. Immunoblots with preimmune sera and/or anti-pig immunoglobulin G were negative.

RESULTS

Cell wall protein preparations from S. epidermidis RP62A grown in vivo and in vitro in various media. Figure 1 is an SDS–12% (vol/vol) polyacrylamide gel which compares du-
plicate lanes of cell wall proteins from cells grown in TSB (lanes 1 and 2), CDM (lanes 3 and 4), and FCS (lanes 5 and 6) and in vivo (lanes 7 and 8). Proteins patterns differ markedly between the duplicate lanes. TSB-grown cells expressed three predominant bands of 40, 43, and 45 kDa, whereas CDM- and FCS-grown cells expressed these proteins and another dominant band at 52 kDa. CDM-grown cells express more distinct low-molecular-mass minor bands. The in vivo-grown cells produced only four major bands, all above 40 kDa (Fig. 1, lanes 7 and 8; see Fig. 4, lane 7). Bands of 43 and 52 kDa, present in CDM- and FCS-grown cells, are also expressed in vivo. The predominant protein of in vivo cells was at 67 kDa. However, this protein migrated to the same point as purified pig albumin and reacted with affinity-purified, anti-pig albumin antiserum (data not shown). For in vitro cultures, the experiments were performed in triplicate, with different isolated colonies from tryptic soy agar plates to inoculate the various media. For in vivo growth, two populations of cells with different starting colonies were used to grow the bacteria for colonization of the silastic devices. In each case, the SDS-PAGE profiles were stable by showing the identical patterns within the given environmental condition (data not shown).

Antigenicity of cell wall proteins. Figure 2 is an immunoblot demonstrating the antigenicity of the cell wall proteins from Fig. 1 against antisera from pigs with i.p. chamber implants (Fig. 2A) versus pigs that received i.m. injection (Fig. 2B). The antisera from pigs with i.p. implants weakly recognized several high-molecular-weight bands and strongly recognized a 43-kDa protein expressed by in vitro-grown cells and a 52-kDa protein produced by in vivo-grown cells. In contrast, the antisera from i.m.-injected pigs did not recognize any in vitro-grown bacterial proteins greater than 38 kDa, and the 52-kDa in vivo-expressed protein was only very weakly antigenic. The banding patterns of proteins between 30 and 40 kDa in both blots are similar. All share major antigens at 32 and 36 kDa and a minor 39-kDa band in TSB-grown cells.

In order to determine the immune responses of pigs with catheter-associated infection, antisera from these pigs were reacted against blotted cell wall protein preparations, as in Fig. 2 (Fig. 3). The antigenic patterns against in vitro-grown proteins were identical. The 32-, 36-, and 39-kDa proteins are antigenic. The 43-kDa protein that reacted strongly with sessile antisera was weakly antigenic in the catheterized pigs. The in vivo-expressed 52-kDa protein, as with the sessile antisera, was strongly antigenic.

The effect of iron limitation and in vivo conditions on the expression of cell envelope proteins of S. epidermidis RP62A. To determine the effect of iron limitation and/or in vivo environment on the expression of envelope proteins, cell envelope preparations were made from S. epidermidis grown in TSB, TSB–Fe, or TSB+Fe or in vivo (Fig. 4). S. epidermidis was reported to express an iron-repressible protein of 130 kDa when grown in spent peritoneal dialysate fluid (25). Therefore, a 9% acrylamide gel was employed in order to explore this molecular mass range (Fig. 4). Proteins of 130 and 106 kDa were expressed in the cell walls and membranes, respectively, of in vivo-grown cells and in TSB–Fe envelopes (Fig. 4, lanes 3, 4, 7, and 8). A diffuse band(s), unique to in vivo-grown cell envelopes at 96 kDa, is also indicated (Fig. 4, lanes 7 and 8). Two bands of 36 and 32 kDa were predominant in the membranes of in vivo-grown cells and were also expressed in TSB–Fe and TSB+Fe cell membranes (Fig. 4, lanes 4, 6, and 8). As in Fig. 1, the pig albumin band is present at 67 kDa (Fig. 4, lanes 7 and 8, open arrowheads). Table 1 provides a summary of unique cell envelope proteins produced under iron limitation or in vivo.

Antigenicity of cell envelope proteins. Figure 5 shows immunoblots of cell wall and cell membrane proteins from TSB–Fe (lanes 1 and 2) and TSB+Fe (lanes 3 and 4) and in vivo (lanes 5 and 6) reacted against antisera from pigs with i.p. chamber implants (Fig. 5A) or antisera from pigs that were injected i.m. (Fig. 5B). Open arrowheads indicate shared antigenic proteins from in vitro-grown cells at 117,
with antisera from pigs from ular mass arrowheads.

Growth conditions and bacterial major proteins are mimicking epidermidis S. major three arrows). These predominant proteins are evident when antigens from i.m.-injected pigs. Figure 6 shows that antigens from the i.m.-injected pigs with catheter-associated infections recognized the common antigens listed above (Fig. 6, open arrows) as well as the 43-, 52-, and 96-kDa proteins, thereby mimicking the antigenic pattern when antigens were used from pigs with i.p. chambers. Table 2 provides a summary of major bacterial antigens recognized under various in vivo conditions.

**DISCUSSION**

It is evident from Fig. 1 that growth conditions have a profound effect on the expression of lysostaphin-extractable cell wall proteins in S. epidermidis RP62A. In TSB-grown cells, three predominant proteins of 40, 43 and 45 kDa are present. This is consistent with the work of Smith and coworkers, who reported that 42-, 48-, and 52-kDa predominant cell wall proteins were expressed in a clinical isolate of S. epidermidis grown in HPD (25). Patrick et al. also found three major cell wall proteins of 37, 41, and 51 kDa in TSB-grown S. epidermidis (23). CDM- and FCS-grown cells produce these predominant proteins as well as a fourth, 52-kDa protein. Sessile cells grown on silastic disks in the pig peritoneum resulted in the repression of many cell wall proteins. Repression of cell wall proteins was also found in HPD-grown cells compared with that in cells grown in nutrient broth (25). Proteins of 47 and 62 kDa as well as a predominant 67-kDa protein were expressed. This protein was found to be albumin, which presumably bound to the bacterium in vivo. The acrylic chamber caused localized chronic inflammation of the peritoneal membrane and subsequent extravasation of blood proteins into the peritoneal cavity. Proteins were free to diffuse in and out of the chamber.

Christensen et al. (7) have demonstrated phase variation of certain phenotypes (e.g., slime production) of RP62A when grown on Memphis agar. In this study, single colonies were used to inoculate different media; therefore, it is possible that the differences in cell wall protein profiles were due to different starting phenotypes and not to environmental conditions. However, it is not known whether phase variation of S. epidermidis extends to cellular protein expression. Whole-cell SDS-PAGE profiles of various coagulase-negative staphylococci, including S. epidermidis, have shown greater than 95% average intraspecies similarity, whereas interspecies similarity ranged between 41.5 and 65% (26). Within a given S. epidermidis strain, SDS-PAGE profiles appear identical even after repeated subculture (23) or, in this study, when different starting colonies were used. These studies suggest that if a strain of coagulase-negative

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**TABLE 1. Molecular masses of unique cell envelope proteins expressed by RP62A**

<table>
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<tr>
<th>Condition</th>
<th>Proteins (kDa)</th>
<th>Cell wall</th>
<th>Membrane</th>
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<tbody>
<tr>
<td>TSB-Fe</td>
<td>130, 106</td>
<td>36, 32</td>
<td></td>
</tr>
<tr>
<td>In vivo</td>
<td>130, 96</td>
<td>106, 96, 36, 32</td>
<td></td>
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</table>

* The proteins are demarcated by arrowheads in Fig. 4.
staphylococci, grown under identical conditions, were to show significantly different electrophoretic protein profiles, it would indicate mixed cultures through contamination rather than the outgrowth of phenotypic variants.

In vivo growth results in cells that are metabolically and phenotypically distinct from their planktonic counterparts (9, 30). There is evidence that in vivo-grown cells may switch off genes not required for cell viability. For example, *Enterococcus faecalis* protein preparations were blotted against hyperimmune sera produced against serum- or nutrient broth-grown cells. When the immunoblot patterns were compared, the antisemur produced against serum-grown cells gave a much less complex blot (1). Moreover, patients with chronic infections, characterized by the presence of tissue-associated bacterial biofilms, produce large amounts of antibodies to a small number of proteins. These same patients produce antibodies to a far greater array of bacterial proteins when undergoing acute exacerbations of septicemia (20). Unfortunately, immunological methods of determining in vivo cell wall expression cannot distinguish whether a given protein is not expressed or simply masked from the immune system. Masking of antigens from the immune system can occur through the production of nonantigenic extracellular structures (e.g., glycopellic acid), by binding of host proteins to the bacterial cells or the lesser ability of the ameboid phagocytes to opsonize sessile, slime-enclosed bacteria (18). The implanted chambers allow direct retrieval of in vivo, sessile cells for determination of macromolecular composition without subculture. The data presented here support an overall repression of protein expression in vivo.

The in vivo environment may also induce the expression of proteins, such as siderophore receptors, in the iron-limited milieu. Wilcox et al. (29) reported the induction of 32- and 36-kDa cytoplasmic proteins in HPD-grown *S. epidermidis*. Since the proteins were iron repressible, the authors speculated that they are part of a siderophore uptake system. We demonstrate that these proteins were highly expressed in in vivo-grown cells and were not expressed in TSB-grown cells, although these proteins were not derepressed to the same level when grown in TSB–Fe and were not completely repressed when iron was added to the medium. It is therefore possible that derepression of the proteins is triggered by one or more undefined in vivo factors found in peritoneal fluid. This derepression may be only partially reversed by the addition of iron. Silver staining was required to show these differences.

A cell wall-associated 130-kDa protein and a 106-kDa cell envelope protein were induced in cells grown in vivo or under iron-restricted conditions. This is consistent with reports of 130 (25)- and 98 (31)-kDa iron-repressible proteins in HPD-grown *S. epidermidis*. The 130-kDa protein was also reported to be cell wall associated, leading to speculation that this is an adhesin (25). In this study, the protein was also expressed in TSB–Fe planktonic cell envelopes, suggesting that it is more likely involved in iron metabolism. This protein does not appear to be antigenic in this system. Bands at 130 kDa from HPD-grown *S. epidermidis* cell wall protein were antigenic in a blot reacted against HPD fluid but not with pooled normal human sera (25). A 96-kDa protein unique to in vivo-grown cells is also present. Since it appears to be predominantly cell wall associated, this is also a candidate for a staphylococcal adhesin. This possibility is being investigated.

Immunoblot of the in vivo-grown cell wall proteins reacted against the three types of antisera displayed an identical background pattern of 117, 93, 60, 36, and 32 kDa. However, an in vitro-expressed protein of 43 kDa and 52- and 96-kDa in vivo-expressed proteins reacted with both antisera from the pigs with peritoneal chamber implants and pericatheter-infected pigs but not with antisera from i.m.- inoculated pigs. With antisera from a chronic, *Staphylococcus aureus* osteomyelitis rat model, immunoblot assays against in vivo chamber- and sessile TSB-grown cellular protein indicated a 210-kDa protein that was absent with both serum- and planktonic TSB-grown antigen (24). Antisera from patients with chronic *E. faecalis* endocarditis reacted against certain in vivo chamber-grown antigens that were not expressed in CDM-grown cells (21). Since the biofilm mode of growth has been implicated in these chronic infections, this indicates that adherence may be a factor which determines the complete antigenicity of in vivo-grown cells. Adherent growth has been shown to directly influence the nutritional microenvironment of bacteria by facilitating the concentration and transportation of nutrients to the cell surface (14). Therefore, the true antigenic complement of bacteria in chronic infections may be a reflection of both mode of growth and in vivo nutritional milieu

![FIG. 6. Immunoblot of cell wall proteins (lanes 1, 3, and 5) and membranes (lanes 2, 4, and 6) separated as in Fig. 4, of TSB–Fe (lanes 1 and 2), TSB–Fe (lanes 3 and 4), and in vivo (lanes 5 and 6)-grown cells. The proteins were reacted against antisemur from the pig with induced catheter-associated infection. Open arrowheads indicate major in vitro-expressed antigens at 117, 93, 60, 36, and 32 kDa (lanes 1 to 4). Closed arrowheads point to the in vivo-produced 52- and 96-kDa proteins (lanes 5 and 6). Molecular mass markers are indicated on the left.](http://iai.asm.org/)

**TABLE 2. Molecular masses of major RP62A antigens recognized from various sources of antisemur in relation to the source of antigen**

<table>
<thead>
<tr>
<th>Source of antisemur</th>
<th>Sources (kDa) of antigen</th>
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<tbody>
<tr>
<td></td>
<td>In vitro</td>
</tr>
<tr>
<td>i.m.</td>
<td>117, 93, 60, 36, 32</td>
</tr>
<tr>
<td>Pericatheter</td>
<td>117, 93, 60, 43, 36, 32</td>
</tr>
<tr>
<td>i.p. chamber</td>
<td>117, 93, 60, 43, 36, 32</td>
</tr>
</tbody>
</table>

* The antigens are demarcated by arrowheads in Fig. 5 and 6.
* Antisera were collected from pigs infected with bacteria at the sites listed.
* Antigen from bacteria grown in TSB–Fe and TSB–Fe media.
* Antigen from cells scraped off the silastic device in the i.p. chamber.
working in concert. To determine the relative importance of each factor requires the advent of noncolonizable implant material.

As an aid to epidemiological studies, a cell wall protein classification scheme for TSB-grown *S. epidermidis* has been proposed (23). Since a biomaterial implant is an absolute requirement for infectivity of this organism (6), perhaps a scheme with surface-adherent cells should be developed. These data suggest that when *S. epidermidis* is allowed to grow, in vivo, on an inert surface, it expresses particular surface proteins which become antigenic. The unique 43- and 52-kDa proteins could not be unequivocally identified by SDS-PAGE, although the 96-kDa protein could (Fig. 4). Current experiments are under way to determine their identity and possible biochemical and antigen relatedness. Understanding these antigens may be important in elucidation of the mechanism of avoidance of immune clearance of *S. epidermidis*.

**ACKNOWLEDGMENTS**

This research is supported by the Natural Science and Engineering Research Council of Canada. K.P.M. is the recipient of a studentship from the Medical Research Council of Canada and a research allowance from the Alberta Heritage Foundation for Medical Research (AHFMR). D.W.M. holds an AHFMR postdoctoral fellowship. We thank G. Domingue and M. Power for helpful discussions.

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