Molecular Cloning of a 32-Kilodalton Lipoprotein Component of a Novel Iron-Regulated *Staphylococcus epidermidis* ABC Transporter

ALAN COCKAYNE,[1,2,3]* PHILIP J. HILL,[1,2,3]† NICK B. L. POWELL,[1,2]
KEITH BISHOP,[1,2] CATE SIMS,[1,3] AND PAUL WILLIAMS[1,2,3]

*Institute of Infections and Immunity,[1] School of Clinical Laboratory Sciences,[2] and
School of Pharmaceutical Sciences,[3] University of Nottingham,
Nottingham NG7 2UH, United Kingdom*

Our previous studies identified two iron-regulated cytoplasmic membrane proteins of 32 and 36 kDa expressed by both *Staphylococcus epidermidis* and *Staphylococcus aureus*. In this study we show by Triton X-114 phase partitioning and tritiated palmitic acid labelling that these proteins are lipoproteins which are anchored into the cytoplasmic membrane by their lipid-modified N termini. In common with those of some other gram-positive bacteria, these highly immunogenic lipoproteins were released from the bacterial cell into the culture supernatants, with release being promoted by growth of the bacteria under iron-restricted conditions. Immunoelectron microscopy with a monospecific rabbit antiserum to the 32-kDa *S. epidermidis* lipoprotein showed that the majority of the antigen was distributed throughout the staphylococcal cell wall. Only minor quantities were detected in the cytoplasmic membrane, and exposure of the lipoprotein on the bacterial surface was minimal. A monoclonal antibody raised to the 32-kDa lipoprotein of *S. aureus* was used in immunoblotting studies to investigate the conservation of this antigen among a variety of staphylococci. The monoclonal antibody reacted with polypeptides of 32 kDa in *S. epidermidis* and *S. aureus* and of 40 kDa in *Staphylococcus hominis*. No reactivity was detected with *Staphylococcus lugdunensis*, *Staphylococcus cohni*, or *Staphylococcus haemolyticus*. The gene encoding the 32-kDa lipoprotein from *S. epidermidis* has been isolated from a Lambda Zap II genomic DNA library and found to be a component of an iron-regulated operon encoding a novel ABC-type transporter. The operon contains three genes, designated *sitA*, *-B*, and *-C*, encoding an ATPase, a cytoplasmic membrane protein, and the 32-kDa lipoprotein, respectively. SitC shows significant homology both with a number of bacterial adhesins, including FimA of *Streptococcus parasanguis* and ScaA of *Streptococcus gordonii*, and with lipoproteins of a recently described family of ABC transporters with proven or putative metal ion transport functions. Although the solute specificity of this novel transporter has not yet been determined, we speculate that it may be involved in either siderophore- or transferrin-mediated iron uptake in *S. epidermidis*.

Staphylococci respond to iron deprivation in vitro and in vivo by increasing the expression of a number of iron-regulated proteins (24, 28, 36, 41). These include a 42-kDa cell wall-associated transferrin binding protein and two cytoplasmic membrane proteins of 32 and 36 kDa whose functions are at present unknown (28, 36). The association of the latter proteins with the cytoplasmic membrane is based on cell fractionation data, but the mechanism of anchorage of these proteins to the cytoplasmic membrane is also currently unclear. In other bacteria, some cytoplasmic membrane proteins are integral to the membrane and are held in place by hydrophobic membrane-spanning regions or hydrophobic N- or C-terminal anchors (31). Other membrane-associated proteins have been shown to be lipoproteins, and these are tethered to the outer surface of the membrane by their lipid-modified N termini (17, 38). Functionally, both groups of proteins may be components of ATP binding cassette (ABC)-type transporters involved in solute acquisition (13, 39). These multicomponent transporters have common structural and organizational features, and functions in addition to solute transport have recently been attributed to some ABC transporter lipoproteins. Studies with gram-positive bacteria show that the lipoproteins SarA and SsaB mediate adherence of *Streptococcus gordonii* and *Streptococcus sanguis*, respectively, to salivary pellicle components (15, 22, 29). FimA from *Streptococcus parasanguis* is involved in attachment of the bacterium to fibrin clots in a rat endocarditis model and to platelet fibrin matrix clots in vitro (14, 40). These functional studies and other antibody binding studies support the surface location of these streptococcal lipoproteins (20, 21, 40). The *Enterococcus faecalis* lipoprotein EfaA also shows sequence homology with streptococcal adhesins, including FimA, SsaB, and ScaA, indicating that EfaA may also be a surface-exposed adhesin (25).

In contrast to the case for other gram-positive organisms, ABC transporters in the staphylococci have been little studied. Proteins that may function as components of an ABC transporter involved in erythromycin resistance have been detected in *Staphylococcus epidermidis* (32), but this system has not been fully characterized and no homologies with such systems in other bacteria have yet been reported. ABC exporters are implicated in the secretion of the lantibiotics epidermin (13) and Pep5 (26) by *S. epidermidis* and of gallidermin (13) by *Staphylococcus gallidermidis*. DNA sequence analysis has also identified an ABC transporter of unknown function in *Staph-*

* Corresponding author. Mailing address: Institute of Infections and Immunity, University Hospital, Queen's Medical Centre, Nottingham NG7 2UH, United Kingdom. Phone: 0115 9249924, ext. 42445. Fax: 0115 970 9923. E-mail: mrzac@mrn1.nottingham.ac.uk.

† Present address: Department of Applied Biochemistry and Food Science, School of Biology, Sutton Bonington Campus, University of Nottingham, Leicestershire LE12 5RD, United Kingdom.

ylococcus aureus (12). Currently the only well-studied staphylococcal lipoprotein is the β-lactamase of *S. aureus* (2, 30), which is found both in a membrane-bound form and in the bacterial culture supernatant (a common finding for other gram-positive lipoproteins [38]). There is currently no published information on transport systems involved in iron acquisition by the staphylococci.

Our ongoing interest in staphylococcal iron uptake mechanisms has led us to further characterize the role of the staphylococcal 32- and 36-kDa iron-regulated cytoplasmic membrane proteins as possible components of iron transport systems. This paper describes the identification of these iron-regulated proteins as lipoproteins and the molecular cloning of the 32-kDa lipoprotein from *S. epidermidis*, which sequence analysis reveals to be a component of a novel ABC-type transporter. Immunoelectron microscopy has also been used to confirm the cellular location of this lipoprotein in *S. epidermidis*, and immunoblotting has been used to investigate the conservation of these lipoproteins among other staphylococcal species.

MATERIALS AND METHODS

Staphylococcal strains and growth conditions. *S. aureus*, *S. epidermidis*, *Staphylococcus hominis*, *Staphylococcus cohni*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, and *Staphylococcus haemolyticus* clinical isolates were obtained from the University and City Hospital NHS Trusts, Nottingham, United Kingdom. *S. aureus* BB (originally isolated from a case of bovine mastitis [8]) and 8325-4 were provided by J. P. Arbuthnott. Strains were maintained by regular subculture on horse blood agar.

For broth culture, strains were grown statically for 18 h at 37°C in RPMI 1640 tissue culture medium containing 2 mg of NaHCO$_3$ per ml. Cultures were incubated in 5% CO$_2$ in air, and where indicated the medium was supplemented with 20 μM Fe$_2$(SO$_4$)$_3$ to produce iron-rich growth conditions.

Polyclonal and monoclonal antibody production. Anti-*S. aureus* BB wall antibodies were raised in adult female BALB/c mice. The cell wall extract was prepared by digestion of whole bacterial cells, grown under iron-restricted conditions, with lysostaphin in the presence of 30% (wt/vol) raffinose (36, 41). Mice were bled 2 weeks after the third immunization. Spleens recovered from these mice were also used to generate hybridomas by fusion with the myeloma cell line NS0 by standard methods. Hybridomas secreting antistaphylococcal antibodies were selected by indirect enzyme-linked immunosorbent assay with *S. aureus* BB wall extract as the antigen, and antigen specificity was confirmed by immunoblotting.

Rabbit monospecific antisera raised against the *S. epidermidis* 32- and 36-kDa iron-regulated cytoplasmic membrane proteins were available from earlier studies (36, 41).

Immunoelectron microscopy. *S. epidermidis* 901 was grown overnight in RPMI 1640. Bacteria were pelleted and fixed by resuspension in 1% (vol/vol) gluteraldehyde in phosphate-buffered saline, pH 7.4 (PBS) at room temperature for 2 h. Fixed bacteria were washed three times in PBS and dehydrated in a graded series of alcohol solutions (25 to 100% [vol/vol] in H$_2$O). Bacterial pellets were then embedded in Lowicryl K4M resin (Agar Scientific, Essex, United Kingdom) by using UV light polymerization, and thin sections were prepared with a Reichert OMU3 ultramicrotome. Sections were transferred to carbon-coated copper grids, and nonspecific binding sites were blocked by incubation with 3% (wt/vol) bovine serum albumin (BSA) in PBS for 30 min at room temperature. Grids were then incubated with either a 1/10 dilution (in PBS plus 1% [wt/vol] BSA) of polyclonal monospecific rabbit antiserum to the *S. epidermidis* 32-kDa lipoprotein or a similar dilution of rabbit serum from a nonimmune animal for 1 h at room temperature. Grids were then washed in PBS and further incubated with a 1/20 dilution (in PBS plus 1% [wt/vol] BSA) of 15-nm-diameter-gold-conjugated protein A (Biocell) for 30 min at room temperature. Following washing in PBS, grids were poststained with 4% (wt/vol) uranyl acetate and lead citrate in H$_2$O and examined in a Jeol Jem100C transmission electron microscope at 80 kV.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) and immunoblotting. All samples were solubilized by being boiled in Laemmli sample buffer (23) for 5 min. Polypeptides were separated by electrophoresis with a 4% (wt/vol) acrylamide stacking gel and a 10% (wt/vol) resolving gel in a Bio-Rad Mini Protean II gel apparatus as previously described (3). Where indicated, gels were stained with silver according to the method of Wray et al. (42). For immunoblotting, polypeptides were transferred to BioTrace NT membranes (Gelman); this was followed by blocking, incubation with primary antibody (a 1/500 dilution overnight for polyclonal antisera or a 1/2 dilution overnight for monoclonal antibodies) and conjugate (a 1/2,000 dilution of antirabbit, antirat, or antimouse peroxidase conjugates for 4 h), and detection of bound antibody as previously described (3).

Triton X-114 extraction. Triton X-114 extractions were performed according to the method of Bordier (6) with minor modifications. Where appropriate, staphylococci were first digested with 100 μl of lysostaphin (80 μg/ml in PBS) for 15 min at 37°C, cooled on ice, and sonicated twice for 30 s at 8 mA on an MSE Soniprep sonicator fitted with a 3-mm-diameter probe. PBS (800 μl) and 10% (vol/vol) Triton X-114 (100 μl in PBS) were then added to each tube, and the tubes were then incubated at 4°C for 2 h. After incubation at 4°C, bacterial preparations were centrifuged (13,000 × g for 10 min at 4°C) to pellet insoluble debris. Supernatants were transferred to fresh tubes and incubated at 37°C for 30 min to allow phase separation. The detergent phase and associated proteins were then pelleted by centrifugation at room temperature, and the aqueous phase was removed and discarded. The detergent pellet was washed once in 1 ml of PBS at 4°C for 1 h and repelleted following incubation at 37°C for 30 min.

Lipoproteins present in culture supernatants were also extracted with Triton X-114. One hundred microliters of 10% (vol/vol) Triton X-114 in PBS was added to 5 ml of filter-sterilized culture supernatant, which was then processed as described above.

All Triton X-114 pellets were diluted 1:1 with H$_2$O prior to solubilization for SDS-PAGE.

[^{3}H]palmitic acid labelling of staphylococcal lipoproteins. Staphylococci were grown in 3-ml volumes of RPMI 1640 in the presence of 0.3 MBq of 9,10-[^{3}H](N)-palmitic acid (Dupont) for 24 h at 37°C. Bacteria were pelleted and lysed by digestion with lysostaphin, and whole cells were analyzed by SDS-PAGE. Alternatively, lipoproteins were extracted from lysostaphin-digested bacterial cells or filtered culture supernatants with Triton X-114 prior to analysis. Following SDS-PAGE, gels were soaked in Amplify (Amersham), dried, and exposed to X-OMAT AR film (Kodak) for 6 weeks.

Construction and screening of genomic DNA libraries. Genomic DNA was prepared from staphylococci by the cetyltrimethylammonium bromide method described by Ausubel et al. (5). A genomic library of random *Eco*RI fragments of *S. epidermidis* 901 DNA was constructed in the phage vector Lambda Zap II (Stratagene) according to the manufacturer's instructions. Recombinant phage were plated on *Escherichia coli* XLI-Blue, and plaques were transferred to nitrocellulose filters. Plaques were screened for expression of staphylococcal antigens by incubation with monospecific rabbit antisera raised to the 32- and 36-kDa *S. epidermidis* iron-regulated cytoplasmic membrane proteins (36) or with mouse anti-*S. aureus* BB cell wall antiserum. Bound antibody was detected by using appropriate species-specific peroxidase-conjugated antibodies, H$_2$O$_2$, and 4-chloro-1-naphthol (3). Reactive plaques were purified, and DNA inserts were recovered by in vivo excision into *E. coli* SOLR. Expression of staphylococcal antigens in *E. coli* SOLR was confirmed by immunoblotting whole bacterial cell lysates prepared from overnight broth cultures supplemented with 50 μg of ampicillin per ml. Recombinant plasmids were recovered by alkaline lysis minipreparation and subjected to restriction analysis. Inserts from positive clones were sequenced with an ABI automated DNA sequencer. Comparisons of the deduced protein primary sequences were performed on Seqnet. The OWL nonredundant protein database, searched by using NEW-SWEEP 2.00 (by Alan Bleasby), Tmbase and Tmpred (19), and PRINTS (4), was used to analyze protein sequences, and FASTA (9) and LASERGENE software (DNAstar Inc.) was used to analyze DNA sequences.

Southern and Northern blot analyses. Staphylococcal chromosomal DNA was digested with *Eco*RI, electrophoresed, and transferred to a Hybond N+ membrane. The blot was incubated with a digoxigenin-labelled probe (Boehringer Mannheim) obtained by random priming of the 5.4-kb *Eco*RI fragment from pW32. Hybridization was performed at 42°C overnight, and blots were washed sequentially in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% (wt/vol) SDS at room temperature and in 0.5× SSC–0.1% (wt/vol) SDS at 68°C according to the protocol of the manufacturer (Boehringer Mannheim). Bound probe was visualized with nitroblue tetrazolium salt color substrate (Boehringer Mannheim) according to the manufacturer's instructions. A 2-kb *Nde*I-*Eco*RI fragment from pW32 containing most of the ABC operon was labelled with digoxigenin and used as a probe for Northern blot analysis. RNA was extracted from *S. epidermidis* 901 grown for 18 h under iron-rich or iron-restricted conditions in RPMI 1640 with a Qiagen Rneasy total RNA kit. Northern blotting was performed as described by Ausubel et al. (5). Digoxigenin-labelled RNA markers (Boehringer Mannheim) were electrophoresed on each gel.

Nucleotide sequence accession number. The DNA sequence of the *S. epidermidis* ABC transporter is available in the GenBank database under accession no. X99127.

RESULTS

Growth of staphylococci under iron-restricted conditions in RPMI 1640. In our previous studies on iron-regulated proteins of staphylococci, we used iron chelators such as ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA) to restrict iron availability in standard broth media or pooled peritoneal dialysate fluid as an iron-restricted growth medium (28, 41). In the present studies we adopted a different approach and used a
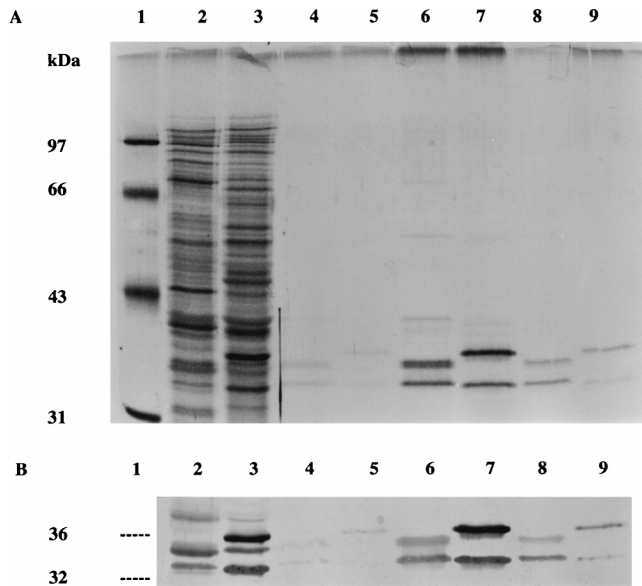
FIG. 1. SDS-PAGE (A) and immunoblots (B) of Triton X-114 extracts prepared from *S. aureus* BB and *S. epidermidis* 901 grown under iron-restricted growth conditions. Lanes: 1, molecular mass markers; 2 and 3, whole-cell polypeptide profiles; 4 and 5, Triton X-114 extracts of intact cells; 6 and 7, Triton X-114 extracts of lysostaphin-digested cells; lanes 8 and 9, Triton X-114 extracts of filtered culture supernatants; 2, 4, 6, and 8, *S. aureus* BB; 3, 5, 7, and 9, *S. epidermidis* 901. The gel in panel A was silver stained, and immunoblots were reacted with a pool of monospecific rabbit antisera to the 32- and 36-kDa iron-regulated cytoplasmic membrane proteins of *S. epidermidis* (36).

commercially available, intrinsically iron-restricted and defined growth medium, RPMI 1640, to investigate the phenotype of staphylococci in response to iron limitation. Our preliminary results indicated that all staphylococcal isolates tested grew readily in this medium, with or without supplementation with 20 μM $Fe_2(SO_4)_3$. Qualitatively, the expression of iron-regulated proteins under iron-restricted conditions was essentially identical to that we had observed in our earlier studies (data not shown). Consequently, we used RPMI 1640 as a more convenient medium for growth of staphylococci for the present studies.

Extraction of iron-regulated staphylococcal cytoplasmic membrane proteins with Triton X-114. Triton X-114 phase partitioning provided preliminary evidence that the iron-regulated cytoplasmic membrane proteins in *S. epidermidis* and *S. aureus* may be lipoproteins. Extraction of lysed bacteria grown under iron-restricted conditions identified a subset of bacterial proteins partitioning into the detergent phase (Fig. 1A). Two major polypeptides of 32 and 36 kDa and of 32 and 35 kDa were detected in extracts of *S. epidermidis* and *S. aureus*, respectively. These polypeptides were not detected in the aqueous phase following detergent partitioning (data not shown). The relationship of these Triton X-114-extractable polypeptides to the previously described staphylococcal iron-regulated cytoplasmic membrane proteins was confirmed by immunoblotting with monospecific rabbit antisera (Fig. 1B). The minor differences between the molecular mass stated for one of the *S. aureus* proteins in the present study and those stated in our previous studies (36, 41) are attributable to minor variations in the electrophoresis technique and the source of molecular mass markers used.

Only trace quantities of these proteins were extractable from lysed bacteria grown under iron-rich growth conditions (data not shown) or from intact iron-restricted cells (Fig. 1A), indi-

cating their regulation by iron availability and the restricted exposure of detergent-soluble forms of these proteins on the surfaces of intact staphylococci.

Triton X-114 extraction of filtered *S. epidermidis* culture supernatants showed that both the 32- and 36-kDa polypeptides were also present in supernatants from iron-restricted cultures but that these polypeptides were not detectable in iron-rich culture supernatants (data not shown). In contrast, small quantities of Triton X-114-extractable polypeptides of 32 and 35 kDa were found in supernatants of iron-rich *S. aureus* cultures (data not shown), although larger amounts of these polypeptides were detected in iron-restricted supernatants (Fig. 1A).

[³H]Palmitic acid labelling studies. Confirmation of the identity of these major Triton X-114-extractable iron-regulated proteins as lipoproteins was provided by results of labelling studies using [³H]palmitic acid. The 32- and 36-kDa polypeptides of *S. epidermidis* and the 32- and 35-kDa polypeptides detected in detergent extracts of *S. aureus* were radiolabelled when the bacteria were grown in RPMI 1640 under iron-restricted conditions (Fig. 2). Labelled polypeptides with these molecular masses were also detected in Triton X-114 extracts of the respective filtered culture supernatants from both staphylococcal species (Fig. 2).

Antigenic relatedness between the staphylococcal lipoproteins. A mouse monoclonal antibody raised against the *S. aureus* BB 32-kDa lipoprotein was used in immunoblotting studies to investigate the conservation of this antigen among a range of staphylococcal species. All bacteria were grown in RPMI 1640 under iron-restricted conditions. A polypeptide of the appropriate size was detected in Triton X-114 extracts of each of four *S. epidermidis* strains and all seven strains of *S. aureus* tested (data not shown). A 40-kDa polypeptide was detected in the single strain of *S. hominis* tested, but no reaction with this antibody was observed by immunoblotting with single strains of *S. lugdunensis*, *S. cohni*, and *S. haemolyticus* (data not shown).

Localization of the 32-kDa lipoprotein in *S. epidermidis* by immunoelectron microscopy. Immunogold labelling of thin sections of iron-restricted bacteria with a rabbit monospecific antibody to the *S. epidermidis* 32-kDa lipoprotein was used to further investigate the distribution of this antigen in the *S. epidermidis* cell. Figure 3A shows that the majority of the 32-kDa lipoprotein is distributed throughout the cell wall of *S. epidermidis*. Smaller quantities of this protein were located in the cytoplasmic membrane, and exposure of the antigen on the cell surface was minimal. Sections incubated with nonimmune rab-
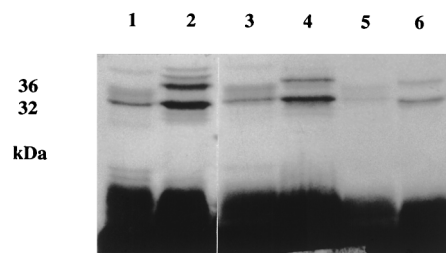

FIG. 2. Autoradiograph showing [³H]palmitate labelling of Triton X-114-extractable lipoproteins of *S. aureus* BB and *S. epidermidis* 901. Lanes: 1 and 2, whole-cell [³H]palmitate-labelled profiles; 3 and 4, Triton X-114 extracts of [³H]palmitate-labelled lysostaphin-digested cells; 5 and 6, Triton X-114 extracts of [³H]palmitate-labelled filtered culture supernatants; 1, 3, and 5, *S. aureus* BB; 2, 4, and 6, *S. epidermidis* 901.
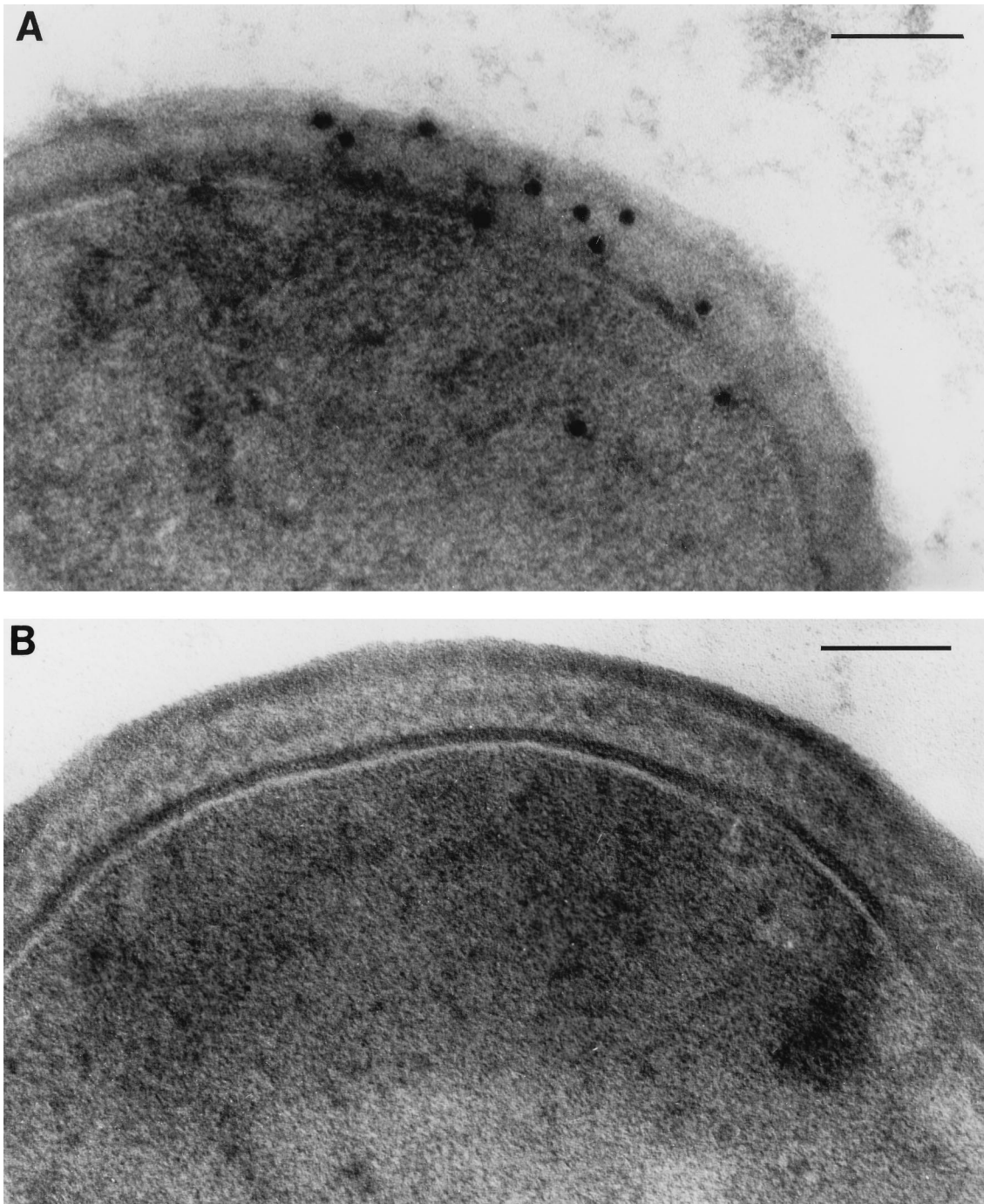
FIG. 3. Electron micrographs of thin sections of *S. epidermidis* 901 cells reacted with monospecific antiserum to the 32-kDa *S. epidermidis* lipoprotein. (A) Sections reacted with monospecific rabbit anti-32-kDa lipoprotein antibody and protein A-gold conjugate; (B) sections reacted with nonimmune rabbit serum and protein A-gold conjugate. Bars, 1 μm.

bit serum showed minimal labelling with protein A-gold (Fig. 3B).

Molecular cloning of the gene encoding the 32-kDa *S. epidermidis* lipoprotein. To further characterize the staphylococcal lipoproteins and investigate their potential functions, we sought to isolate the genes encoding these antigens from staphylococcal genomic DNA libraries. Initial antibody screening of an *S. epidermidis* 901 Lambda Zap II library with monospecific

antisera to the 32- and 36-kDa iron-regulated cytoplasmic membrane lipoproteins failed to identify reactive plaques. Since our previous studies had indicated some antigenic cross-reactivity between iron-regulated proteins in different staphylococcal species (36), we rescreened the library with anti-*S. aureus* BB wall antibodies. This antiserum was raised against native staphylococcal antigen, in contrast to the denatured antigen used in the preparation of the monospecific anti-32- and anti-36-kDa
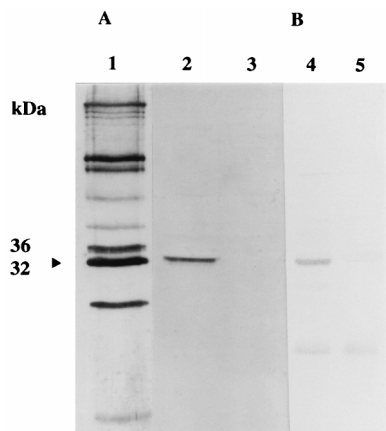
FIG. 4. Immunoblots of recombinant *E. coli* SOLR pW32 reacted with anti-*S. aureus* BB wall antiserum (A) or monospecific anti-32-kDa *S. epidermidis* lipoprotein antiserum (B). Lanes: 1, BB wall extract; 2 and 4, *E. coli* SOLRpW32 whole-cell extract; 3 and 5, *E. coli* SOLR whole-cell extract. The position of the 32-kDa lipoprotein antigen is indicated by the arrowhead.

FIG. 6. Northern blot showing transcript analysis of the *S. epidermidis* ABC transporter operon and differential transcription under iron-rich and iron-restricted growth conditions. Lanes: 1, molecular size markers; 2, RNA extracted from iron-restricted culture; 3, RNA extracted from iron-rich culture. The arrowhead indicates the position of the single 2.7-kb RNA transcript detected in *S. epidermidis* 901.

rabbit sera. This second screen identified several plaques reactive with antistaphylococcal antibodies. Following plaque purification and in vivo excision of one of these plaques, a polypeptide of approximately 32 kDa was detected on immunoblots of an *E. coli* SOLR lysate probed with anti-*S. aureus* BB wall antiserum (Fig. 4A). The identity of the polypeptide as the *S. epidermidis* 32-kDa iron-regulated cytoplasmic membrane lipoprotein was confirmed by immunoblotting with monospecific anti-32-kDa antiserum (Fig. 4B). The plasmid encoding the 32-kDa lipoprotein was designated pW32.

Characterization of the cloned DNA fragment. Restriction endonuclease mapping and subcloning of pW32 localized the gene encoding the 32-kDa lipoprotein to a 2.43-kb *Hpa*I-*Eco*RI fragment. The plasmid containing this fragment was designated pW33. The entire DNA fragment was sequenced on both strands following synthesis of appropriate DNA primers, and three open reading frames encoding putative polypeptides of 28, 35, and 30 kDa were identified. We have designated the genes encoding these polypeptides *sitA*, *-B*, and *-C*, respectively (*sit* is for staphylococcal iron transport). The organization of these genes in pW33 is shown in Fig. 5.

RNA transcript analysis by Northern blotting showed a single, iron-repressible RNA transcript of ~2.7 kb, indicating that the operon containing *sitA*, *-B*, and *-C* is transcribed as a polycistronic mRNA (Fig. 6).
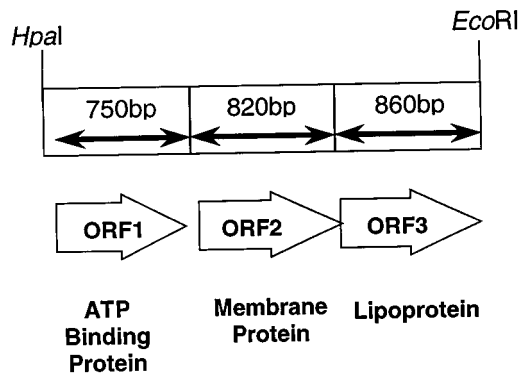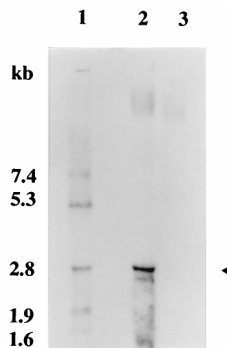

FIG. 5. Organization of the *S. epidermidis* ABC transporter operon.

Southern blotting was used to investigate conservation of this operon among other staphylococcal strains. Probing with the 5.4-kb *Eco*RI insert from pW32 identified single copies of these genes in genomic DNAs from *S. epidermidis* 901 and 138; however, no signal was detected when DNA from *S. aureus* BB or 8325-4, *S. lugdunensis*, *S. cohni*, or *S. warneri* was similarly probed (data not shown). In addition, although these genes could be amplified by PCR with appropriate sequencing primer pairs and *S. epidermidis* genomic DNA as the template, no products could be obtained when DNAs from the other staphylococcal isolates were used (data not shown).

The cloned *S. epidermidis* DNA locus shows significant homology with bacterial ABC transporters. Sequence comparisons indicated significant homologies at the DNA and protein levels between *sitA*, *-B*, and *-C* and their products and sequences of previously identified bacterial ABC transporters. Sequence comparisons yielding the highest homologies at the amino acid level are shown in Table 1.

SitA shows significant homology with known or theoretical ATP binding proteins in a number of bacterial species, including *Streptococcus gordonii* and *Streptococcus parasanguis*. *S. epidermidis* SitA contains consensus ATP binding motifs, including a P loop (GPNGAGKS) (33). Secondary-structure analysis failed to identify potential membrane-spanning regions in this protein, suggesting a cytoplasmic location for the mature protein. Interestingly, SitA also shows homology with an ATP binding protein, FecE, involved in iron(III) dicitrate transport in the gram-negative pathogen *Haemophilus influenzae*.

SitB has significant homology with several ABC transporter membrane proteins, including those of *Streptococcus gordonii* and *Streptococcus parasanguis*. Other strong homologies include those with MntB, a protein involved in Mn^{2+} transport in *Synechocystis* spp., and YfeC, a putative iron transport protein in *Yersinia pestis*. Secondary-structure analysis identified seven hydrophobic transmembrane-spanning regions in the staphylococcal polypeptide, supporting its likely identity as a cytoplasmic membrane protein. This *S. epidermidis* membrane protein lacks an ATP binding motif, a feature considered to be characteristic of ABC importers rather than exporters (13).

SitC, the 32-kDa *S. epidermidis* lipoprotein, shows significant homology with other ABC transporter lipoproteins, including EfaA, ScaA, and FimA in *Enterococcus faecalis*, *Streptococcus gordonii*, and *Streptococcus parasanguis*, respectively. These proteins are all thought to play a role in bacterial adherence, and all contain recently described conserved motifs that define the

TABLE 1. Sequence homologies of the *S. epidermidis* ABC transporter operon proteins with similar proteins in databases

Protein type (*S. epidermidis* protein)	Accession no.	% Identity	Organism(s)	Protein/function[a]
ATP binding protein (SitA)	X99127	100	*S. epidermidis*	ATP binding protein/Fe transport?
	L11577	43	*Streptococcus gordonii*	ScaA-associated ATP binding protein/?
	M26130	46	*Streptococcus parasanguis*	FimA-associated ATP binding protein/?
	U55214	39	*Treponema pallidum*	TroB/?
	U52850	38	*Erysipelothrix rhusiopathiae*	EwlA-associated ATP binding protein/?
	L45003	34	*H. influenzae*	FecE/iron(III) dicitrate transport
	L34630	33	*Synechocystis* spp.	MntA/Mn transport
Membrane protein (SitB)	X99127	100	*S. epidermidis*	Membrane protein/Fe transport?
	M26130	48	*Streptococcus parasanguis*	FimA-associated membrane protein/?
	L11577	46	*Streptococcus gordonii*	ScaA-associated membrane protein/?
	L45001	39	*H. influenzae*	HI0359 membrane protein/?
	L34630	39	*Synechocystis* spp.	MntB membrane protein/Mn transport
	U50597	38	*Y. pestis*	YfeC membrane protein/Fe transport?
Lipoprotein (SitC)	X99127	100	*S. epidermidis*	Lipoprotein/Fe transport?
	L19055	50	*Streptococcus pneumoniae*	PapA lipoprotein/adhesion?
	U03756	49	*Enterococcus faecalis*	EfaA/adhesion?
	M26130	50	*Streptococcus parasanguis*	FimA/saliva binding protein
	U46542	48	*Streptococcus crista*	ScbA/adhesion or transport?
	L11577	48	*Streptococcus gordonii*	ScaA/coaggregation adhesion

[a] A question mark indicates that the function of the protein is presently unknown.

family of bacterial adhesins designated ADHESINFAMILY or related periplasmic binding proteins (4). The staphylococcal lipoprotein has a consensus prelipoprotein signal peptide cleavage sequence (17) (ILAACG), with the cysteine residue at position 18, a finding which confirms our biochemical analysis, and also contains an ATP binding motif (GWFEKAL DQAGKSTKDKN) (33).

DISCUSSION

The data presented here extend our previous observations on iron-regulated proteins in the staphylococci (36, 41). The iron-regulated cytoplasmic membrane proteins in *S. epidermidis* and *S. aureus* have been identified as lipoproteins, and the 32-kDa *S. epidermidis* lipoprotein has been shown to be a component of a novel, iron-regulated ABC transporter which shows organizational and sequence homologies with previously described transporters in several gram-positive and gram-negative bacterial pathogens.

At present it is unclear which specific solute(s) is bound by the 32-kDa lipoprotein in *S. epidermidis*. However, the iron-regulated nature of this ABC transporter provides further support for our previous suggestion that this staphylococcal cytoplasmic membrane lipoprotein may be involved in iron transport. This suggestion is supported by recent work by Dintilhac et al. (10, 11), who examined the DNA sequence of the *S. epidermidis* ABC transporter reported here and found structural similarities with a new family of solute binding proteins (cluster 9) that is distinct from the eight other families previously described by Tam and Saier (39). Members of cluster 9 share the property of proven or hypothetical binding of metallic cations, including Fe, Mn, and Zn. Our own recent unpublished data suggest that the *S. epidermidis* operon may also be regulated by Mn^{2+} availability, raising the possibility that the 32-kDa *S. epidermidis* lipoprotein may be able to bind and transport a variety of metal ions. We are currently attempting to generate *S. epidermidis* mutants to address this possibility.

The spatial distribution of the 32-kDa lipoprotein within the cell wall of *S. epidermidis* as shown by immunoelectron micros-

copy is consistent with those of other solute binding lipoproteins in gram-positive bacteria. These proteins are anchored to the cytoplasmic membrane by a lipid-modified N terminus, with the solute-binding component projecting into or in some cases through the cell wall where they are exposed on the bacterial surface. However, the observation that SitC is located primarily within the cell wall of *S. epidermidis* initially appears to be at variance with results from our earlier cell fractionation studies which showed that this protein was associated with the staphylococcal cytoplasmic membrane (36, 41). This apparent anomaly can be resolved if, following removal of the cell wall by lysostaphin treatment, SitC remains tethered to the cytoplasmic membrane by its lipid tail. The lipoprotein would then be pelleted with the cytoplasmic membrane fraction of the lysed staphylococcal cells.

This distribution would potentially allow the 32-kDa lipoprotein to function in both siderophore-dependent and -independent iron acquisition pathways. The lipoprotein could act as a ferric siderophore receptor either exposed on the cell surface or within the porous matrix of the cell wall, into which low-molecular-mass molecules such as siderophores readily penetrate. The involvement of lipoproteins in ferrihydroxamate siderophore iron uptake has been previously reported for *Bacillus subtilis* (35), and more recent studies with *Corynebacterium diphtheriae* IRP1 suggest that this iron-regulated lipoprotein may also function as a siderophore receptor (34). Alternatively, the 32-kDa *S. epidermidis* lipoprotein may be involved in shuttling iron from receptor-bound transferrin or lactoferrin across the cell wall to the cytoplasmic membrane prior to uptake into the cell. Experiments to identify the solute specificity of the 32-kDa lipoprotein in *S. epidermidis* are under way.

Our immunoblotting data obtained by using a monoclonal antibody specific for the 32-kDa lipoprotein are consistent with our earlier studies which showed that this antigen was conserved among *S. epidermidis* and *S. aureus* isolates but was generally not expressed by other staphylococcal species (36, 41). The other staphylococci do, however, produce one or more major lipoproteins in the 30- to 40-kDa size range as shown by

SDS-PAGE analysis of Triton X-114 extracts (7), and we speculate that these lipoproteins may also be components of ABC transporters in these species. Based on these data, it also seems likely that the ~36-kDa and 35-kDa iron-regulated membrane lipoproteins produced by *S. epidermidis* and *S. aureus*, respectively, may also be components of other ABC transporters which may also have a role in iron acquisition. We are currently attempting to clone the genes encoding these lipoproteins to confirm this possibility.

Despite the strong antigenic and potential functional homology between the 32-kDa lipoproteins of *S. epidermidis* and *S. aureus*, Southern blot and PCR analyses surprisingly failed to detect any homology between the genes encoding these proteins at the DNA level under the experimental conditions used. A previous study has indicated that even within the highly conserved staphylococcal gene encoding HSP60, species-specific variations exist which are sufficient to allow species differentiation by hybridization techniques (16). It is therefore conceivable that such species-specific variation within the staphylococcal ABC transporter gene sequences may also exist and may account for our findings.

Interestingly, the 32-kDa *S. epidermidis* lipoprotein also shows significant sequence homology with a number of proteins known to be adhesins in other gram-positive bacteria. In these other organisms, the lipoproteins are clearly surface exposed and available for interaction with appropriate tissue or other surface receptors (20, 37). The limited exposure of the 32-kDa antigen on the surface of *S. epidermidis* as assessed by electron microscopy suggests, however, that it is unlikely that this lipoprotein plays a significant role in the adhesion of *S. epidermidis*. However, the observation that in both *S. epidermidis* and *S. aureus*, significant quantities of membrane lipoproteins are released into culture supernatants under iron-restricted growth conditions in vitro and in vivo (7) may indicate an extracellular function for these lipoproteins. The mechanism of release of the lipoproteins from the staphylococcal surface is presently unclear, but our Triton X-114 phase partitioning and [³H]palmitate labelling studies indicate that the released lipoproteins remain acylated. Similar findings have been reported for extracellular lipoproteins from *Streptococcus mutans* (37). A possible toxic role for the staphylococcal lipoproteins is supported by recent studies which indicate that membrane proteins of L forms of *S. aureus* may stimulate cytokine release from mammalian cells in in vitro assays and may be lethal when administered to D-galactosamine-treated mice (1). The relationship between the lipoproteins described in the present studies and the toxic membrane proteins of 30 and 36 kDa isolated by Akashi et al. (1) is presently unclear but merits further investigation, since other bacterial lipoproteins have previously been shown to induce cytokine release from mammalian cells (15).

In addition to their potential toxic activity, the extracellular lipoproteins also induce a significant humoral antibody response in rats with chamber implants inoculated with staphylococci (27), and variable levels of antibodies to these lipoproteins are detectable in human sera from individuals with staphylococcal infection (36). This observation raises the serodiagnostic potential of these lipoproteins for some types of staphylococcal disease.

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