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The Elastin-binding Protein of *Staphylococcus aureus* (EbpS) Is Expressed at the Cell Surface as an Integral Membrane Protein and Not as a Cell Wall-associated Protein*

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The elastin-binding proteins EbpS of *Staphylococcus aureus* strains Cowan and 8325-4 were predicted from sequence analysis to comprise 486 residues. Specific antibodies were raised against an N-terminal domain (residues 1–267) and a C-terminal domain (residues 343–486) expressed as recombinant proteins in *Escherichia coli*. Western blotting of lysates of wild-type 8325-4 and Newman and the corresponding *ebpS* mutants showed that EbpS migrated with an apparent molecular mass of 83 kDa. The protein was found exclusively in cytoplasmic membrane fractions purified from protoplasts or lysed cells, in contrast to the clumping factor ClfA, which was cell-wall-associated. EbpS was predicted to have three hydrophobic domains H1-(205–224), H2-(265–280), and H3-(315–342). A series of hybrid proteins was formed between EbpS at the N terminus and either alkaline phosphatase or β-galactosidase at the C terminus (EbpS-PhoA, EbpS-LacZ). PhoA and LacZ were fused to EbpS between hydrophobic domains H1–H2 and H2–H3, and distal to H3. Expression of enzymatic activity in *E. coli* showed that EbpS is an integral membrane protein with two membrane-spanning domains H1 and H3. N-terminal residues 1–205 and C-terminal residues 343–486 were predicted to be exposed on the outer face of the cytoplasmic membrane. The ligand-binding domain of EbpS is known from previous studies to be present in the N terminus between residues 14–34 and probing whole cells with anti-EbpS1–267 antibodies indicated that this region is exposed on the surface of intact cells. This was also confirmed by the observation that wild-type *S. aureus* Newman cells bound labeled tropoelastin whereas the *ebpS* mutant bound 72% less. In contrast, the C terminus, which carries a putative LysM peptidoglycan-binding domain, is not exposed on the surface of intact cells and presumably remains buried within the peptidoglycan. Finally, expression of EbpS was correlated with the ability of cells to grow to a higher density in liquid culture, suggesting that EbpS may have a role in regulating cell growth.

Staphylococcus aureus is an important Gram-positive bacterial pathogen. It is equipped with an arsenal of virulence factors, including cytolytic toxins, superantigens, extracellular enzymes, and cell-surface-associated proteins (1, 2). The wide variety of virulence factors reflects the organism's ability to cause many infections ranging from superficial skin lesions such as abscesses and impetigo to invasive and more serious infections such as osteomyelitis, septic arthritis, pneumonia, and endocarditis (3). Strains causing nosocomial infections are often resistant to most of the antibiotics available for treatment (*e.g.* MRSA[1]).

The organism can express an array of surface proteins that promote bacterial interactions with components of the extracellular matrix (ECM) (2, 4, 5). These are collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Binding to the ECM allows bacteria to adhere to and to colonize host tissue. Surface proteins that interact with collagen (6), fibrinogen (7, 8), fibronectin (9), and von Willebrand factor (10) have been characterized. The MSCRAMMs have many common structural features, including a cleavable signal sequence at the N terminus, a conserved Leu-Pro-*X*-Thr-Gly (LP*X*TG) pentapeptide sequence at the C terminus, a stretch of hydrophobic residues, and a positively charged tail (11). Importantly, these MSCRAMMs are covalently attached to cell wall peptidoglycan by the membrane-associated enzyme sortase that recognizes the LP*X*TG motif and joins the Thr residue of the motif to the nascent cross-bridge of the peptidoglycan precursor prior to its incorporation into the wall polymer (11).

Elastin, along with microfibrillar components, are a major component of the elastic fiber ECM (12). Elastin plays a crucial role in maintaining the structural integrity and function of tissues in which reversible extensibility or deformability is required (13). Thus elastin and elastic fibers are present in abundance in tissues that require elasticity such as the lung, skin, and major blood vessels. Physiologic elastin, like interstitial collagen and other matrices, is an insoluble polymeric fiber. Elastin is comprised of cross-linked tropoelastin monomers that are first secreted as soluble precursors from cells (12). Lysine residues in the cross-linking domain of secreted tro-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U48826 and AF400161.

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[1] The abbreviations used are: MRSA, methicillin-resistant *S. aureus*; IPTG, isopropyl-β-D-thiogalactopyranoside; ECM, extracellular matrix; MSCRAMM, microbial surface components recognizing adhesive matrix molecules; Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Tc, tetracycline; AP, alkaline phosphatase; PBS, phosphate-buffered saline; ClfA, clumping factor A; PhoA, alkaline phosphatase; TSB, typical soy broth.

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poelastin are rapidly cross-linked both inter- and intramolecularly by the enzyme lysyl oxidase (14).

Because *S. aureus* infects many tissues that are rich in elastin, we previously investigated whether *S. aureus* interacts specifically with elastin. These studies revealed that *S. aureus* binds to soluble tropoelastin via a cell-surface-associated 25-kDa protein named the elastin-binding protein of *S. aureus* EbpS. EbpS binds to the N-terminal 30-kDa region of elastin, which does not contain the VGVAPG hexapeptide recognition motif for the mammalian elastin receptor (15, 16). The EbpS elastin binding site is also different from the charged C-terminal region in elastin where microfibrillar components associate for elastic fiber assembly (17). These findings suggest that the EbpS binding site in elastin is available for *S. aureus* binding and that this interaction may promote bacterial colonization to facilitate pathogenesis.

In contrast to the fibrinogen-, collagen-, and fibronectin-binding proteins, the elastin-binding protein EbpS was reported to lack a signal sequence and the C-terminal LP*X*TG motif present in *S. aureus* MSCRAMMs that are used to link surface molecules covalently to the cell wall peptidoglycan by sortase (15, 18). This suggested novel mechanisms of secretion from the cell and attachment to the cell wall. Here we report that the *ebpS* gene product is an integral membrane protein. The N terminus, which contains the elastin binding site, is exposed to the extracellular milieu. Using LacZ and PhoA fusions we showed that the EbpS protein has two transmembrane domains, and the C terminus as well as the N terminus is located on the outer face of the cytoplasmic membrane. A mutant of *S. aureus* lacking EbpS exhibited decreased binding to radiolabeled tropoelastin, a defect that was restored by the presence of the wild-type *ebpS* gene on a complementing plasmid. These findings clarify how EbpS is expressed on the cell surface and demonstrate that EbpS is the cell-surface protein of *S. aureus* that mediates binding to soluble tropoelastin.

MATERIALS AND METHODS

Growth of Bacteria

Escherichia coli cells bearing recombinant plasmids were grown in L broth or on L agar incorporating ampicillin (Ap, 100 μg/ml), chloramphenicol (Cm, 25 μg/ml) Ranamycin (Va, 25μg/ml) or spectinomycin at 25 μg/ml, where appropriate. *S. aureus* was routinely grown in trypticase soy broth (TSB) or agar incorporating chloramphenicol (Cm, 5–10 μg/ml), erythromycin (Em, 10 μg/ml), or tetracycline (Tc, 2 μg/ml) as appropriate. A chemically defined medium CDM (19) was employed for growth yield experiments.

DNA Manipulations

Standard methods were used for DNA manipulation used in constructing plasmids and for Southern blot hybridization (20). *S. aureus* genomic DNA was isolated using the AGTC bacterial genomic DNA purification kit (Edge BioSystems) adapted for use with staphylococci by incorporation of lysostaphin (200 μg/ml) (21).

Molecular Cloning and Sequencing EbpS

DNA of *S. aureus* 8325-4 was digested with *Pst*I and fractionated on a sucrose gradient (20). Fragments of 5–6 kb were cloned into pBluescript KS+ and transformed into *E. coli* XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB*$^+$ *lacI*^{q} *ZΔM15* Tn*10* (Tc^r)] (Stratagene). Transformants were screened by colony blotting using a probe corresponding to the 609 bp of the *S. aureus* strain Cowan (ATCC12598) *ebpS* coding sequence labeled by PCR with digoxigenin (Roche Molecular Biochemicals). The 5.2-kb *Pst*I fragment on one positively hybridizing clone pFR5.2 was sequenced by MWG Biotech.

Construction of ebpS::Em^r Mutation by Allele Replacement

Two sets of primers were used to PCR amplify the 8325-4 *ebpS* locus from pFR5.2 in two segments, which were cloned together into pUC18 (22) resulting in a deletion of 116 bp (Δ*ebpS*) located between 538 and 654 bp in the coding sequence. A 3′-fragment of approximately 1.4 kb was amplified by F5′-CCCAAGCTTTGAATGGTTGCTGCCGTTTC incorporating a *Hin*dIII, site (underlined) and R5′-TCCCCGCGGGATGTTTCTGATTGGTTAGCAC containing a *Sac*II site (underlined). A 3′-fragment of approximately 1.5 kb was amplified by F5′-TCCCCGCGGCTGGTGCAATGGCTGCTTC incorporating a *Sac*II site and R5′-CCGGAATTCCTTCTCGGTAATAAACTAGCG incorporating an *Eco*RI site. The fragments were cleaved with *Hin*dIII and *Sac*II or *Eco*RI and *Sac*II, as appropriate, and ligated with pUC18 cleaved with *Hin*dIII and *Eco*RI to form pFR1. The *ermC* gene of pE194 (23) was amplified on a 1-kb fragment by F5′-TCCCCGCGGAGGTGTCACAAGACACTC and R5′-TCCCCGCGGAGCTCGTGCTATAATTATAC incorporating *Sac*II sites (underlined), and cloned into the *Sac*II site in the middle of the *ebpS* gene in pFR1 forming *ebpS*::Em^r (pFR2). The temperature-sensitive *S. aureus* plasmid pTS2 (expresses resistance to chloramphenicol) (24) was cloned into the *Hin*dIII site located between the two flanking *ebpS* sequences of pFR2 forming pFR3 (*ebpS*::Em^r). This plasmid was transformed into *S. aureus* RN4220 (25) selecting Em^r at 30 °C. Recombinational exchange between pFR3 and the chromosomal *ebpS* locus was detected (21) resulting in strain DU5979 (RN4220 *ebpS*::Em^r). The fidelity of the exchange was tested by Southern blotting. Genomic DNA was cleaved with *Eco*RI and *Hin*dIII, probed with a DIG-labeled 787-bp 5′-*ebpS* fragment amplified with primers F5′ and R5′, which revealed that a single 3.15-kb fragment from the wild-type was replaced with a 4.0-kb fragment in the mutant. The *ebpS*::Em^r mutation was transduced to 8325-4 (forming DU5980), to 8325-4 *spa*::Tc^r (10) to form DU5982, and to Newman *spa*::Ka^r (DU5971) forming DU5983.

Construction of pCU1ebpS$^+$ and pCL84ebpS$^+$ for Complementation Analysis

The wild-type *S. aureus* 8325-4 *ebpS* gene with 300 bp of 5′-DNA, which should be sufficient to carry the promoter, and 200 bp of 3′-DNA, was cloned from plasmid pFR5.2 into the shuttle plasmid pCU1 (26) (Ap^r in *E. coli*; Cm^r in *S. aureus*) by PCR using *Pfu* polymerase and primer F5′-CCGGAATTCGGATCAATGTAGACGGAAG containing an *Eco*RI site (underlined) and R5′-CCCAAGCTTGACAAGACCTTGTTAAATAAC containing a *Hin*dIII site (underlined). pCU1*ebpS*$^+$ was transformed into *S. aureus* RN4220 selecting for resistance to Cm and transduced using phage 85 (21) to other strains of *S. aureus*.

A single-copy integrating vector plasmid pCL84 (27) was also used for complementation tests. pCL84 expresses spectinomycin resistance in *E. coli* and Tc^r in *S. aureus*. It carries the *att* site of phage L54a but lacks a replicon that functions in *S. aureus*. When transformed into *S. aureus* CYL316 (27), which overexpresses the L54a integrase, the plasmid integrated into the chromosomal *att* site located in the *geh* gene. The *ebpS* gene was amplified using the primers for cloning in pCU1 given above, except that the reverse primer carried an *Eco*RI site. Plasmid pCL84*ebpS*$^+$ and the pCL84 empty vector control were transformed into CYL316 selecting for Tc^r where they integrated into the phage L54a *att* site forming DU5984 and DU5985, respectively. The integrated plasmids were then transduced by phage 85 into strain 8325-4 *ebpS*::Em^r forming DU5986 (8325-4 *ebpS*::Em^r *geh*::pCL84*ebpS*$^+$, Tc^r) and DU5987 (8325-4 *ebpS*::Em^r *geh*::pCL84,Tc^r).

Expression of Recombinant EbpS Proteins

Recombinant EbpS proteins were expressed with 6xHis affinity tags at their N termini using the expression vector pQE30 (Qiagen). rEbpS1–267, carrying the first 267 residues of EbpS from strain 8325-4, was constructed by PCR amplification using pFR5.2 as the template and primers F5′-CGCGGATCCATAGAAAGGAAG**G**TGGCT**G**TG incorporating a *Bam*HI site (underlined) and with the A of the two ATG codons changed to G (in boldface) to avoid internal initiation of translation and R5′-CCCAAGCTTTTATGGAATAACGATTTGTTGACC incorporating a *Hin*dIII site (underlined). The PCR fragment was cleaved with *Bam*HI and *Hin*dIII and cloned into pQE30 cleaved with the same enzymes. The primers amplified 6 codons upstream from the methionine codon defined by N-terminal sequencing of the 25-kDa form of EbpS purified from *S. aureus* Cowan. Thus rEbpS1–267 contains 17 extra residues at the N terminus, the first 11 provided by the pQE30 vector and 6 from the open reading frame that extends upstream from the *ebpS* ATG start codon.

The pQE30 plasmid expressing the C-terminal truncate rEbpS343–486 was also constructed by PCR using primers F5′-CGCGGATCCAACAATCATAATAATGGTAC incorporating a *Bam*HI site (underlined) and R5′-CCCAAGCTTTTATGGAATAACGATTTGTTG incorporating a *Hin*dIII site (underlined) to facilitate cloning. The resulting plasmids pQEebpS1–267 and pQEebpS343–486 were transformed into *E. coli* M15 *lac ZΔM15 ara gal mtl* (pREP4 *lacI*^{q}, Ka^r) (Qiagen) for protein expression.

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The recombinant proteins were purified using Ni^{2+}-chelate chromatography. rEbpS1–267 was insoluble and formed inclusion bodies, which were dissolved in 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0, buffer and purified as described previously (18). rEbpS343–486 was soluble and was purified as described previously (28).

Construction of ebpS-phoA and ebpS-lacZ Fusions

The entire 1461-bp 8325-4 *ebpS* open reading frame was PCR-amplified from plasmid pFR5.2 using *Pfu* polymerase and primers F5′-CATGCCATGGCTAATAATTTTAAAGATGACTTTG containing a *Nco*I site (underlined) and R5′-CCCAAGCTTTTATGGAATAACGATTTGTTG containing a *Hin*dIII site (underlined). The resulting PCR fragment was ligated into the expression vector pKK233-2 (29) forming plasmid pKK233*ebpS*. This construct served as a template for generating a series of *ebpS-phoA* and *ebpS-lacZ* fusions. The *ebpS* gene was truncated at the 3′-end by PCR amplification. The same forward primer was used in each amplification. The forward primer was complementary to DNA located 5′ to the *trc* promoter in pKK233-2 and contained an *Eco*RI site. When combined with reverse primers containing a 5′-*Sal*I site that were complementary to different sequences within *ebpS*, it was possible to generate a set of *ebpS-lacZ* and *ebpS-phoA* fusions by cloning between *Eco*RI and *Sal*I sites in the fusion expression vectors pBAF (*phoA*) (30) and pLKC480 (*lacZ*) (31).

Assays for Alkaline Phosphatase and β-Galactosidase

Alkaline phosphatase (AP) activity was initially tested on LA plates incorporating 100 μg/ml Ap, 1 mM IPTG, and 40 μg/ml of the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.). β-Galactosidase activities were detected on LA incorporating 100 μg/ml Ap, 25 μg/ml Cm, 2 mM IPTG, and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Melford Laboratories). To quantify AP activity, the strain CC118 (*araD139* Δ(*ara*, *leu*)*7697* Δ*lacX74 phoA20 galE galK thi rpsE argE*(Am) *recA1*) (32) harboring pBAF*ebpS-phoA* fusions were grown to $A_{600\ nm}$ of 0.4 at 30 °C with aeration in L broth containing 100 μg/ml Ap and 0.4% (w/v) glucose. Similarly, to quantify β-galactosidase activity, strain TG1 (20) (pDIA17) also harboring plasmid pLKC480*ebpS-lacZ* fusions were grown in Luria broth incorporating 100 μg/ml Ap and 25 μg/ml Cm. pDIA17 is a derivative of pACYC184 with the *lacI* gene cloned into the *Bam*HI site allowing expression from the *tet* promoter.[2] IPTG was added to a final concentration of 3 mM (*phoA* fusions) or 2 mM (*lacZ* fusions), and cultures were grown for a further 4 h. AP activity was assayed by the method of Brickman and Beckwith (33) with the addition of iodoacetamide (4 mg/ml) to the Tris-HCl buffer, pH 8.0, used to resuspend cells. LacZ activity was measured by the method of Miller (34).

Anti-EbpS Antibodies

Antibodies were raised in young New Zealand White rabbits to rEbpS1–267 and rEbpS343–486, and the immunoglobulin fraction was purified (35). Antibodies to rEbpS343–486 were affinity-purified to remove antibodies that cross-reacted with other *S. aureus* proteins. rEbpS343–486 was coupled to CNBr-activated Sepharose (Amersham Biosciences, Inc.) according to the manufacturer's instructions. Antibody that bound to the Sepharose was eluted with 50 mM glycine, 0.5 M NaCl, pH 2.7.

Cell Fractionation Experiments

Protoplasts—*S. aureus* Newman *spa* cultures were grown to stationary phase in TSB with aeration ($A_{600\ nm}$ of 12). Cells were harvested by centrifugation at 7000 × *g* at 4 °C for 15 min and washed with PBS. Cells were concentrated by resuspension in lysis buffer (50 mM Tris-HCl, 20 mM $MgCl_2$, pH 7.5) supplemented with 30% raffinose at an $A_{600\ nm}$ of 100. Cell wall proteins were solubilized by incubation with lysostaphin (200 μg/ml) in the presence of protease inhibitors (mini Complete, Roche Molecular Biochemicals). Protoplasts were recovered by centrifugation at 6000 × *g* for 20 min, and the supernatant was taken as the wall fraction. Protoplasts were washed once in raffinose buffer and resuspended in ice-cold lysis buffer. Protoplasts were lysed by vortexing and repeated pipetting. Lysis was confirmed by phase-contrast microscopy. The membrane fraction was obtained by centrifugation at 40,000 × *g* for 60 min at 4 °C followed by washing and resuspension in lysis buffer. The supernatant became the cytoplasmic fraction. The culture supernatant was filtered through a 0.45-μm filter, and proteins were precipitated by 1:20 volume ice-cold 100% (w/v) trichloroacetic acid on ice for 30 min. Precipitated proteins were pelleted at 18,000 × *g* for 15 min at 4 °C. The pellet was washed once in ice-cold acetone, air-dried, and dissolved in Laemmli final sample buffer (36). An equivalent of 1 A_{600} unit of each fraction was analyzed by SDS-PAGE and Western immunoblotting. Fractions were assayed for isocitrate dehydrogenase (37).

Mechanical Disintegration—*S. aureus* Newman *spa* was grown to stationary phase in TSB with aeration. Cells were collected by centrifugation, washed in PBS, and resuspended in lysis buffer (see above) at an $A_{600\ nm}$ of 150. The suspension was transferred to a blue cap Fast RNA tube and shaken in a FP120 Fastprep (Savant) cell disintegrator at speed 6 for 40 s. This was repeated 10 times with cooling on ice for 1 min between cycles. Lysates were centrifuged at 4 °C for 2 min at 3000 × *g* to remove whole cells. The supernatant was then centrifuged at 15,000 × *g* for 10 min to sediment cell wall material. Membranes were sedimented at 40,000 × *g* for 60 min, washed in ice-cold lysis buffer, and resuspended in lysis buffer.

Preparation of a Membrane Fraction of E. coli

E. coli strains harboring *ebpS-lacZ* and *ebpS-phoA* fusions were induced with IPTG. Cells were collected by centrifugation and resuspended in PBS containing 10 mM $MgCl_2$, protease inhibitors (40 μl/ml) (mini Complete, Roche Molecular Biochemicals), and lysozyme (200 μg/ml) (chicken egg white, Sigma). Cell suspensions were transferred to a blue cap Fast RNA tube and shaken in a FP120 FastPrep cell disrupter at speed 4.5 for 40 s. Shaking was repeated five times with cooling. Lysates were spun at 40,000 × *g* for 1 h at 4 °C. The membrane-containing pellets were washed in PBS with 10 mM $MgCl_2$, pelleted as above, and finally resuspended in PBS.

Western Immunoblotting

Samples were prepared for electrophoresis by boiling for 5 min in final sample buffer (0.125 M Tris-HCl, 4% (w/v) sodium dodecyl sulfate, 20% glycerol, 10% (v/v) β-mercaptoethanol, 0.002% (w/v) bromphenol blue) and analyzed in 10% (w/v) acrylamide gels. Gels were transferred to polyvinylidene difluoride Western blotting membranes (Boehringer) by the semidry system (Bio-Rad). Membranes were blocked for 15 h at 4 °C with 5% (w/v) skim milk. Rabbit polyclonal anti-rEbpS1–267 and anti-rEbpS343–486 antibodies were used at a 1:1500 dilution. Bound antibody was recognized by incubation with protein A-peroxidase (Sigma, 1:2000) and visualized using the Chemiluminescence Detection System (Roche Molecular Biochemicals). Rabbit anti-*Micrococcus luteus* F_1/F_0-ATPase, which cross-reacts with the *S. aureus* protein, was a gift from P. Owen, Trinity College, Dublin. Mouse anti-β-galactosidase polyclonal antibodies (Sigma) were used at a dilution of 1:50. Rabbit polyclonal antibodies to alkaline phosphatase (Abcam Ltd.) were used at a dilution of 1:10,000.

Whole Cell Dot Blotting

S. aureus Newman *spa*, Newman *spa ebpS*, and Newman *spa ebpS* (pCU-*ebpS*$^+$) were grown to stationary phase in TSB with aeration ($A_{600\ nm}$ of 12). Cells were harvested by centrifugation, washed in PBS, and resuspended at an $A_{600\ nm}$ of 100 units. Ten microliters from this suspension were dotted onto a nitrocellulose membrane (Protran) and dried for 30 min. The membrane was blocked with 5% (w/v) skim milk in PBS for 1 h. Western blots were probed with anti-rEbpS1–267 or anti-rEbpS343–486 antibodies at a 1:1500 dilution.

Tropoelastin Binding to S. aureus

Full-length recombinant human tropoelastin (20 μg) (38) was iodinated with 1 mCi of $Na^{125}I$ for 10 min at room temperature in an Iodogen-coated microcentrifuge tube. Binding of ^{125}I-recombinant human tropoelastin to *S. aureus* was measured as described previously (15, 18).

Bacterial Dry Weight Determination

S. aureus strains were grown for 18 h in 20 ml of CDM in a 250-ml flask in an orbital shaking incubator at 200 rpm. Cells from an 8-ml volume of culture were washed in 50 mM Tris-HCl, pH 7.5, and twice in distilled water before being transferred to pre-weighed glass tubes. The cells were dried in an oven at 105 °C for 24 h. Then the tubes were weighed again, and the dry weight of the cells was obtained by subtracting the weight of the tube.

RESULTS

Sequence Analysis of the ebpS Gene—The *ebpS* gene of strain Cowan was previously reported to be 606 bp in length and to encode a protein of 202 residues with an apparent molecular

[2] P. Bertin, Institute Pasteur, personal communication.

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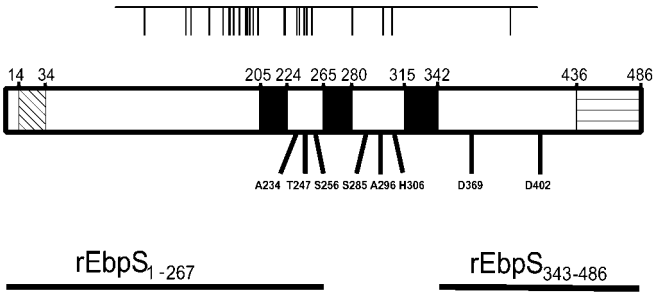

FIG. 1. **A schematic diagram illustrating the primary structure of the EbpS protein.** The *diagonal cross-hatched box* indicates the 21-residue ligand-binding domain in the N terminus. The *black boxes* in the center of the molecule represent hydrophobic domains, the *first* and *third* of which are predicted to span the cytoplasmic membrane. The C-terminal LysM domain is denoted by *horizontal lines*. The *vertical lines* show the positions where the amino acid sequences differ between the proteins expressed by strains Cowan and 8325-4 and EbpS sequences predicted from six publicly available genome sequences. The positions of fusions between EbpS and PhoA or LacZ are indicated *below*.

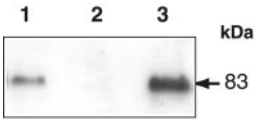

FIG. 2. **Western immunoblotting analysis showing EbpS.** Lysostaphin-generated lysates of *S. aureus* 8325-4 (*lane 1*), 8325-4 *ebpS* (*lane 2*), and 8325-4 *ebpS* (pCU1*ebpS*$^+$) (*lane 3*) were separated by SDS-PAGE and probed with anti-rEbpS343–486 antibodies.

mass of 25 kDa (18). We identified, by Southern hybridization using an *ebpS*-specific probe, a 5.2-kb *Pst*I fragment that carries the *ebpS* locus of strain 8325-4. The fragment was cloned into pBluescript KS+ and sequenced. This revealed that *ebpS* comprises an open reading frame of 1461 bp, which encodes a 486-residue protein with a predicted molecular mass of 53.2 kDa, the first 202 residues of which are 93% identical to those of the Cowan protein. The Cowan *ebpS* gene carried on plasmid pEBPS-1 (18) was sequenced again and shown also to be 1461 bp in length and to specify a protein of 486 residues with a predicted molecular weight of 53.08. The Cowan and 8325-4 EbpS proteins have 25 residue differences (95% identity). Most of the differences (11/25) are clustered between residues 171 and 205 (Fig. 1). The EbpS protein of strain COL (available at www.tigr.org/tdb/mdb/mdbinprogress.html) is identical to that of strain 8325-4 reported here and to that of strain 8325 (Fig. 1) (www.genome.ou.edu/staph.html) whereas the Cowan EbpS protein is identical to that of strain EMRSA-16 (www.sanger.ac.uk/Projects/S_aureus). In contrast, the EbpS proteins of N315 and Mu50 (39) are identical to each other and are different from the Cowan protein at the 9th residue position and from the 8325 protein at the 19th residue position. The locations of the residue differences between the various EbpS proteins are summarized in Fig. 1.

We conclude that a sequencing error was made in the Cowan *ebpS* DNA sequence in the original study by erroneous insertion of a G at the third position of codon 201. This created a translational frameshift leading to apparent termination two codons downstream.

The EbpS protein is distinct from typical *S. aureus* MSCRAMMs, because it lacks a secretory signal sequence at the N terminus and an LPXTG motif and hydrophobic domain at the C terminus (11). The ligand-binding domain of EbpS is located between residues 14–34 at the N terminus (15) (Fig. 1). Three hydrophobic domains occur in the center of the molecule between residues 205 and 224, 265 and 280, and 315 and 342. The first two hydrophobic domains are 75% identical and are preceded by six identical residues. Only the first and the third domain were predicted to span the cytoplasmic membrane by the program TMHMM (41). A 50-residue LysM domain occurs at the C terminus. The LysM domain is widespread in prokaryotes and is proposed to bind to peptidoglycan (42). It is found mainly in cell wall-degrading enzymes and is presumed to anchor catalytic domains to their substrates. It is also found in other envelope-associated proteins such as protein A of *S. aureus* and intimin of enteropathogenic *E. coli*.

The *ebpS* loci of *S. aureus* strains 8325-4, EMRSA-16, and COL show significant organizational similarity to a discrete region of the *Bacillus subtilis* and *Enterococcus faecalis* genomes centering on the *ybpE* and *alyS* genes, respectively. Proteins EbpS, YbpE (*B. subtilis*), and AlyS (*E. faecalis*) each possess LysM domains. AlyS is an autolysin with *N*-acetylmuramoyl-1-alanine amidase activity. The open reading frames that flank *ebpS*, *ybpE*, and *alyS* have organizational similarity. In *B. subtilis* this region (*recQ-cmk*) is thought to be part of a supraoperon mostly specifying enzymes involved in amino acid synthesis (43). This, combined with the atypical MSCRAMM structure, might suggest that EbpS has a function other than promoting bacterial attachment to the ECM.

Identification of the EbpS Protein by Western Immunoblotting—To facilitate analysis of the EbpS protein in *S. aureus*, a site-specific mutation was isolated in the *ebpS* gene by allele replacement. The *ebpS*::Em^r mutation contains an insertion of a 1-kb fragment encoding erythromycin resistance. The mutation was isolated by allele replacement in strain RN4220 and was then transduced into protein A-deficient (*spa*) mutants of strains 8325-4 and Newman. Protein A mutants were used to facilitate detection of the EbpS protein by Western immunoblotting. A shuttle plasmid pCU1*ebpS*$^+$ was transferred into each of the mutants for complementation experiments.

Two recombinant truncated derivatives of EbpS were expressed with 6 histidine residues at the N terminus (6xHis affinity tag) (Fig. 1). rEbpS1–267 and rEbpS343–486 were purified by Ni^{2+}-chelate chromatography and used to immunize rabbits. Western immunoblotting analysis was performed with proteins solubilized after lysis of *S. aureus* cells. Strains 8325-4 *spa* and Newman *spa* were each compared with their isogenic *ebpS*::Em^r mutants for immunoreactive protein bands that could be ascribed to the *ebpS* gene. A polypeptide of 83-kDa apparent molecular mass was present in each wild-type strain but was missing in the mutants. Furthermore, a protein of the same size was expressed in the mutant strains carrying the pCU1*ebpS*$^+$ complementing plasmid. Fig. 2 shows Western immunoblots of proteins from strain 8325-4 probed with anti-rEbpS343–486 antibodies. The same-sized protein was detected with anti-rEbpS1–267 antibodies (not shown). This clearly demonstrates that the 8325-4 *ebpS* gene product is a 83-kDa protein.

Strain Cowan expressed an immunoreactive protein with an apparent molecular mass of 79 kDa, which we assume to be the product of the *ebpS* gene (data not shown). An *ebpS* mutant of Cowan was not available for comparison. The slightly faster migration of the putative Cowan EbpS protein can be attributed to the amino acid differences compared with the 8325-4 protein. It is likely that the previously reported 25-kDa EbpS protein of strain Cowan (18) is a proteolytic degradation product.

EbpS Is Associated with the Cytoplasmic Membrane—To determine the cellular location of the EbpS protein, *S. aureus* Newman *spa* cells that had been grown to stationary phase were sedimented and resuspended in a buffer containing the osmotic stabilizer raffinose. Lysostaphin was used to solubilize the cell wall peptidoglycan to release cell-wall-associated proteins. The stabilized protoplasts were sedimented, lysed, and

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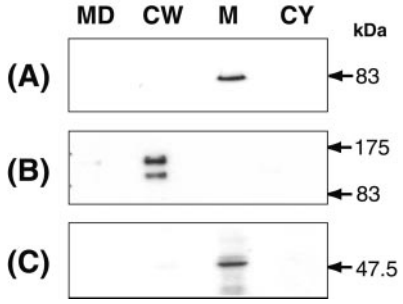

FIG. 3. **Western immunoblotting analysis of cellular fractions of *S. aureus* Newman *spa*.** The cell wall (*CW*) fraction contains proteins that were solubilized from lysostaphin-generated stabilized protoplasts. The cytoplasmic membrane (*M*) and cytoplasmic (*CY*) fractions were prepared by lysis of protoplasts. Fraction *MD* contains proteins concentrated from the growth medium. *A*, EbpS detected by anti-EbpS343–486 antibodies. *B*, ClfA detected with anti-ClfA antibodies. *C*, ATPase detected with anti-*M. luteus* ATPase antibodies. Molecular mass markers are shown in kDa.

separated into a membrane and cytoplasmic fraction by centrifugation. Each fraction was analyzed by Western immunoblotting using anti-rEbpS1–267 and anti-rEbpS343–486 antibodies. The clumping factor A (ClfA) protein was used as a marker for cell-wall-associated proteins. ClfA is anchored covalently to peptidoglycan by sortase (11). The F_1/F_0-ATPase was used as a marker for the cytoplasmic membrane (44). The presence of ClfA and ATPase in each fraction was monitored by Western immunoblotting using specific antibodies. Membrane and cytoplasmic fractions were also tested for isocitrate dehydrogenase activity, which is a cytoplasmic protein marker (49).

Fig. 3*A* clearly demonstrates that the 83-kDa EbpS protein of strain Newman *spa* is exclusively associated with the cytoplasmic membrane fraction. It was not detected in the culture supernatant, the cell wall, or the cytoplasmic fraction. When probed with anti-rEbpS1–267 antibodies, an immunoreactive protein of 83 kDa was also revealed, along with a smaller protein detected in the membrane fraction but not in other fractions. We assume that the smaller protein is a truncate lacking residues from the C terminus, because it was absent from (i) the corresponding fraction of an *ebpS*::Em^r mutant and (ii) the same material probed with anti-rEbpS343–486 antibodies (data not shown). In contrast, ClfA was associated exclusively with the cell wall fraction (Fig. 3*B*). This shows that the protoplast membrane fraction was not contaminated significantly with peptidoglycan. The anti-ATPase antibodies only reacted with a protein of 47.4 kDa in the membrane fraction (Fig. 3*C*), indicating that the membrane did not contaminate the other fractions. Of the total cellular isocitrate dehydrogenase activity, 83.8% was associated with the cytoplasm fraction whereas the cytoplasmic membrane fraction had 16.2% ($n = 3$). This indicates that there was little contamination of the membrane fraction by cytoplasmic proteins.

A membrane fraction of *S. aureus* Newman was prepared after mechanical disintegration of cells and differential centrifugation. Western immunoblotting showed that this fraction lacked the cell-wall-associated protein ClfA but did contain the 83-kDa EbpS protein (data not shown). This confirms that EbpS is firmly associated with the cytoplasmic membrane and establishes that it did not become associated with the protoplast membrane after solubilization.

Analysis of EbpS-PhoA and EbpS-LacZ Fusion Proteins—The *ebpS* gene was amplified by PCR and cloned into the *E. coli* expression vector pKK233-2 such that the entire *ebpS* open reading frame was joined to the ribosome binding site and ATG codon of the vector, allowing regulatable expression from the *trc* promoter of the vector in hosts expressing the repressor LacI. Western immunoblotting analysis of whole cell lysates revealed that an 83-kDa immunoreactive protein was expressed after induction with IPTG (data not shown). This protein was shown to be associated with the envelope fraction after mechanical disruption of cells and centrifugation (data not shown). To determine if the protein was in the membrane and not in an inclusion body, inner and outer membrane fractions were separated by isopycnic centrifugation. Western blotting with anti-EbpS antibodies showed immunoreactive proteins only in the cytoplasmic membrane fraction (data not shown). These were smaller than the 83-kDa protein detected in lysates, presumably due to proteolytic degradation during the prolonged isolation procedure.

Confident that EbpS entered the cytoplasmic membrane of *E. coli*, a series of fusions were constructed between EbpS and alkaline phosphatase (PhoA) or β-galactosidase (LacZ) downstream from the three hydrophobic domains (Fig. 1) to study protein topology. If the PhoA fusion is located distal to a transmembrane domain that spans the membrane from the cytoplasmic side to the periplasmic side, the PhoA domain is placed in the periplasm where it becomes enzymatically active (45). However, if the fusion is linked to a transmembrane domain that spans the membrane from the periplasmic side to the cytoplasmic side, the PhoA domain is located in the cytoplasm and cannot become enzymatically active. Conversely an EbpS-LacZ fusion will only be enzymatically active if it occurs distal to a transmembrane domain that spans the membrane in the outside-inside direction. The large tetrameric LacZ protein is only active in the cytoplasm, because it is too large to be secreted into the periplasmic space.

Using pKK233*ebpS* as the template, truncated derivatives of the *ebpS* gene lacking varying lengths of 3′-DNA were amplified by PCR along with the pKK233-2 promoter and ribosome binding site and cloned into the *phoA* vector pBAF and the *lacZ* vector pLKC480 to produce *ebpS-phoA* and *ebpS-lacZ* fusions, respectively. Both fusions were expressed from the imported *trc* promoter. The pLKC480*ebpS-lacZ* fusions were controlled by LacI expressed by co-resident plasmid pDIA17 to avoid problems of toxicity that occurred even when small amounts of the proteins were expressed in a leaky lacIq host. Three fusions were made in the putative loop regions between hydrophobic domains H1–H2 and H2–H3, and two fusions were made at the C terminus of EbpS downstream from H3 (Fig. 1).

The alkaline phosphatase or β-galactosidase activities of the fusions (as appropriate) are shown in Fig. 4. Only the C-terminal fusions at Asp-369 and Asp-402 showed detectable PhoA activity indicating that these fusions promoted exposure of PhoA in the periplasm, whereas those occurring between hydrophobic domains H1–H2 and H2–H3 are located in the cytoplasm. This was in direct contrast to the LacZ fusions at the same residues, where the proximal fusions between H1–H2 and H2–H3 gave high levels of β-galactosidase, whereas the C-terminal fusions were almost devoid of activity. Western immunoblotting analysis with anti-PhoA antibodies indicated that each of the EbpS-PhoA fusions expressed an immunoreactive protein of between 100 and 150 kDa (Fig. 5*A*). The size of the proteins increased in proportion to the length of EbpS sequence incorporated. Similar results were obtained with anti-EbpS1–267 antibodies (data not shown). This shows that the lack of enzymatic activity was not due to lack of protein expression. Each of the EbpS-LacZ fusions expressed a single major immunoreactive protein (Fig. 5*B*) apart from the inactive Asp-369 and Asp-402 fusions. In the last two samples, several smaller immunoreactive bands were sometimes detected, which indicates that extensive degradation was occurring. After induction, cells bearing these two mutants grew very

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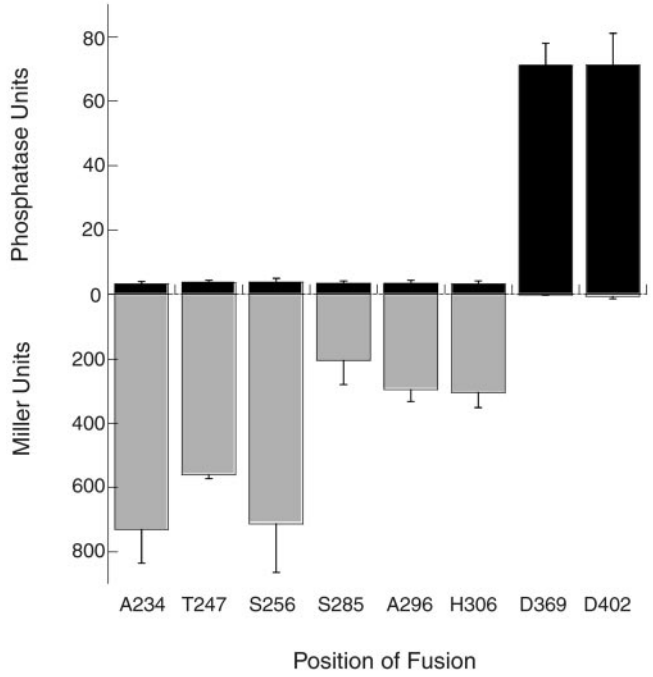

FIG. 4. **Enzymatic activity expressed by EbpS-PhoA and EbpS-LacZ fusions.** Alkaline phosphatase (AP) activities of EbpS-PhoA fusions were expressed in *E. coli* CC118 (mean specific activities of three independent experiments). Background levels of AP activity from CC118 (pBAF) were subtracted. β-Galactosidase activities of EbpS-LacZ fusions were expressed in *E. coli* TG1 (pDIA17) (mean specific activities of three independent experiments). Background enzyme activity from TG1(pDIA17/pLKC480) was subtracted.

slowly, which is consistent with synthesis of a toxic protein.

The EbpS-PhoA and EbpS-LacZ fusion data allows two models to be proposed for the membrane topography of EbpS (Fig. 6). In both models, the N-terminal and the C-terminal domains are located on the outer face of the cytoplasmic membrane whereas the central part of the protein is on the cytoplasmic face. The middle hydrophobic domain H2 might be membrane-associated but does not traverse the membrane.

Whole Cell Immunoblotting—Whole cell immunoblotting was used to determine if the N-terminal domain of EbpS is surface-exposed. *S. aureus* strains Newman *spa*, Newman *spa ebpS*, and Newman *spa ebpS* (pCU-*ebpS*$^+$) were grown to stationary phase and probed with anti-rEbpS1–267 and anti-rEbpS343–486 antibodies. EbpS was not detected on the surface of strains Newman *spa* or its *ebpS* isogenic mutant by either antibody but was recognized on the surface of the pCU-*ebpS*$^+$-bearing strain by anti-rEbpS1–267 antibodies (data not shown). Because EbpS is expressed at an ~8-fold higher level in the complemented strain compared with wild-type (data not shown), we assumed that elevated expression of the protein was required to allow detection of EbpS on the cell surface by antibody. This may explain the lack of detection of EbpS by anti-rEbpS1–267 antibodies with the wild-type cells. The failure of anti-rEbpS343–486 antibodies to bind suggests that the C terminus of EbpS is not surface-exposed.

EbpS Promotes Binding of S. aureus to Soluble Elastin Peptides and to Tropoelastin—Previous studies with *S. aureus* Cowan demonstrated that EbpS promotes the interaction of *S. aureus* cells with the soluble elastin precursor tropoelastin and with soluble degradation products of elastin (α-elastin) (15, 18). We have compared the ability of *S. aureus* Newman, Newman *ebpS*::Em^r, and Newman *ebpS*::Em^r bearing the complementing plasmid pCU1*ebpS*$^+$ to bind ^{125}I-labeled tropoelastin (Fig. 7). The *ebpS* mutant showed a 72% reduction in ^{125}I-tropoelastin binding. Binding was reduced to background levels by incubation with excess cold elastin peptides, reaching the same level as the *ebpS* mutant incubated with cold peptides. The complementing plasmid pCU1*ebpS*$^+$ restored elastin binding to the same level as the parental strain. These data indicate that EbpS is the dominant elastin-binding factor of *S. aureus* Newman but also suggest that the bacteria express a second elastin-binding moiety.

Growth of an ebpS Mutant Is Impaired—Preliminary observations indicated that broth cultures of the *ebpS*::Em^r mutant of strain 8325-4 grew less well than the wild-type. To show unambiguously that the loss of EbpS caused the growth defect, the *ebpS* mutant was complemented with a single-copy integrating plasmid pCL84 bearing a wild-type copy of *ebpS*, and growth was compared with the *ebpS* mutant carrying the empty vector.

No difference in the rate of cell division in the exponential phase of growth (division time) was detected when the EbpS$^+$ and EbpS$^-$ isogenic pair were compared. However, a consistent difference was noted between the densities of the cultures at stationary phase after 18 h growth (Table I). The difference in growth yield was confirmed by dry weight measurements. These data suggest that the primary role of EbpS might not be to promote bacterial attachment to soluble elastin but that it might be involved in sensing the environment or in nutrient transport.

DISCUSSION

The elastin-binding protein EbpS of *S. aureus* strain Cowan was originally reported to be a surface-associated protein of 25 kDa comprising 202 residues (18). Here we demonstrate that the *ebpS orf* of strain Cowan in fact comprises 1461 bp and encodes an EbpS protein of 486 residues. The EbpS protein of 8325-4 is the same size and is 95% identical. The EbpS protein of strain COL is identical to that of strain 8325-4 whereas the EbpS proteins of Cowan and EMRSA-16 are also identical to each other. The EbpS proteins predicted from genome sequences of strain N315, Mu50, and MSSA are also 486 residues. It is now clear that an error occurred in the original DNA sequence of the Cowan *ebpS* gene, which placed a frameshift in codon 201 and caused an apparent termination two codons downstream.

By careful analysis of proteins solubilized during lysis of protoplasts, we have shown that the EbpS proteins of *S. aureus* Newman and 8325-4 migrate in SDS-PAGE with an apparent molecular mass of 83 kDa. Using antibodies raised against recombinant EbpS protein fragments, the EbpS protein expressed by strains 8325-4 and Newman was identified by Western immunoblotting by comparison with the appropriate *ebpS*::Em^r mutant and the mutant complemented with a plasmid carrying the wild-type 8325-4 *ebpS* gene. In these experiments, lysis was achieved quickly and in the presence of protease inhibitors so it is most likely that the 25-kDa EbpS protein described previously (18) was a ligand-binding N-terminal truncate that was cleaved from the body of EbpS by bacterial proteases during the extraction procedure.

Analysis of the cell envelope of EbpS$^+$ *S. aureus* indicated that EbpS is strongly associated with the cytoplasmic membrane. When stable protoplasts of *S. aureus* were formed by lysostaphin digestion of peptidoglycan, the EbpS protein remained firmly associated with the membrane fraction, whereas ClfA, a cell-wall-associated protein anchored to peptidoglycan by the LPDTG motif and sortase, was completely solubilized. It could be argued that EbpS was originally in the wall fraction but became associated with the membrane during protoplast formation. This is unlikely, because EbpS was also detected in a membrane fraction prepared by mechanical disintegration

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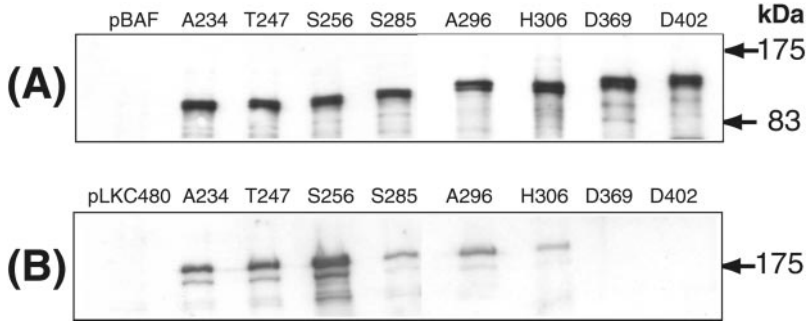

FIG. 5. **Western immunoblotting analysis of EbpS-PhoA and EbpS-LacZ fusions.** *A*, EbpS-PhoA fusion proteins in the total membrane fraction of *E. coli* CC118 (pBAF*ebpS-phoA*) detected with polyclonal anti-PhoA antibodies. *B*, EbpS-LacZ fusions proteins in total envelope fraction of *E. coli* TG1 (pDIA17, pLKC480*ebpS-lacZ*) detected with anti-β-galactosidase antibodies.

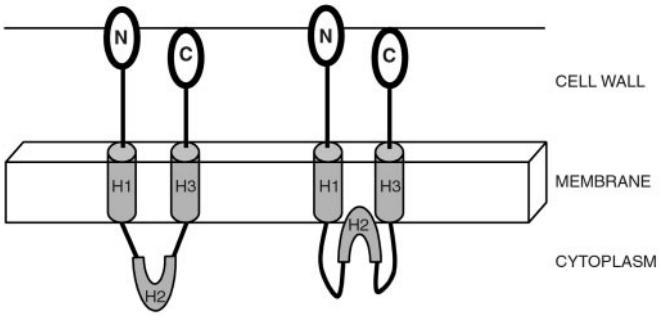

FIG. 6. **Schematic figure showing the deduced topology of EbpS in the cytoplasmic membrane.** The hydrophobic domain H2 could be in one of two possible orientations.

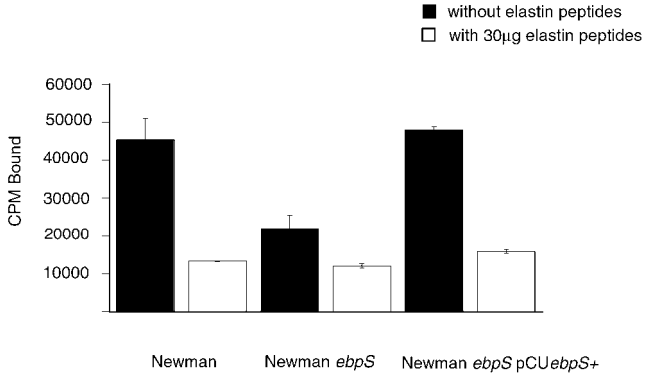

FIG. 7. **Binding of radioiodinated human tropoelastin to stationary phase strain Newman *spa* and isogenic mutants.** *S. aureus* cells (1×10^8 colony-forming units) were incubated with 40 ng of ^{125}I-labeled elastin peptides in the absence or presence of excess unlabeled elastin peptides (30 μg) for 1 h at room temperature. Radioactivity associated with cells is presented as the mean cpm bound ± S.D. of triplicate determinations.

TABLE I
Optical densities and dry weights of S. aureus strains grown in chemically defined media (CDM)

Data were duplicate measurements from three independent cultures of each strain. Pairwise Student's *t* tests were performed between EbpS$^+$ and EbpS$^-$ strains.

Strain	$A_{600\ nm}$	Dry weight
		g/liter
8325-4	5.83 ± 0.10 [a]	1.79 ± 0.02 [a]
8325-4 *ebpS*	4.65 ± 0.14	1.50 ± 0.04
8325-4 *ebpS* (pCL84:*ebpS*)	5.98 ± 0.08 [a]	1.80 ± 0.02 [a]
8325-4 *ebpS* (pCL84)	4.75 ± 0.10	1.50 ± 0.05

[a] Indicates p = <0.0001.

and differential centrifugation.

Examination of the amino acid sequence and analysis by secondary structure prediction algorithms suggested three hydrophobic domains in EbpS, two of which were predicted to span the cytoplasmic membrane. The N terminus of the protein must be located on the extracellular face of the membrane because (i) residues 14–34 comprise the ligand-binding domain, (ii) intact bacterial cells bind to elastin via the N-terminal domain, and (iii) antibodies to the N-terminal recombinant protein rEbpS1–267 bound to intact *S. aureus* cells.

EbpS was shown to be an integral membrane protein with two transmembrane domains by analyzing EbpS-PhoA and EbpS-LacZ expressed in *E. coli*. Initially the wild-type EbpS protein was expressed from the ATG vector pKK233-2. Cell fractionation experiments showed that EbpS was associated with the cytoplasmic membrane of *E. coli*. Expression of alkaline phosphatase activity and β-galactosidase activity by the fusions is consistent with the model shown in Fig. 6, where both the N-terminal domain lying proximal to the hydrophobic region and the C-terminal domain located distal to the hydrophobic region are exposed on the outer face of the cytoplasmic membrane. However, only the N terminus is surface-exposed as indicated by whole cell immunoblots performed with anti-EbpS1–267 and EbpS343–486 antibodies where a positive reaction was only obtained with the former. Comparison of the predicted amino acid sequences of the EbpS proteins from seven different strains reveals that amino acid substitutions are mainly clustered between residues 170–258 (Fig. 1). The hydrophobic domain H1 is identical in all strains, suggesting that its sequence is important for function.

The elastin-binding protein was previously suggested to be a microbial surface protein recognizing adhesive matrix molecules (MSCRAMM). These proteins typically promote bacterial attachment to components of the extracellular matrix and are likely to be important in the pathogenesis of tissue and wound infection by promoting bacterial adhesion and colonization. *S. aureus* expresses surface protein MSCRAMMs that promote attachment to collagen, fibronectin, fibrinogen, and von Willebrand factor. In each case the bacterial MSCRAMM promotes adhesion of bacterial cells to the pure ligand immobilized on an inert surface and also binding of bacterial cells to the soluble ligand. Each of these MSCRAMMs is a member of the family of proteins that contain the LPXTG motif and are covalently anchored to peptidoglycan by sortase. However, not all surface proteins that bind to host proteins are in the LPXTG family. Thus the MHCII analogous protein (46), also called extracellular adhesive protein (47), and coagulase (48) are loosely associated with the cell wall and are found in large amounts in the growth media (coagulase) or can be released from cells by treatment with LiCl (Map). The internalin B protein of *Listeria monocytogenes* is attached to the bacterial cell surface by a specific interaction with bacterial lipoteichoic acid mediated by C-terminal repeats (49). EbpS is unique among MSCRAMMs by lacking a cleavable N-terminal signal sequence associated with secreted proteins and is an integral membrane protein. We have shown clearly that EbpS promotes the binding of soluble elastin products and the tropoelastin precursor.

One can only speculate about the *in vivo* significance of

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EbpS. Similar to other *S. aureus* MSCRAMMs, EbpS may function to facilitate colonization of injured tissues abundant in elastin, such as skin and lung, whose elastic fiber is exposed and available for bacterial recognition. *S. aureus* expresses a cysteine protease with potent elastolytic activity (50). Elastin peptides are chemotactic for neutrophils as a signal of tissue damage (40), so it is possible that EbpS is a part of a signal transduction system providing early warning of an impending neutrophil influx. In this regard it is interesting to note that the EbpS protein located in the membrane of *S. aureus* reacts with the bi-functional cross-linking reagent dithiobis(succinimidyl propionate) to form a single multimeric protein of ~250 kDa,[3] which might be consistent with the protein being involved in transport or signaling, functions where it is unlikely to be active as a monomer. The fact that the EbpS mutants exhibited a reduction in yield, when growing in defined synthetic medium, suggests that the protein might also have a role in nutrient transport or signal transduction. If this is the case, it is likely that the cytoplasmic domain of EbpS located between residues 225 and 314 interacts with one or more other proteins in the cytosol. We propose to test this hypothesis by bio-panning methods.

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