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Identification of Antigenic Components of *Staphylococcus epidermidis* Expressed during Human Infection

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A spectrum of in vivo-expressed *Staphylococcus epidermidis* antigens was identified by probing a bacterio-phage lambda library of *S. epidermidis* genomic DNA with human serum from infected and uninfected individuals. This analysis resulted in identification of 53 antigen-encoding loci. Six antigenic polypeptides were expressed from these loci and purified. These polypeptides were the propeptide, mature amidase, and repeat sequence domains of the major autolysin AtlE, GehD (lipase), and two members of a conserved family of surface proteins (ScaA [AaE] and ScaB). AtlE, ScaA, and ScaB all exhibit human ligand binding capacity. Screening a bank of human serum samples revealed that there were significant increases in the amounts of reactive immunoglobulin G in infected individuals compared to the amounts in healthy individuals for the repeat sequence and mature amidase domains of AtlE, ScaB, and GehD. Vaccination of mice with recombinant antigens stimulated an immune response which in vitro opsonized *S. epidermidis*. In this study we identified prospective candidate antigens for prophylaxis or immunotherapy to control disease.

Staphylococcus epidermidis is a common cause of nosocomial infections, which have significant rates of morbidity and mortality (26) and can be difficult to treat with conventional antibiotics (39). An inhabitant of human skin, this bacterium is the most common organism causing infection of cerebrospinal fluid shunts and endocarditis of prosthetic valves and catheters (20). Resistance to a wide range of antibiotics is now common in *S. epidermidis*, and many isolates are resistant to erythromycin, clindomycin, chloramphenicol, tetracycline, and methicillin (2, 3, 18, 34). Recently, isolates with reduced susceptibility to vancomycin have been observed (19). Thus, there have been renewed efforts to identify suitable immunotherapeutic or prophylactic targets. Production of an effective subunit vaccine or monoclonal antibody requires identification of surface antigens that are recognized by the immune system and, critically, are expressed during infection.

Identification of suitable targets for opsonizing anti-*S. epidermidis* antibodies provides a potential alternative to antimicrobial chemotherapy. Different techniques have been used to identify candidate antigens in a range of pathogenic bacteria, including the closely related organism *Staphylococcus aureus*.

Bioinformatics have been used to identify putative surface and secreted proteins in *Streptococcus pneumoniae* and *Neisseria meningitidis* (37, 44). However, this method does not eliminate the need for expression and purification of suitable amounts of protein in a heterologous system. In a study of *S.*

pneumoniae carriage using an experimental human colonization model McCool et al. identified a serum immunoglobulin G (IgG) and secretory IgA response to pneumococcal surface protein A as a result of colonization; individuals who did not become colonized after inoculation had preexisting antibodies to this protein (31).

Using randomly fragmented DNA of *S. aureus* in a bacterial expression library, which was subsequently screened with serum from infected patients, resulted in identification of a range of antigenic proteins. However, this required appropriate cloning of all expressed genes in frame with the fusion protein, and the target proteins had to be amenable to expression in *Escherichia coli* (11). More recently, antigenic components of *S. epidermidis* have been identified by Western blotting of cellular extracts with serum from experimentally infected rabbits (40).

In a recent study Clarke et al. (9) used a bacteriophage lambda expression library of *S. aureus* DNA, which did not require in-frame cloning into vector DNA, that was screened with convalescent patient serum to identify candidate antigens for prophylaxis. Recombinant polypeptides were then used to determine specific antibody titers in a large collection of human sera by an enzyme-linked immunosorbent assay (ELISA). Significantly higher titers of antibodies to several proteins were observed in individuals who were not nasal carriers of *S. aureus* at the time of serum collection than in individuals who were carriers. This led to the hypothesis that an increased antibody titer protected against nasal carriage, which was confirmed by protection studies performed with cotton rats.

In recent years, studies of surface proteins of *S. aureus* have led to development of several potentially efficacious immunological therapeutic and prophylactic strategies for control of this organism (15). A donor serum with a high anti-ClfA IgG titer has been shown to have the potential to reduce sepsis caused by *S. aureus* and mortality in infants with very low birth weights (6, 42). Currently, there is much research in which

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
S. epidermidis strains		
138	Clinical isolate	32
Hay	ATCC 55133	ATCC
E. coli strains		
XL-1 Blue MRF′	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* (F′ *proAB lacI^{q}ZΔM15* Tn5 [Tc^r])	Stratagene
XLOLR	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA thi-1 recA1 gyrA96 relA1 lac* (F′ *proAB lacI^{q}ZΔM15* Tn5 [Tc^r]) Su$^-$ λ^r	Stratagene
BL21(DE3)	F$^-$ *ompT gal* (*dcm*) (*lon*) *hsdS$_B$* (r$_B^-$ m$_B^-$)	Novagen
Plasmids		
pET21-a	His$_6$-tagged overexpression vector; Ap^r	Novagen
pET24-d	His$_6$-tagged overexpression vector; Km^r	Novagen
pMA	pET24-d containing internal fragment encoding AtlE amidase domain; Km^r	This study
pML	pET24-d containing internal fragment encoding GehD; Km^r	This study
pRSN	pET21-a containing internal fragment encoding AtlE repeat sequence; Ap^r	This study
pPP	pET24-d containing internal fragment encoding AtlE propeptide; Km^r	This study
pScaA	pET24-d containing internal fragment encoding ScaA; Km^r	This study
pScaB	pET24-d containing internal fragment encoding ScaB; Km^r	This study

workers are trying to develop humanized anti-ClfA monoclonal antibodies for treatment of *S. aureus* infections (10, 21, 36), which could be used for passive immunotherapy. The collagen adhesin, Cna, has been proposed as a target for anti-adhesive antibody therapy (43) and as a vaccine component (33). It has also been suggested that FnBPA and FnBPB could be targets for passive immunotherapy (16, 38).

In this study we identified antigenic components of *S. epidermidis* using a bacteriophage lambda expression library screened with sera from patients with confirmed *S. epidermidis* and *S. aureus* infections. The relative titers of serum IgG reactive to the antigens were determined by ELISA. Polyclonal antibodies generated from these recombinant polypeptides were shown to be opsonic in vitro, demonstrating their potential therapeutic efficacy.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani medium, using selection with ampicillin (100 μg/ml) or kanamycin (50 μg/ml) when appropriate. *S. epidermidis* was grown in brain heart infusion medium (Oxoid).

Construction of *S. epidermidis* expression libraries. Genomic DNA of *S. epidermidis* 138 was partially digested with Sau3A to generate 2- to 10-kb fragments which were ligated into bacteriophage lambda DNA and packaged into phage particles by using a Lambda ZAP Express kit (Stratagene) according to the manufacturer's instructions.

Human sera. Eighteen sera were used to screen the genomic expression library. Since one element of this study was to identify antibodies which cross-react with proteins from different species of staphylococci, a variety of sources of sera were used. Of the 18 samples used in the screening process, 4 were obtained from hospital workers, 4 were obtained from community volunteers, 4 were obtained from patients with *S. epidermidis* infections, and 6 were obtained from patients with *S. aureus* infections. All samples were collected at the Royal Hallamshire Hospital, Sheffield, United Kingdom; the individuals gave informed consent, and the study was approved by the local IRB (SSREEC/02/299). For ELISA experiments, serum samples from 15 individuals with confirmed *S. epidermidis* infections, collected at Aston University, Birmingham, United Kingdom, were used along with 10 samples from community volunteers collected at the Royal Hallamshire Hospital, Sheffield, United Kingdom.

Screening of expression libraries with human sera. Bacteriophage were propagated, and plaque lifts were made on Immobilon-NC membranes (Millipore) and probed according to the manufacturer's instructions with human sera diluted

1:1,000 and then with alkaline phosphatase-conjugated anti-human IgG gamma chain-specific monoclonal antibodies (Sigma) diluted 1:50,000. Additional rounds of screening resulted in pure phages from which phagemids were excised (according to the manufacturer's instructions).

Phagemid DNA was purified using QIAprep spin miniprep columns, and DNA was sequenced using primers T3 and T7. BLAST-N searches using sequences from either end of the insert were carried out against the *S. epidermidis* ATCC 12228 genome (http://www.tigr.org).

E. coli XLOLR containing excised phagemids was grown overnight in the presence of 10 μM isopropyl-β-D-thiogalactopyranoside (IPTG), cells were boiled in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) buffer, and the proteins were separated by 12% (wt/vol) SDS–PAGE prior to Western blotting and probing with human serum, which were carried out by using standard laboratory methods, as described previously (8).

Cloning and overexpression of recombinant polypeptides. Oligonucleotides were used to PCR amplify antigen-encoding genes (Table 2). DNA fragments were cloned into pET24-d or pET21-a. Clones were verified by DNA sequencing. His$_6$-tagged recombinant polypeptides were expressed, purified, and verified by N-terminal sequencing.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]
T3	AATTAACCCTCACTAAAGGG
T7	GTAATACGACTCACTATAGGG
Amidase F	GCGCGCC<u>CCATGG</u>TATCTAGTCAAAAAACATCATC
Amidase R	GCGCGCC<u>CTCGAG</u>TGATGGTGCAGTTAAATAGTA
RSN F	GCGCGCC<u>CATATG</u>ACAACATCTACAAAACCGTC
RSN R	GCGCGCC<u>CTCGAG</u>TGATGGTGCAGTTAAATAGTA
ML F	GCGCGCC<u>CCATGG</u>CGCAAGCTCAATATAA
ML R	GCGCGCC<u>CTCGAG</u>CTTACGTGTAATACCATCTAACTC
ScaA F	GCGCGCC<u>CCATGG</u>CAACAACGCATACAGT
ScaA R	GCGCGCC<u>CTCGAG</u>ATGAATAAATTTGTAATTTCTCACT
ScaB F	GCGCGCC<u>CCATGG</u>CTTCTACACAACATAAGGTTC
ScaB R	GCGCGCC<u>CTCGAG</u>GTGGATAAATGCATATGAAGA

[a] Restriction endonuclease sites are underlined.

TABLE 3. Identification of *S. epidermidis* loci encoding antigens recognized by human sera[a]

Locus	Locus coordinates	Gene in locus (cloned region amino acids)	Designation, putative function, and/or homology
1	735135–739142	SE0750 (45–1053)	AtlE, major autolysin
2	1281499–1283139	SE1250 (1–137)	Conserved hypothetical protein
		SE1251 (1–127)	Conserved hypothetical protein
		SE1252 (1–85)	Glycyl-tRNA synthetase
3	2430659–2432743	SE2349 (1–422)	Xanthine permease
		SE2350 (1–192)	Xanthine phosphoribosyltransferase
4	2142357–2144445	SE2098 (40–350)	Alcohol dehydrogenase
		SE2099 (1–255)	Conserved hypothetical protein
5	469921–471793	SE0477 (1–368)	CsbB stress response protein
		SE0478 (190–322)	Histidine kinase
6	428348–430455	SE0432 (113–336)	Low-affinity inorganic phosphate transporter
		SE0433 (1–266)	Secretory antigen SsaA-like protein
7	2401382–2403540	SE2318 (343–501)	Poly(glycerol-phosphate) alpha-glucosyltransferase
		SE2319 (1–324)	Aae, autolysin
8	1431038–1433713	SE1394 (1–156)	Conserved hypothetical protein
		SE1396 (1–200)	30S ribosomal protein S4
9	2421512–2423462	SE2337 (1–342)	PfoR, regulatory protein
		SE2339 (1–103)	Conserved hypothetical protein
10	2192554–2194685	SE2146 (1–151)	Transcription regulator
		SE2147 (1–378)	Amino acid transporter
11	1640739–1643296	SE1587 (315–511)	High-affinity proline permease
		SE1588 (1–402)	Conserved hypothetical protein
12	465589–468072	SE0473 (1–390)	*N*-Acetylglucosamine-6-phosphate deacetylase
		SE0474 (1–139)	Conserved hypothetical protein
13	1979057–1981257	SE1945 (1–439)	L-Lactate permease LctP-like protein
		SE1946 (1–178)	Conserved hypothetical protein
14	1925763–1928363	SE1889 (37–295)	Conserved hypothetical protein
		SE1890 (1–474)	Phosphotransferase system sucrose-specific IIBC component
		SE1891 (1–124)	RpiR, transcription regulator
15	980757–978852	SE0971 (309–514)	Conserved hypothetical protein
		SE0972 (1–121)	Conserved hypothetical protein
		SE0973 (1–164)	Conserved hypothetical protein
16	1941916–1943617	SE1905 (1–293)	Dehydrogenase
		SE1906 (198–372)	Amino acid amidohydrolase
17	129349–135120	SE0137 (1–581)	Cap5B, capsular polysaccharide synthesis enzyme
		SE0138 (1–115)	ArsD, arsenic resistance operon
		SE0139 (1–106)	Arsenic resistance operon repressor-like protein
		SE0140 (1–294)	Putative membrane protein
18	1513472–1516321	SE1464 (1–365)	*O*-Succinylbenzoic acid-coenzyme A ligase
		SE1466 (1–167)	Conserved hypothetical protein
19	175644–179837	SE0185 (155–643)	GehD
		SE0186 (1–132)	Conserved hypothetical protein
		SE0187 (1–86)	Conserved hypothetical protein
		SE0188 (1–293)	Cobalamin synthesis-related protein
20	756962–759182	SE0768 (1–494)	PurF
		SE0769 (1–207)	PurM

Continued on following page

TABLE 3—*Continued*

Locus	Locus coordinates	Gene in locus (cloned region amino acids)	Designation, putative function, and/or homology
21	804022–806137	SE0812 (153–372) SE0813 (1–355)	Conservedhypotheticalprotein Pyruvate carboxylase
22	1658497–1660726	SE1602 (132–309) SE1603 (1–459)	Manganese-dependent inorganic pyrophosphatase Aldehyde dehydrogenase
23	2248831–2250776	SE2197 (374–489) SE2198 (23–514)	Alkaline phosphatase III precursor Conserved hypothetical protein
24	334901–337600	SE0331 (933–1065) SE0332 (1–500)	Fbe Poly(glycerol-phosphate) alpha-glucosyltransferase
25	1837430–1839195	SE1779 (1–348) SE1780 (157–289)	Alkanal monooxygenase alpha chain Conserved hypothetical protein
26	2283238–2285302	SE2228 (105–646) SE2229 (1–141)	Phosphotransferase system fructose-specific II component Mannose-6-phosphate isomerase
27	1602487–1603234	SE1548 (79–328)	Glutamate-1-semialdehyde aminotransferase
28	2086731–2089154	SE2044 (1–452) SE2045 (326–513)	Gluconate permease Gluconokinase
29	2345106–2346500	SE2272 (230–695)	Cell division protein
30	2376544–2377407	SE2303 (1–61)	Spermidine acetyltransferase
31	263229–263653	SE0263 (165–306)	Drp35 protein
32	2492025–2494409	SE2411 (1–127) SE2412 (1–119) SE2413 (1–53) SE2414 (1–279) SE2415 (125–239)	Conserved hypothetical protein Conserved hypothetical protein Hypothetical protein DNA-binding Spo0J-like protein Glucose-inhibited division protein B
33	2454565–2456586	SE2380 (143–391) SE2381 (1–439)	Putative cystathionine beta-lyase Conserved hypothetical protein
34	2481293–2483748	SE2400 (201–252) SE2401 (1–626) SE2402 (1–63)	VraD VraE Conserved hypothetical protein
35	1369989–1372476	SE1340 (138–186) SE1341 (1–355) SE1342 (156–427)	DNA-3-methyladenine glycosidase Conserved hypothetical protein Glutamate-1-semialdehyde 2,1-aminomutase
36	585999–587077	SE0594 (1–18) SE0595 (1–117) SE0596 (1–118)	Thioredoxin Arsenate reductase Glycine cleavage system protein H
37	1247787–1250204	SE1211 (1–310) SE1212 (1–341) SE1213 (88–185)	Glutamate *N*-acetyltransferase *N*-Acetylglutamate gamma-semialdehyde dehydrogenase Elongation factor EF-P
38	2387434–2389445	SE2309 (1–311) SE2310 (1–273)	Conserved hypothetical protein LysR-type transcriptional regulator
39	1940963–1942972	SE1903 (1–66) SE1904 (1–203) SE1905 (4–293)	Conserved hypothetical protein Conserved hypothetical protein Dehydrogenase
40	465589–467653	SE0472 (591–650) SE0473 (1–390)	Phosphotransferase system fructose-specific IIABC component *N*-Acetylglucosamine-6-phosphate deacetylase

TABLE 3—*Continued*

Locus	Locus coordinates	Gene in locus (cloned region amino acids)	Designation, putative function, and/or homology
41	813245–815819	SE0819 (1–308)	Glycerophosphoryl diester phosphodiesterase
		SE0820 (1–39)	Hypothetical protein
		SE0821 (1–83)	Conserved hypothetical protein
		SE0822 (1–129)	Conserved hypothetical protein
42	177914–179837	SE0187 (46–86)	Conserved hypothetical protein
		SE0188 (1–293)	CobW
		SE0189 (193–460)	Ferrous iron transporter protein B
		SE0190 (1–61)	Hypothetical protein
43	469456–469924	SE0475 (113–269)	Plant metabolite dehydrogenase
44	152788–156004	SE0165 (1–252)	Two-component response regulator
		SE0166 (1–512)	Two-component sensor histidine kinase
		SE0167 (24–324)	BitC
45	2044647–2046972	SE2007 (282–505)	Beta-lactamase
		SE2008 (1–278)	Lipoprotein precursor
		SE2009 (133–310)	2-Dehydropantoate 2-reductase
46	972873–974051	SE0966 (125–264)	Conserved hypothetical protein
		SE0967 (1–215)	Putative 2-oxoacid ferredoxin oxidoreductase alpha subunit
47	240928–242180	SE0244 (38–207)	Conserved hypothetical protein
		SE0245 (1–124)	Lipase precursor
48	1845438–1846232	SE1786 (1–153)	Galactose-6-phosphate isomerase LacB subunit
		SE1787 (36–142)	Galactose-6-phosphate isomerase LacA subunit
49	1016461–1018676	SE1011 (1–306)	Homoserine kinase
		SE1012 (1–268)	Conserved hypothetical protein
50	169874–172149	SE0180 (1–451)	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase
		SE0181 (1–301)	8-Amino-7-oxononanoate synthase
51	890136–893481	SE0894 (1–247)	Putative PP2C protein phosphatase
		SE0895 (1–667)	Protein kinase
52	2070338–2072937	SE2032 (1–272)	Glucose 1-dehydrogenase
		SE2033 (1–353)	Exopolyphosphatase
53	319239–321290	SE0318 (1–358)	Branched-chain amino acid aminotroansferase-like protein
		SE0319 (1–85)	Putative 4-diphosphocytidyl-2C-methyl-D-erythritol synthase

[a] The library was screened with four sera obtained from patients with confirmed *S. epidermidis* bacteremia, six sera obtained from patients with confirmed *S. aureus* bacteremia, four sera obtained from hospital workers, and four sera obtained from community volunteers. The locus, clone, and gene coordinates correspond to the coordinates in the *S. epidermidis* ATCC 12228 genome (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org_search = &org = ntse02). Functions were assigned according to the ATCC 12228 genome database.

ELISA. Total IgG contents in serum were determined by serial dilution (1:100 to 1:100,000) in phosphate-buffered saline (PBS). Purified human IgG (1 mg/ml to 10 ng/ml; Calbiochem) was used as the standard. One hundred microliters of a diluted sample was coated onto wells of a 96-well microtiter plate at 4°C overnight. The plates were washed three times with PBST (PBS containing 0.05% [vol/vol] Tween 20) and blocked with 5% (wt/vol) bovine serum albumin in PBST for 2 h at room temperature. After three washes with PBST, alkaline phosphatase-conjugated anti-human IgG gamma chain-specific monoclonal antibodies (Sigma) diluted 1:50,000 in PBST-bovine serum albumin were added, and the preparations were incubated for 1 h at room temperature. After three washes with PBST, antibodies were detected using the Sigma Fast *p*-nitrophenyl phosphate system (Sigma) at A_{405} (Victor plate reader [Wallac]).

To quantify specific antibodies to each antigen in serum samples, 100-μl portions (10 μg/ml in PBS) of recombinant protein were added to 96-well microtiter plates (4°C, overnight). The plates were washed and blocked as described above. One hundred microliters of a randomized 1:100 dilution serum sample (consistently within a linear range) was added to each well (1 h) at room temperature. The plates were developed as described above. The results were standardized for total IgG concentration in each serum sample.

Opsonization of *S. epidermidis*. Antibodies were raised by inoculating 8-week-old female BALB/c mice with appropriate recombinant antigens. The opsono-phagocytic (bacterial killing) activities of specific antisera were determined with an assay by using *S. epidermidis* Hay in the presence of polymorphonuclear leukocytes (PMNs) and complement, as described previously (14). Briefly, PMNs were isolated from freshly obtained human blood using PMN separation medium (Robbins Scientific) according to manufacturer's instructions. Reference antibody was mixed with ca. 2×10^6 PMNs, human complement, and ca. 3×10^4 bacteria in a sterile 96-well plate in a final volume of 100 μl. Immediately after mixing a sample of bacteria was removed from each well and plated onto tryptic agar plates with 5% (vol/vol) sheep blood (T_0 sample). The assay plate was incubated for 2 h at 37°C on an orbital shaker. A second sample was removed from each well and plated as described above (T_2 sample). The plates were incubated at 37°C for 20 to 24 h, and the percentage of bacterial killing was calculated using the following formula: $(T_0 - T_2/T_0) \times 100$.
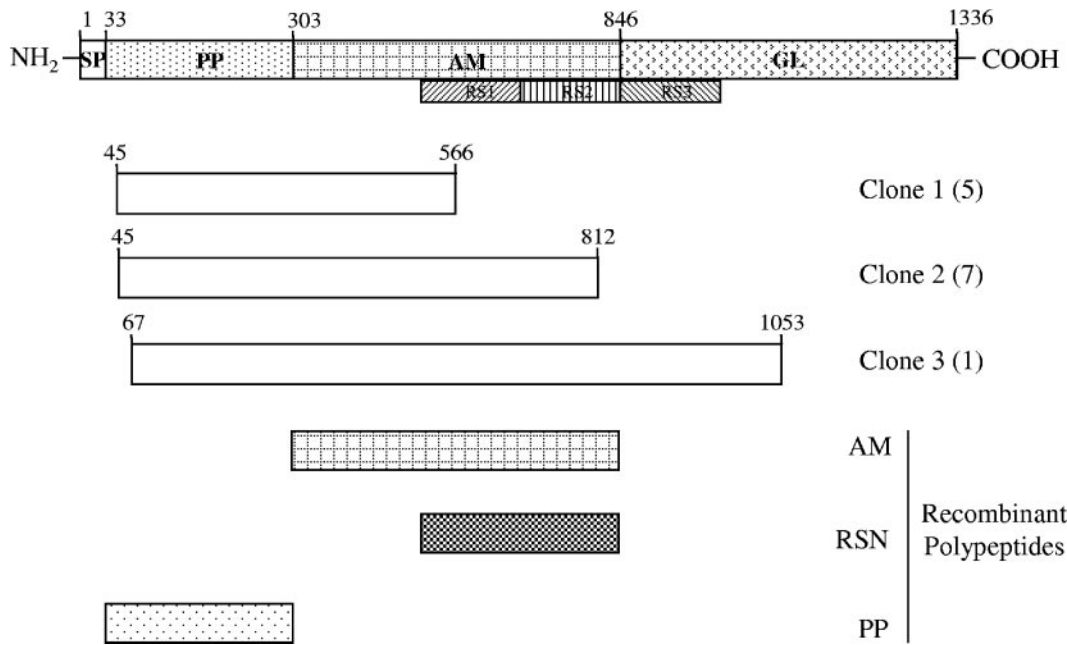
FIG. 1. Organization of *atlE* clones isolated during the screening process. The full-length nascent protein with putative signal peptide (SP), propeptide (PP), amidase (AM), and glucosaminidase (GL) domains is shown, and repeat sequence (RS) areas are also indicated. The clones isolated (with numbers in parentheses) and recombinant peptides are also indicated. The numbers indicate the positions of the relevant amino acid residues.

Ligand binding assays. Ligand binding was assayed by Western blotting, using biotinylated human serum proteins as described previously (8). Human fibrinogen, human lactoferrin, and bovine submaxillary mucin were purchased from Sigma. Human fibronectin was obtained from ICN.

RESULTS AND DISCUSSION

Identification of *S. epidermidis* antigens. In order to identify *S. epidermidis* antigens expressed during human infection, a bacteriophage lambda expression library of *S. epidermidis* 138 was probed with sera collected at the Royal Hallamshire Hospital (Sheffield, United Kingdom). Expression does not necessarily require correct in-frame cloning of insert DNA and allows production of insoluble proteins or proteins toxic to *E. coli*. Eighty-seven clones were isolated, and they corresponded to 53 different loci (comprising a contiguous DNA region from single or overlapping clones) containing 121 known or putative open reading frames of *S. epidermidis* (Table 3).

Antigenic regions of AtlE, the major autolysin of *S. epidermidis*, were identified by using overlapping clones due to the random nature of library construction (Fig. 1). Information concerning the genes in each clone is shown in Table 3. Corresponding DNA can be obtained by using clone coordinates from the *S. epidermidis* ATCC 12228 genome (http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org_search = &org = ntse02).
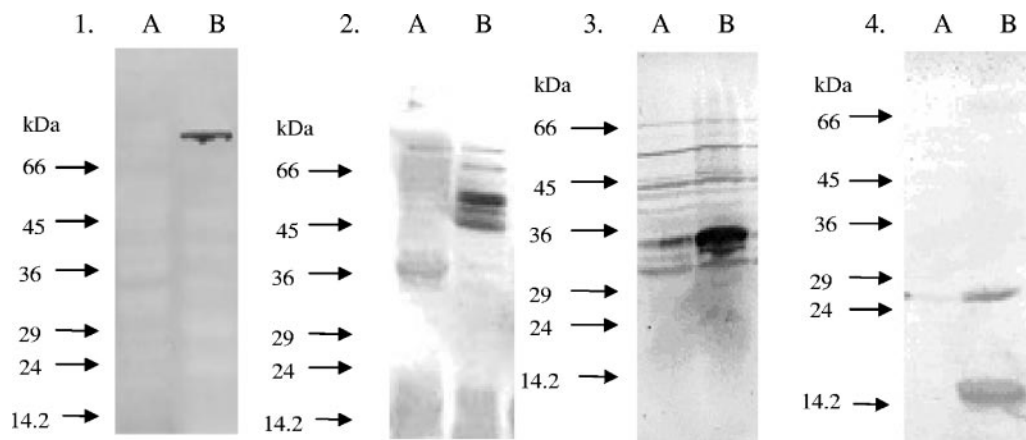

FIG. 2. Western blot analysis of antigen-encoding clones. All membranes were blotted with human sera, which identified the original clone to confirm its suitability for overexpression. In all blots, lane A contained a negative control (*E. coli* XLOLR/pBK-CMV). The proteins encoded by the clones in the blots were Atl Clone3 (blot 1), GehD (blot 2), ScaA (blot 3), and ScaB (blot 4).
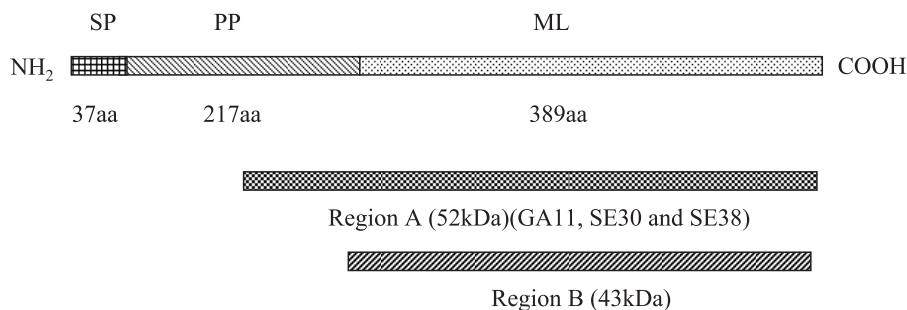
FIG. 3. Structural organization of GehD. The signal peptide (SP), propeptide (PP), and mature lipase (ML) domains are indicated, along with their lengths in amino acid (aa) residues (adapted from reference 30). Region A is the region that is present in the three identical clones identified in the serum screen. Region B encompasses the mature lipase which was used for overexpression.

In order to select suitable antigens for further study, *E. coli* phagemid clones that were representative of each individual locus were screened by Western blotting using the serum that originally identified the clone. This revealed four representative clones that exhibited high levels of reactivity (Fig. 2), corresponding to loci 1, 6, 7, and 19. In these loci there were three likely candidate antigens due to the predicted surface or secreted location, and these antigens were used for further study (AtlE, GehD, ScaA [AaE], and ScaB). All *atlE* clones isolated from the screen contained at least some of the amidase domain, the repeat sequences, and the propeptide region (Fig. 1). For this reason, recombinant peptides corresponding to the amidase domain, repeat sequences, and propeptide were generated and used in Western blotting experiments with patient serum. The amidase domain and the repeat sequence, but not the propeptide, reacted with serum (results not shown). Thus, the amidase domain and repeat sequence were used in ELISA. Clones expressing GehD represented the entire mature lipase domain and part of the propeptide (Fig. 3); hence, the mature lipase was overexpressed and used in further experiments.

AtlE, the major autolysin of *S. epidermidis*, exhibits significant structural homology with Atl, Aas, and AtlC, the major autolysins of *S. aureus*, *Staphylococcus saprophyticus*, and *Staphylococcus caprae*, respectively, and consists of both amidase and glucosaminidase domains (1, 17, 22, 25, 35). Interestingly, a recent study showed that the glucosaminidase domain of Atl is associated with *S. aureus* infection (9). In this study, we did not identify the glucosaminidase domain of AtlE as an antigen, although this may have been due to the cloning strategy employed. Thus, we confined our study to the propeptide, amidase, and central repeat sequence domains (22). In order to determine the importance of the three domains, the propeptide, mature amidase, and repeat sequence domains were overexpressed and purified (Fig. 4).

The N-terminal sequences for the recombinant proteins were checked and were found to be as follows: VSSQKT (AtlE amidase), TTSTKP (AtlE repeat sequence), AEQPQNQS (AtlE propeptide), ATTHTVK (ScaA), and AQAQYKN (GehD). All sequences were as predicted, confirming the fidelity of the cloning process. However, the N-terminal sequence of ScaB was SGGTATQ and was 127 amino acids downstream of the translation start of the recombinant protein. This corresponds to a truncated (ca. 16-kDa) form of the

protein which is produced (Fig. 4) and is most likely the result of proteolytic processing in *E. coli*.

scaA and scaB are members of a multigene family. Screening of the library with human sera resulted in identification of two proteins, SE0433 (locus 6) and SE2319 (locus 7), which are closely related to each other. Sequence analysis using CLUSTALW (http://npsa-pbil.ibcp.fr) revealed 45% identity for the entire length of both proteins and more than 60% identity in the 110-amino-acid C-terminal domains. Both proteins have apparent signal peptides, which result in mature proteins whose molecular masses are 25 kDa (SE0433) and 32 kDa (SE2319). Interestingly, these two proteins are both members of a 10-protein family in *S. epidermidis*, based on homology of the ca. 110-amino-acid C-terminal domain. We designated these proteins *s*taphylococcal *c*onserved *a*ntigen *A* (ScaA), ScaB, ScaC, ScaD, ScaE, ScaF, ScaG, ScaH, ScaI, and ScaJ (Fig. 5). SE2319 and SE0433 are ScaA and ScaB, respectively. ScaA, ScaB, and ScaE all contain one or more LysM domains, which were originally identified in enzymes that degrade bacterial peptidoglycan but are also found in many other bacterial proteins that are associated with cell walls (5). The LysM domain has a peptidoglycan-binding fold (27), and al-
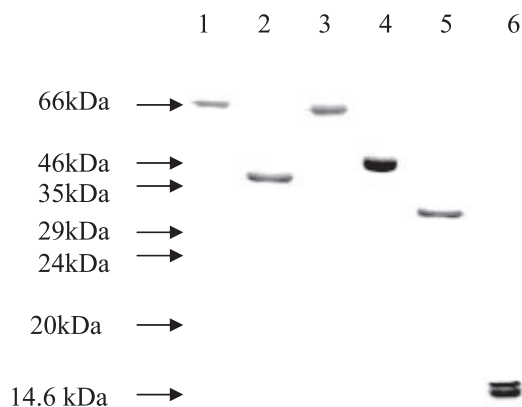

FIG. 4. Coomassie blue-stained 12% (wt/vol) SDS–PAGE gel containing the six purified polypeptides. Lane 1, mature amidase domain of AtlE; lane 2, repeat sequence domain of AtlE; lane 3, propeptide domain of AtlE; lane 4, GehD; lane 5, ScaA; lane 6, ScaB.
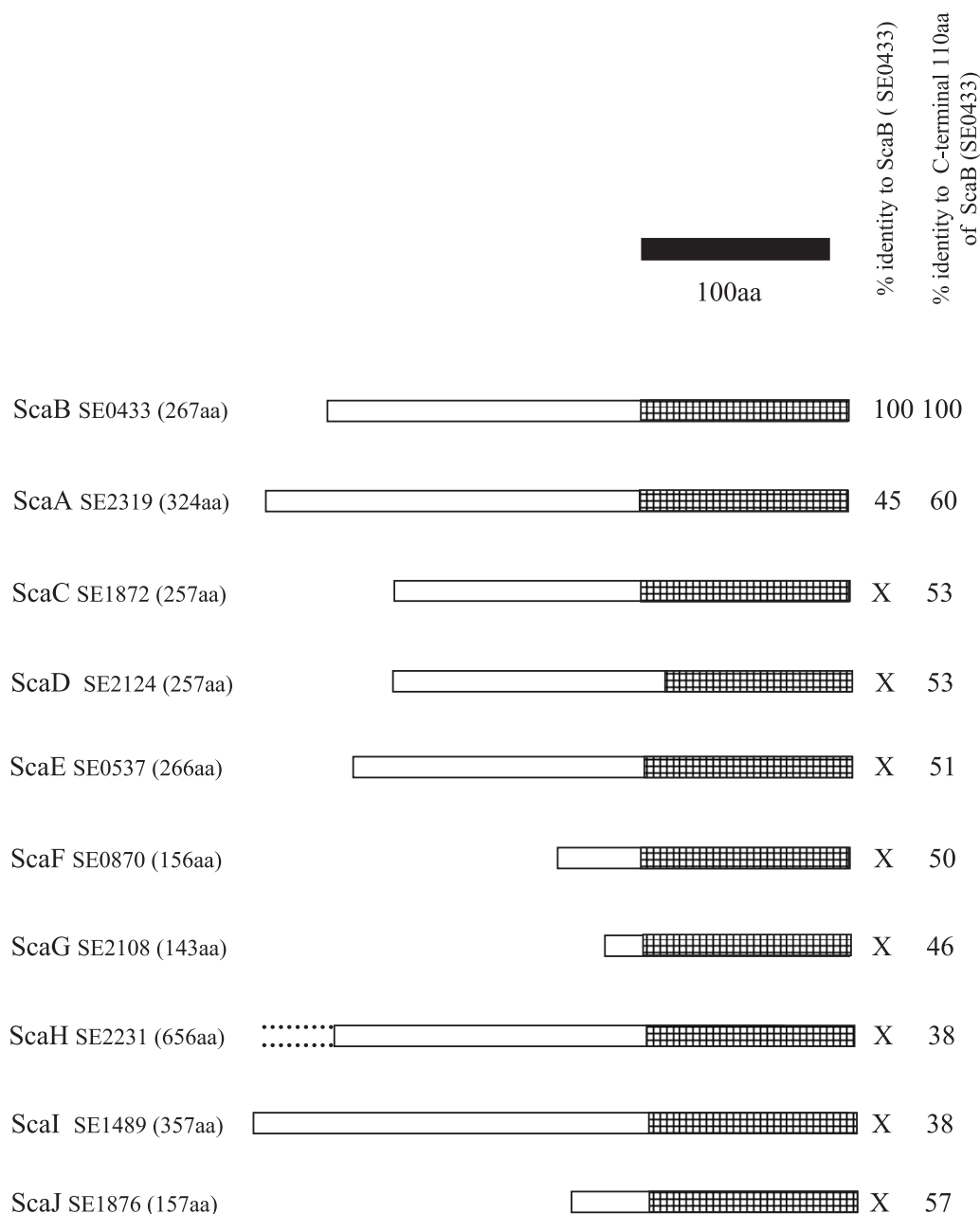
FIG. 5. Comparison of the ScaA-J family of *S. epidermidis*. The hatched bars indicate the 110-amino-acid (aa) C-terminal domain. The solid bar indicates 100 amino acids. X, significant homology (≥20% identity) occurs only in the C-terminal domain.

though a potential binding site in a shallow groove on the surface of the protein has been identified, the precise component to which it binds remains unknown (4).

ScaB exhibits 56% identity with SsaA of *S. epidermidis*. In *S. aureus*, SsaA has a virulence role specific to the pathogenesis of staphylococcal infections (29).

During this study it was shown that SE2319 (ScaA) is a novel autolysin, which has been designated Aae (23), and its paralogue in *S. aureus* has also been identified (24) and has been shown to be a virulence factor (28). While it is not essential that vaccine or antibody targets have such characteristics, these are attractive attributes as it is important that

antigens are expressed on the bacterial cell surface during the infectious process so that the immune system of the host can mount a response. *S. aureus* possesses a 10-member Sca protein family with homology in the C-terminal domain. Open reading frames encoding homologous C-terminal domains are also to be found in the genomes of *S. pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus mutans*, *Enterococcus faecalis*, *Lactococcus lactis*, and *Bifidobacterium longum* (results not shown). Vaccination against, or therapeutic antibodies reactive to, such proteins may provide targets for use against a broad spectrum of gram-positive bacteria.

TABLE 4. Statistical analysis of specific antibody levels
in *S. epidermidis* patient sera and community sera

Protein	Median absorbance at 450 nm (interquartile range)[a]		P value
	S. epidermidis infection (n = 15)	Healthy (n = 10)	
AtlE amidase	0.650 (0.110–1.004)	0.260 (0.002–0.342)	0.004
AtlE repeat sequence	0.546 (0.152–0.999)	0.165 (0–0.2329)	0.002
GehD	0.071 (0.030–0.310)	0.032 (0.014–0.048)	0.0001
ScaB	0.137 (0.017–0.260)	0.058 (0–0.130)	0.027

[a] The interquartile range indicates the minimum and maximum values for reactions of sera with each antigen.

Human antibody response to *S. epidermidis* antigens. An antibody response to a given antigen is indicative of its expression in vivo and of its potential for use in a subunit vaccine or as a target in passive immunotherapy or prophylaxis. The immune response against the selected antigens was quantified by ELISA using four recombinant proteins and 30 serum samples, including 20 samples of disease-associated sera (15 *S. epidermidis* infections and 5 *S. aureus* infections) and 10 serum samples from healthy blood donors (5 non-*S. aureus* nasal carriers and 5 *S. aureus* nasal carriers). Two of the original six polypeptides (Fig. 4) were dropped from the study; AtlE propeptide was not included as it did not react with serum in the Western blotting stage, and ScaA was not included because of its homology to ScaB.

The statistical significance of differences in serum antibody levels between the diseased and healthy patient groups was tested using the Kruskall-Wallis test. A comparison of the results for diseased and healthy individuals revealed that all four recombinant antigens had significantly higher ($P \leq 0.05$) reactive IgG titers in serum samples obtained from diseased individuals than in serum samples obtained from healthy individuals (Table 4). This indicates that all four proteins are produced in vivo and are thus immunological markers of infection that have potential diagnostic utility.

Opsonization of *S. epidermidis*. Different dilutions of serum from immunized mice were used to obtain measurable killing of *S. epidermidis* Hay (Table 5). Antibodies raised against AtlE or ScaB are opsonic in vitro. The antibodies raised against GehD, a lipase with collagen binding activity (7, 30), did not opsonize *S. epidermidis*. The mature processed form of GehD is found predominantly in the extracellular milieu, and it has been proposed that some of this form is present on cell walls (7). Thus, a relatively small amount of the protein on the cell

TABLE 5. Opsonization using antibodies raised
against recombinant antigens[a]

Antigen	Dilution	Avg % killed	SD
AtlE amidase	1/20	80	15
AtlE repeat sequence	1/20	55	41
GehD	1/10	0.4	1
ScaB	1/10	42	35

[a] *S. epidermidis* Hay was mixed with PMNs isolated from freshly obtained human blood and complement. After incubation for 2 h at 37°C, the surviving bacteria were counted, and the percentage of killed bacteria was determined. The results indicate the average killing efficacy of serum obtained from five animals.
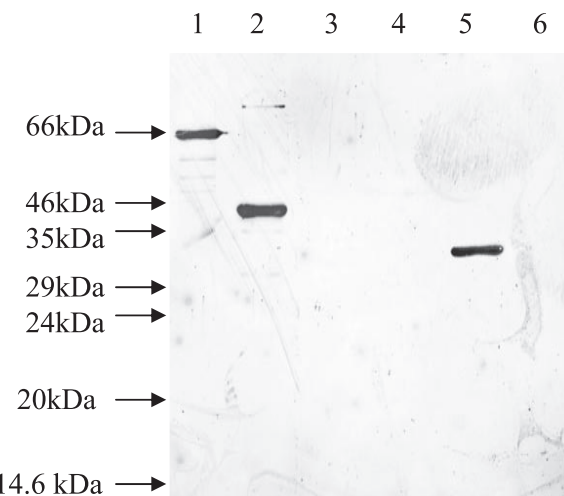

FIG. 6. Screening for fibrinogen binding by purified recombinant polypeptides, using the Western affinity blotting technique. All lanes contained ca. 1 µg of protein. Lane 1, mature amidase domain of AtlE; lane 2, repeat sequence domain of AtlE; lane 3, propeptide domain of AtlE; lane 4, GehD; lane 5, ScaA; lane 6, ScaB.

wall may result in an insufficient amount of antigen binding to trigger opsonization. Therefore, it is tempting to speculate that either AtlE or ScaB or both could be efficacious vaccines or passive immunotherapy targets and thus be useful for prevention or treatment of infections caused by many staphylococcal species due to the presence of paralogues in these organisms. Historically, polyclonal antibodies developed for use in passive immunotherapy of *S. aureus* infections have been limited by serotype specificity (12, 13) and recognize only 75% to 80% of all *S. aureus* clinical isolates (41). It is unlikely that targets such as AtlE or ScaB would pose such a problem, since several staphylococcal species possess AtlE paralogues and since ScaB is conserved in many gram-positive species.

Ligand binding activity of antigenic surface proteins. Bacterial colonization is a crucial step in pathogenicity, and its onset is determined by an interaction between the invading organism and its host. To study the possible involvement of the purified recombinant proteins in adhesion, these proteins were analyzed by performing binding assays with a selection of plasma proteins (fibrinogen, fibronectin, mucin, and lactoferrin), Western blotting, and probing with biotinylated ligands (Fig. 6 and Table 6).

The mature amidase fragment of AtlE bound all four plasma proteins. Interestingly, the repeat sequences of mature ami-

TABLE 6. Ligand binding capabilities of recombinant proteins
(fibrinogen, fibronectin, lactoferrin, and mucin)

Antigen	Ligand binding capability of:			
	Fibrinogen	Fibronectin	Mucin	Lactoferrin
AtlE mature amidase	+	+	+	+
AtlE repeat sequence	+	+	+	+
AtlE propeptide	−	−	−	−
GehD	−	−	−	−
ScaA	+	+	+	+
ScaB	−	+	−	+

dase exhibited such binding, which corresponds to findings for Aas and AtlC, whose central repeat sequences have been shown to mediate fibronectin binding (1, 25). Here we found that in AtlE, the repeat sequences also mediated binding to fibrinogen, mucin, and lactoferrin. The propeptide fragment of AtlE did not exhibit binding to any of the plasma proteins. ScaA bound all four of the plasma proteins, but the very highly homologous molecule ScaB bound to only fibronectin and lactoferrin, although a reduction in ligand binding capacity may have resulted from the proteolytic processing of recombinant ScaB. Alternatively, sequence differences between the two proteins may provide clues concerning the binding properties. Previous studies have shown that ScaA (AaE) binds fibrinogen and fibronectin (23) and that its *S. aureus* paralogue, AaA, binds fibrinogen, fibronectin, and vitronectin (24). Although GehD has been shown to bind to collagen (7), it did not bind any plasma proteins used in this study.

In this study we identified many antigenic *S. epidermidis* proteins. Some of these proteins are potential targets for immunotherapy, which provides a novel strategy for control of *S. epidermidis* infection, and could potentially reduce the toll of infection and have a significant impact on human health. Interestingly, a subset of *S. epidermidis* proteins are also conserved in *S. aureus* (9) and in other genera. These antigens may have important functions in disease.

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