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Is the GehD Lipase from *Staphylococcus epidermidis* a Collagen Binding Adhesin?*

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The opportunistic human pathogen *Staphylococcus epidermidis* is the major cause of nosocomial biomaterial infections. *S. epidermidis* has the ability to attach to indwelling materials coated with extracellular matrix proteins such as fibrinogen, fibronectin, vitronectin, and collagen. To identify the proteins necessary for *S. epidermidis* attachment to collagen, we screened an expression library using digoxigenin-labeled collagen as well as two monoclonal antibodies generated against the *Staphylococcus aureus* collagen-adhesin, Cna, as probes. These monoclonal antibodies recognize collagen binding epitopes on the surface of *S. aureus* and *S. epidermidis* cells. Using this approach, we identified GehD, the extracellular lipase originally found in *S. epidermidis* 9, as a collagen-binding protein. Despite the monoclonal antibody cross-reactivity, the GehD amino acid sequence and predicted structure are radically different from those of Cna. The mature GehD circular dichroism spectra differs from that of Cna but strongly resembles that of a mammalian cell-surface collagen binding receptor, known as the α_1 integrin I domain, suggesting that they have similar secondary structures. The GehD protein is translated as a preproenzyme, secreted, and post-translationally processed into mature lipase. GehD does not have the conserved LPXTG C-terminal motif present in cell wall-anchored proteins, but it can be detected in lysostaphin cell wall extracts. A recombinant version of mature GehD binds to collagens type I, II, and IV adsorbed onto microtiter plates in a dose-dependent saturable manner. Recombinant, mature GehD protein and anti-GehD antibodies can inhibit the attachment of *S. epidermidis* to immobilized collagen. These results provide evidence that GehD may be a bi-functional molecule, acting not only as a lipase but also as a cell surface-associated collagen adhesin.

Staphylococcus epidermidis is now recognized as an important nosocomial pathogen. In the past 20 years it has emerged as a frequent cause of infections associated with indwelling devices such as catheters, artificial heart valves, and orthopedic implants (1). In certain populations such as low birth weight infants and immuno-compromised patients *S. epidermidis* can be a prominent source of morbidity and mortality (2).

The molecular mechanisms of pathogenesis of *S. epidermidis* disease are not well understood, but as with most infections, bacterial adherence to host surfaces is recognized as the first crucial step in the infection process and a prerequisite for colonization. A two-step process of *S. epidermidis* adherence is often described in which the first step is bacterial attachment to the biomaterial, and the second step includes microbial proliferation, intercellular adhesion, and biofilm formation. Almost all *S. epidermidis* strains are able to attach to native abiotic surfaces (3–6). However, any foreign material implanted into the human body is quickly coated with various plasma proteins such as fibrinogen, fibronectin, and vitronectin (7, 8), and *Staphylococcus aureus*, which is also a common cause of biomaterial centered infections, appears to adhere to this protein coat via adhesins of the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs)[1] type.

Analysis of the adherence behavior of *S. epidermidis* suggests that this organism also expresses MSCRAMMs. In fact, a gene encoding a fibrinogen binding MSCRAMM (*sdrG*, also called *fbe*) was cloned and sequenced from *S. epidermidis* (9). SdrG, a 119-kDa MSCRAMM, has a structural organization similar to the clumping factor (ClfA) from *S. aureus* and specifically recognizes the N-terminal region of the fibrinogen Bβ chain (10). In addition, the autolysin AtlE, necessary for *S. epidermidis* attachment to polystyrene, was shown to specifically bind to biotin-labeled vitronectin (11). These data indicate that *S. epidermidis*, similarly to *S. aureus*, may express specific MSCRAMMs that mediate cell attachment to host protein-conditioned surfaces.

In the present communication, we report that the GehD (12) lipase binds to collagen type I, II, and IV and may mediate the adherence of *S. epidermidis* cells to immobilized collagens. We identified GehD probing a *S. epidermidis* expression library with labeled collagen type I and monoclonal antibodies generated against the *S. aureus* collagen-binding protein, Cna. Staphylococcal lipases have been implicated as possible virulence factors in localized infections such as absesses (13–15), and there is evidence that they are highly expressed during infection in a murine model (16). The contribution of these

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[1] The abbreviations used are: MSCRAMM, microbial surface components recognizing adhesive matrix molecules; PBS, phosphate-buffered saline; mAb, monoclonal antibody (Ab); BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay.

enzymes to virulence is not clearly understood, although lipases may be important for the colonization and persistence of organisms on the skin (17).

Another class of proteins that function as collagen binding adhesion receptors are the mammalian integrins. These proteins mediate the attachment of eukaryotic cells to the extracellular matrix. The integrins are transmembrane $\alpha\beta$ heterodimeric proteins that mediate cell-cell and cell-matrix interactions of mammalian cells. In this extensive family of proteins, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are the primary collagen binding integrins. Within the α subunit of the collagen binding integrins, the ligand binding region is called I domain (33). Our data predict that mature GehD may adopt a structure that resembles that of the integrin α_1 I-domain.

The data described here show that the GehD lipase binds to collagens and may promote *S. epidermidis* attachment to immobilized collagens. Our data indicate that the GehD lipase may be a bifunctional molecule, acting as a glycerol ester hydrolase and a collagen adhesin.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—*S. epidermidis* strains 146, 9491, 12228, 14852, and 14990 were obtained from the ATCC collection. *S. epidermidis* 9, 2J24 (*gehC*::*ermC*), and KIC82 (*gehD*::*ermC*) were created by Christopher M. Longshaw (12). *S. aureus* Cowan 1 spa::*tet*^{R} strain was generously donated by T. Foster (University of Dublin, Ireland). All strains were grown in brain heart infusion or tryptic soy broth media (Difco) at 37 °C overnight. For the monoclonal antibody reactivity assays, bacteria were harvested and re-suspended in phosphate-buffered saline (PBS), pH 7.4 (140 mM NaCl, 270 μM KCl, 430 μM Na_2HPO_4, 147 μM KH_2PO_4) 0.02% sodium azide, washed, and adjusted to a cell density of 10^{10} cells/ml using a standard curve relating the A_{600} to the cell number determined by counting cells in a Petroff-Hausser chamber. The cells were then heat-killed at 88 °C for 10 min.

For all other assays, overnight cultures were diluted 1:1000 into fresh tryptic soy broth media, and the resultant culture was incubated until it reached logarithmic growth phase (A_{600} 0.3–0.6). Bacteria were then harvested by centrifugation and used in attachment or Western assays.

Library Construction—A *S. epidermidis* 9491 λZAP Express (Stratagene) expression library was constructed as follows. *S. epidermidis* 9491 chromosomal DNA was partially digested with *Mbo*I, and the fragments corresponding to 3–11 kilobases were isolated and purified. The purified fragments were ligated to the ZAP Express® (Stratagene) vector, predigested with *Bam*HI, and dephosphorylated with CIAP (calf intestinal alkaline phosphatase). The resultant ligation product was packaged into phage particles using the Gigapack III Gold (Stratagene)-packaging extract. The obtained library was amplified and screened using the *Escherichia coli* XL1-Blue MRF′ strain. Clones of interest were excised from the λ ZAP Express® phage using the ExAssist® helper phage to generate the pBK-CMV phagemid vector packaged as filamentous phage particles. The filamentous phage stock was used to infect the *E. coli* XLOLR strain. The resultant colonies carrying the excised pBK-CMV phagemid vector were used for subsequent subcloning and dideoxy sequencing of the cloned inserts.

A DNA fragment encoding the mature domain of the GehD lipase was PCR-amplified from *S. epidermidis* 9491 genomic DNA. The oligonucleotides primers 5′-TTT GAA TTC TGC GCA AGC TCA ATA TAA and 5′-TTT GCG GCC GCT ATC GCT ACT TAC GTG TAA were used to amplify the fragment designated as mature GehD. Constructs generated by PCR were cloned into the pETBlue-2 System using the *E. coli* NovaBlue strain as a cloning host and the *E. coli* Tuner (DE3) pLacI strain as the expression host.

Large scale expression and preparation of recombinant proteins were as described previously using HiTrap nickel-chelating chromatography (10). Protein concentrations were determined from the absorbance at 280 nm as measured on a Beckman Du-70 UV-visible spectrophotometer. The molar extinction coefficient of the proteins was calculated using the method of Pace *et al.* (18).

Labeling of Proteins—Purified collagen I (Vitrogen®, Cohesion, Palo Alto CA) was labeled with digoxigenin-3-*O*-methylcarbonyl-ϵ-aminocaproic acid-*N*-hydroxy-succinimide ester (digoxigenin) (Roche Molecular Biochemicals) according to the manufacturer's instructions.

To label recombinant proteins with biotin, 7.5 mg of sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin; Pierce) was dissolved in 100 μl of dimethyl sulfoxide (Me_2SO) and combined with 0.5 mg of recombinant protein in PBS. The total reaction (1 ml volume) was incubated in an end-over-end rotator at room temperature for 2 h then dialyzed against PBS and stored at 4 °C.

Library Screens—Digoxigenin-labeled collagen or mAbs 11H11 and 1F6 (19) were used to screen the *S. epidermidis* 9491 λZAP Express (Stratagene) expression library. The library was plated using standard methods according to the vector manufacturer's instructions (Stratagene). After blocking additional protein binding sites on the filter lifts with a solution containing 3% (w/v) bovine serum albumin (BSA) in TBST (0.15 M NaCl, 20 mM Tris-HCl, 0.05% (v/v) Tween 20, pH 7.4), digoxigenin-labeled collagen (0.5 μg/ml in TBST) was incubated with the filters. The bound digoxigenin-labeled collagen was incubated with anti-digoxigenin Fab conjugated to alkaline phosphatase (1:5000 in TBST, Roche Molecular Biochemicals). When mAbs 11H11 or 1F6 (1:500 in TBST) were used as probes, goat anti-mouse antibodies (1:4000 in TBST) conjugated to alkaline phosphatase (Bio-Rad) were used as secondary antibodies. Clones expressing collagen-binding proteins were identified by developing the membranes with 5-bromo-4-chlor-3-indoyl phosphate *p*-toluidine salt and *p*-nitro blue tetrazolium chloride (Bio-Rad).

Enzyme-linked Immunosorbent Assay (ELISA)—To test the reactivity of the mAbs generated against bacterial surface proteins, microtiter wells (Dasit, Milan, Italy) were coated overnight at 4 °C with 2 μg of human fibronectin in 100 μl of 50 mM sodium carbonate, pH 9.5, to provide a surface for bacterial attachment. The wells were washed five times with 10 mM sodium phosphate buffer, pH 7.4, containing 0.13 M NaCl and 0.1% (v/v) Tween 20 (PBST), and additional protein binding sites were blocked with a solution of 2% (w/v) BSA in PBS. Suspensions of 1×10^8 cells of *S. epidermidis* or *S. aureus* Cowan 1 *spa*::*tet*^{R}) whole cells were added and incubated for 2 h at room temperature followed by 5 washes with PBS to remove unbound cells. Solutions of 2 μg of each monoclonal antibody in 100 μl of 2% (w/v) BSA in PBS were added, incubated for 2 h at room temperature, washed extensively with PBST, and detected with a 1:500 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Dako, Gostrup, Denmark). The conjugated enzyme was incubated with *o*-phenylenediamine dihydrochloride (Sigma) as a substrate, and the color development absorbance was monitored at 492 nm using a microplate reader (Bio-Rad).

To test protein-protein interactions, microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 1 μg of type I collagen in 100 μl of PBS/well overnight at 4 °C. Wells were then washed 3 times with PBS and blocked with 1% (w/v) bovine serum albumin in PBS for 1 h before the addition of varying concentrations of the biotinylated recombinant protein. After incubation at room temperature for 2 h with gentle shaking, the wells were extensively washed with PBS containing 0.05% (v/v) Tween 20 (PBST). Streptavidin-alkaline phosphatase conjugate (Roche Molecular Biochemicals) was diluted 10,000-fold with blocking buffer and added to the wells. After incubation at room temperature for 45 min, the wells were washed with PBST. For color development, 100 μl of 1.3 M diethanolamine, pH 9.8, containing 1 mg/ml *p*-nitrophenyl phosphate (Sigma) was added to the wells. Absorbance at 405 nm ($A_{405\ nm}$) was measured using a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA) after 1 h of incubation at room temperature. Experiments were performed in triplicate and repeated with independently prepared protein preparations. Binding to BSA-coated wells was considered as background level and subtracted from binding to collagen. Data were presented as the mean value ± S.E. of $A_{405\ nm}$ from a representative experiment ($n = 3$). The effect of antibodies as inhibitors of proteins binding to collagen was examined as described above except that biotinylated proteins were mixed with antibodies at varying ratios and added to the wells.

Circular Dichroism—The secondary structural composition of recombinant proteins was examined by CD spectroscopy. Far UV CD data were collected using a Jasco J720 spectropolarimeter calibrated with a 0.1% (w/v) *d*-10-camphorsulfonic acid solution using a bandwidth of 1 nm and integrated for 4 s at 0.2-nm intervals. All sample concentrations were less than 30 μM in 20 mM Tris-HCl buffer, pH 7.4. Spectra were recorded at ambient temperatures in 0.2-mm path length cuvettes. Thirty scans were averaged for each spectrum, the contribution from the buffer was subtracted, and quantitation of secondary structural elements was performed by deconvolution software provided by University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School (Piscataway, NJ) and D. Greenwood (Softwood Co., Brooksfield, CT). These deconvolution programs (SELCON and VARSLC1) are derived from databases of known protein structures.

Surface Plasmon Resonance Spectroscopy—Analyses were performed

using the BIAcore 1000 system (BIAcore AB, Uppsala, Sweden) as described previously (20). The Cna protein was tested in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4). The α_1 I domain was tested in HBS containing 5 mM β-mercaptoethanol, and mature GehD was tested in both HBS and glycine buffer (50 mM glycine, pH 7.4). Data from the equilibrium portion of the sensorgrams were used for analysis and calculation of the K_D and n.

Preparation of Polyclonal Antibodies—Purified mature GehD was dialyzed in PBS, pH 7.4, before being sent to Rockland Immunochemicals, Inc. (Gilbertsville, PA) for immunization in rabbits and production of polyclonal antisera. IgGs were purified from both immune and preimmune serum by chromatography using protein A-Sepharose (Sigma).

Bacterial Adherence Assays—Microtiter plates (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 1 μg of type I collagen in 100 μl of PBS/well overnight at 4 °C. Wells were then washed 3 times with PBS and then blocked with 1% (w/v) bovine serum albumin in PBS for 1 h before the addition of bacteria. Early log-phase *S. epidermidis* cultures (A_{600} of 0.5) were added, and the plates were incubated for 2 h at room temperature. After gentle washes, adherent cells were fixed with 100 μl of 25% (v/v) aqueous formaldehyde and incubated at room temperature for at least 30 min. The plates were then washed gently, stained with crystal violet, then washed again and read on an ELISA plate reader at 590 nm.

To study inhibition of collagen binding by IgGs, *S. epidermidis* suspensions were preincubated with serial dilutions of purified IgGs in PBS for 2 h at room temperature. The cell suspensions were then transferred to ELISA plates coated with 1 μg of collagen/well, and their ability to attach to collagen was tested as described above.

SDS-PAGE and Western Ligand Blot—For whole-cell SDS-PAGE (21), 2×10^7 *S. epidermidis* (previously treated with lysostaphin) cells or *E. coli* cells were boiled in 2% (w/v) SDS for 3–5 min under reducing conditions and subjected to electrophoresis through a 10% acrylamide gel at 150 V for 45 min. The separated proteins were stained with Coomassie Brilliant Blue.

For Western ligand blot assays, whole cell lysates, or purified proteins were transferred from the polyacrylamide gel onto a nitrocellulose membrane in a semi-dry electroblot system (Bio-Rad). Additional binding sites on the membrane were blocked by incubating in 2% (w/v) BSA in TBST for 2 h at room temperature or overnight at 4 °C followed by three 10-min washes in TBST. The membrane was then incubated at room temperature with 0.5 μg of digoxigenin-labeled collagen/ml TBST for 1 h, washed, and incubated with 1:5000 anti-digoxigenin Fab alkaline-phosphatase conjugate (Roche Molecular Biochemicals) in TBST for 1 h. The membrane was washed, and collagen-binding proteins were visualized with 150 μg of 5-bromo-4-chlor-3-indoyl phosphate *p*-toluidine salt/ml and 300 μg of *p*-nitro blue tetrazolium chloride/ml (Bio-Rad) in carbonate bicarbonate buffer (14 mM Na_2CO_3, 36 mM $NaHCO_3$, 5 mM $MgCl_2 \cdot 6H_2O$, pH 9.8).

RESULTS

Adherence of S. epidermidis 9491 to Extracellular Matrix Proteins—The clinical isolate *S. epidermidis* 9491 was chosen as a prototype strain in our search for new MSCRAMMs. We tested its ability to adhere to immobilized bovine collagen type I, human fibrinogen, and human fibronectin. Each protein was immobilized in microtiter wells, and the bacteria attached to the wells were detected using crystal violet. The results presented in Fig. 1 show that *S. epidermidis* 9491 has the ability to attach to collagen, fibrinogen and fibronectin. Although previous studies have shown that *S. epidermidis* attachment to human fibrinogen is mediated by proteins such as Fbe and SdrG (9, 10), the bacterial components that mediate attachment to collagen or fibronectin were up to this point not identified.

Binding of Monoclonal Antibodies to S. epidermidis Strains—A panel of 22 monoclonal antibodies was previously generated against the *S. aureus* MSCRAMM Cna-(151–318) (19). We explored the possibility that at least some of these mAbs would cross-react with collagen-binding proteins on *S. epidermidis* by examining a panel of strains (*S. epidermidis* 146, 9491, 12228, 14852, and 14990). Two monoclonals, 11H11 and 1F6, cross-reacted with whole cells of all the *S. epidermidis* strains tested. Both of these antibodies were raised against the ligand binding central region of Cna-(151–318). Furthermore,

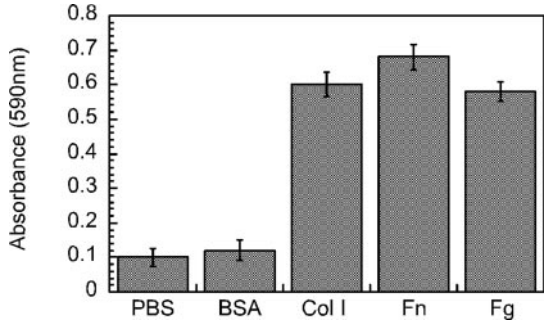

FIG. 1. ***S. epidermidis* can bind to immobilized extracellular matrix proteins.** Log-phase bacterial cultures were washed and incubated in microtiter wells coated with 1 μg of BSA, collagen I (*coll*), fibronectin (*Fn*), and fibrinogen (*Fg*). Attached *S. epidermidis* cells were detected using crystal violet. Values represent the means and S.E. of triplicate wells. This experiment was repeated several times with similar results.

these antibodies were shown to inhibit collagen binding to Cna and recognize conformationally dependent epitopes, presumably located in the ligand binding site of Cna-(151–318) (19). As expected, all of the anti-Cna mAbs bind to *S. aureus* Cowan 1 cells. The *S. epidermidis* strains are recognized only by two antibodies. These results suggest that *S. epidermidis* exposes on its surface proteins that form epitopes similar to those present on Cna and that these proteins are recognized by 1F6 and 11H11.

Construction of an Expression Library and Identification of a New Collagen-binding Protein—We constructed an expression library ligating *Mbo*I partially digested, size-selected genomic DNA from *S. epidermidis* 9491 to *Bam*HI-digested λZAP Express II® vector. Using mAbs 1F6 and 11H11 as well as digoxigenin-labeled collagen, we screened ~690,000 plaques. We isolated three clones that reacted with each mAb and labeled collagen. DNA sequencing of the excised phagemids revealed that 2 of the clones were identical, and the third had an additional 36 bp of upstream sequence. Further sequence analysis revealed that the cloned DNA immediately downstream of the T7lac sequence from the phagemid is 97% identical to the previously identified *S. epidermidis* second lipase gene, *gehD* (12) (Fig. 2*A*).

Purification and Characterization of Recombinant, Mature GehD—Previous studies of GehD and other staphylococcal lipases have shown that they are transcribed and translocated as 650–700-amino acid precursors that are processed post-translationally to extracellular mature lipases of about 360 amino acids with a size of ~45 kDa (12). To simulate the native protein in the mature form, we used the PCR to construct recombinant mature GehD (Fig. 2*A*). The PCR product encoding mature GehD was cloned into the expression vector pET-Blue-2 (Novagen). The protein was expressed as a C-terminal polyhistidine (His tag) fusion and purified by nickel-chelating chromatography. Mature GehD appears as a single polypeptide at ~45 kDa when analyzed by SDS-PAGE (Fig. 2*B*, *lane 2*).

Primary and Secondary Structure of Mature GehD—Amino acid sequence comparisons did not reveal any significant similarities between the linear amino acid sequences of Cna and mature GehD. Furthermore, the CD spectra of mature GehD shown in Fig. 3*A* is very different from that of Cna (Fig. 3*C*). Deconvolution of the mature GehD data using the SELCON and VARSLC1 programs revealed that the predicted overall secondary structure of mature GehD consists of ~26.5% α-helix, 20.6% β-sheet, and 52.9% coil. This secondary structure composition differs markedly from that of the reported crystal structure of Cna-(151–318): 8% α-helix, 53% β-sheet, and 39% coil (29).

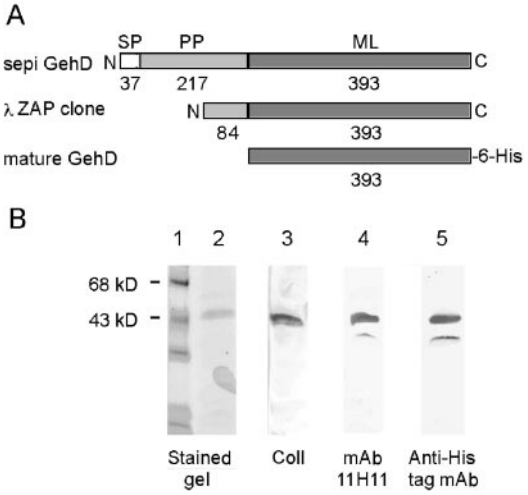

FIG. 2. **Mature GehD binds to collagen.** *A*, schematic representation of the GehD lipase and recombinant constructs. *Sepi GehD*, *S. epidermidis* 9 GehD; λ *ZAP clone*, truncated GehD preproenzyme obtained from phage isolated from the *S. epidermidis* 9491 library; *mature GehD*, recombinant clone created by PCR. The signal peptide (*SP*), propeptide (*PP*), and mature lipase (*ML*) domains are indicated, with their lengths in amino acid residues *below*. *6-His*, six-histidine tag for purification purposes. *B*, recombinant mature GehD was overexpressed and purified using standard techniques. Mature GehD was separated by SDS-PAGE. *Lanes 1* and *2* were stained with Coomassie Brilliant Blue, *lanes 3–5* were transferred to a nitrocellulose membrane and probed with digoxigenin-labeled collagen (*Coll*) or monoclonal antibodies.

In contrast, the mature GehD CD spectra strongly resembles that of a mammalian cell-surface collagen binding receptor known as the α_1 integrin I domain (Fig. 3*B*). The secondary structure composition of this domain is 33.2% α-helix, 20.7% β-sheet, and 46.1% coil, which is comparable with that of mature GehD.

Recombinant Mature GehD Binds to Collagen—The collagen binding activity of the recombinant, mature GehD was analyzed by Western ligand blot. Purified protein was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with digoxigenin-labeled collagen, mAbs 11H11 (anti-Cna), or 7E8 (anti-His tag) (Fig. 2*B*, *lane 4* and *5*, respectively). In this assay, the recombinant, mature GehD binds collagen and both antibodies. It should be noted that a second lower mass polypeptide is detected in *lanes 4* and *5*. This is a contaminating polypeptide that is recognized by the secondary anti-mouse antibody used to detect mAbs11H11 and anti-His.

The collagen binding activity of the recombinant, biotin-labeled mature GehD was also assessed by a solid phase, ELISA-type assay. Mature GehD bound in a concentration-dependent, saturable manner to collagens I, II, and IV coated on microtiter wells (Fig. 4), whereas the binding to wells coated with albumin was minimal. From the ELISA-type assay we estimated that half-maximum binding occurred at about 0.25 μM mature GehD. In addition, we examined the ability of unlabeled mature GehD to inhibit the binding of biotinylated mature GehD to immobilized collagen. Unlabeled GehD could inhibit the binding of the labeled protein to immobilized collagen, whereas a fibrinogen binding recombinant protein from *S. epidermidis* (SdrG) had no inhibitory effect (data not shown). This suggests that both biotin-labeled and unlabeled mature GehD bind with similar affinity to immobilized collagen.

We also tried to characterize the binding of mature GehD to collagen by surface plasmon resonance. In this assay, soluble recombinant mature GehD is run over a sensory chip coated with type I collagen. Using a Hepes-based buffer system, we could calculate a K_D of 4 μM for the interaction. Using a glycine buffer we recorded equilibrium data and calculated a K_D of 3 μM and 1 binding site for mature GehD per collagen monomer. However, not only was the interaction of mature GehD with collagen dependent on the buffer system used, but the collagen binding activity declined as the purified mature GehD was stored for long periods of time. Clearly, these are aspects of the mature GehD binding to collagen that we do not understand at the present, and the K_D values reported above must be taken with caution.

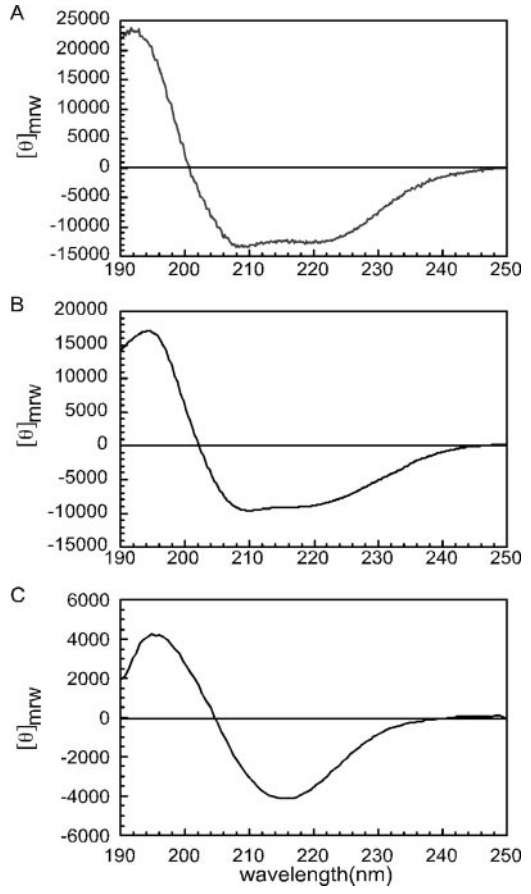

FIG. 3. **Far-UV CD spectra of recombinant, collagen adhesins.** *A*, mature GehD; *B*, α_1 I domain, *C*, Cna-(151–318). The predicted secondary structure composition of each protein is reported under "Discussion." Mean residue weight ellipticity (*θ mrw*) is reported in degrees·cm²/dmol.

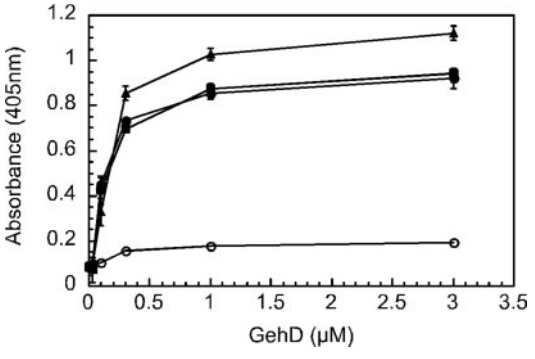

FIG. 4. **Binding of recombinant, mature GehD to immobilized collagens.** Microtiter wells were coated with 1 μg of collagen I (●), II (■), IV(▲), or BSA (○). Increasing concentrations of biotinylated, recombinant GehD were incubated in the wells for 1 h at room temperature. Bound protein was detected with alkaline phosphatase-conjugated streptavidin followed by development with *p*-nitrophenylphosphate substrate. Values represent the means and S.E. of triplicate wells. This experiment was repeated three times with similar results.

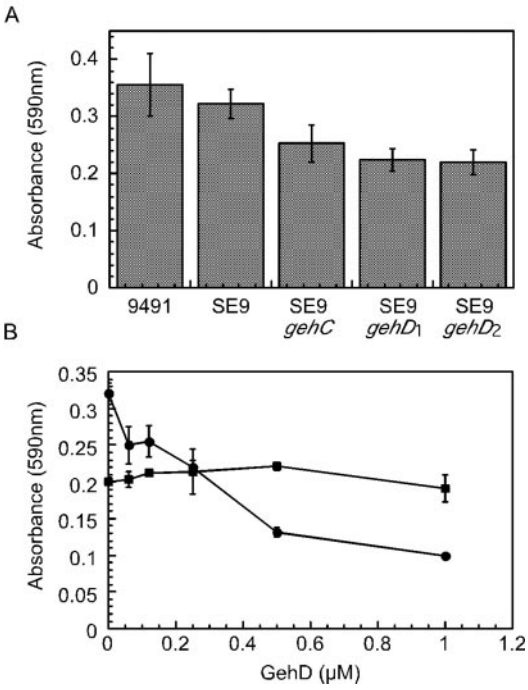

FIG. 5. **Attachment of *S. epidermidis* strains to collagen type I.** *A*, attachment of whole cells onto immobilized collagen. Microtiter wells were coated with 1 μg of type I collagen, washed, and blocked for 1 h at room temperature with BSA. Log-phase *S. epidermidis* 9491, *S. epidermidis* 9, *S. epidermidis* 9 *gehC*::*ermC*, and *S. epidermidis* 9 *gehD*::*ermC* cultures were washed and added to the coated wells. *S. epidermidis* 9 $gehD_1$ and *S. epidermidis* 9 $gehD_2$ represent two identical, individually isolated clones. Attached cells were detected staining the cells with crystal violet and measuring their absorbance at 590 nm. Values represent the means and S.E. of triplicate wells. This experiment was repeated several times with similar results. *B*, microtiter wells were coated with 1 μg of collagen type I, washed, and preincubated for 1 h at room temperature with increasing concentrations of recombinant, mature GehD. Log-phase *S. epidermidis* 9 (●) and *S. epidermidis* 9 *gehD*::*ermC* (■) cultures were washed and added to the coated wells. Attached cells were detected staining the cells with crystal violet and measuring their absorbance at 590 nm. Values represent the means and S.E. of triplicate wells. This experiment was repeated several times with similar results.

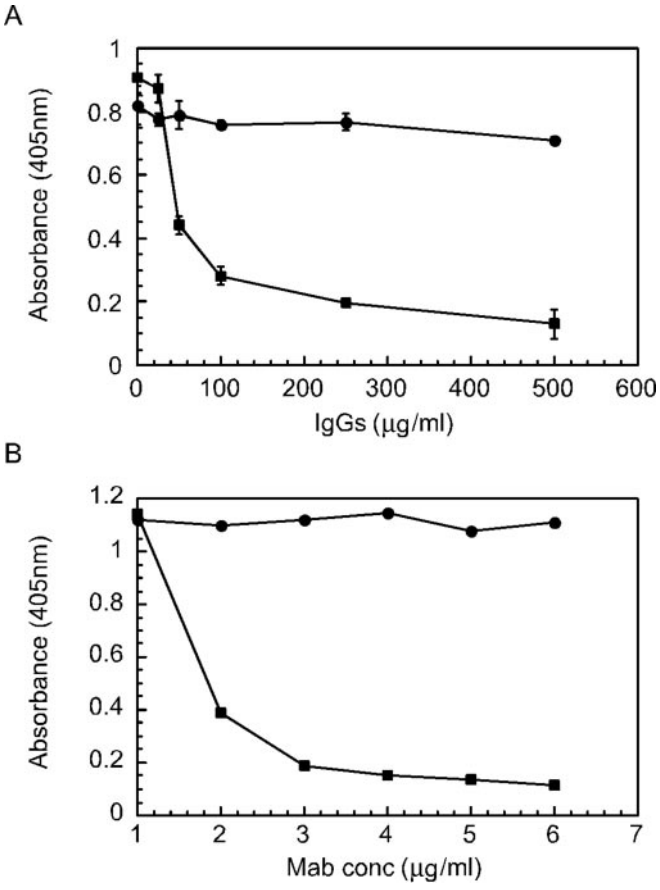

FIG. 6. **Inhibition of mature GehD binding to immobilized collagen.** *A*, recombinant, biotinylated mature GehD was preincubated with anti-GehD (■) or preimmune (●) antibodies before it was added to microtiter wells coated with 1 μg of collagen type I. Biotinylated, bound protein was detected with avidin conjugated to alkaline phosphatase. Values represent the means and S.E. of triplicate wells. *B*, recombinant, biotinylated mature GehD was preincubated with mAb 11H11 (■) (anti-Cna) or mAb 13G12 (●) (anti-FnbpA) antibodies before it was added to microtiter wells coated with 1 μg of collagen type I. Biotinylated, bound protein was detected with avidin conjugated to alkaline phosphatase. Values represent the means and S.E. of triplicate wells.

Purified Mature GehD and Antibodies Can Block the Attachment of S. epidermidis to Collagen—We used a microtiter well attachment assay to study the adherence of *S. epidermidis* to collagen. Two independent, identical clones of *S. epidermidis* carrying a deletion of the *gehD* gene show a decreased ability to attach to immobilized collagen when compared with their isogenic strain, *S. epidermidis* 9. However, the *gehD* mutant strain has a significant residual collagen adherence. A similar strain carrying a deletion in the *gehC* gene has a slight decreased ability to attach to collagen when compared with its isogenic strain (Fig. 5*A*). These data suggests that there may be more than one cell surface adhesin mediating cell attachment to collagen.

The effects of purified, recombinant mature GehD on bacterial adherence were examined in experiments in which collagen-coated microtiter wells were preincubated with increasing concentrations of recombinant mature GehD for 1 h before whole *S. epidermidis* were added. Purified mature GehD inhibited the attachment of *S. epidermidis* 9491 to collagen in a concentration-dependent manner, but it does not affect the already decreased attachment of a *gehD* null strain (Fig. 5*B*). We generated polyclonal antibodies against the recombinant, mature GehD protein and assessed their ability to interfere with the binding of GehD to collagen. Purified anti-mature GehD IgGs effectively inhibit the binding of biotin-labeled mature GehD to immobilized collagen, whereas purified, preimmune IgGs had no noticeable effect (Fig. 6*A*). A similar effect was observed when monoclonal antibodies were used. The monoclonal 11H11 generated against Cna effectively blocked the binding of mature GehD, whereas an unrelated monoclonal, 13G12, did not inhibit (Fig. 6*B*). In addition, we tested the specificity of these antisera using *E. coli* and *S. epidermidis* cell extracts. The anti-mature GehD purified IgGs recognize a polypeptide of ~45 kDa in cell lysates of both *E. coli* expressing the *gehD* gene or *S. epidermidis*. This 45-kDa polypeptide is not present in the *gehD* mutant cell lysates (not shown). These data show that anti-GehD IgGs are specific for mature GehD.

We therefore used these antibodies in a microtiter well attachment assay to test their ability to inhibit the attachment of whole *S. epidermidis* cells to immobilized collagen. *S. epidermidis* cells were preincubated with increasing concentrations of purified anti-mature GehD antibodies before the cell suspensions were added to collagen-coated microtiter wells. Attached cells were detected using crystal violet. Purified, anti-mature GehD antibodies effectively inhibit the attachment of *S. epidermidis* to collagen. Preimmune purified IgGs had no noticeable effect (not shown). The same purified IgGs do not seem to affect the already decreased attachment of the *gehD* null strain (Fig. 7*A*). A similar effect was observed when the monoclonal 11H11 was used to preincubate the bacterial cells (Fig. 7*B*).

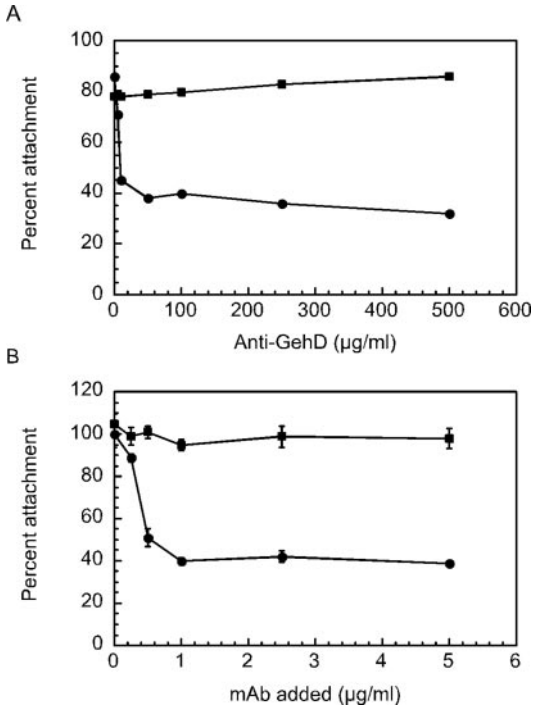

FIG. 7. **Antibodies inhibit the attachment of *S. epidermidis* to collagen type I.** *A*, log-phase *S. epidermidis* 9 (●) and *S. epidermidis* 9 *gehD*::*ermC* (■) cultures were washed and preincubated with anti-mature-GehD before addition to wells coated with type I collagen. Attached cells were detected staining the cells with crystal violet and measuring their absorbance at 590 nm. Preimmune IgGs did not inhibit the attachment (data not shown). Values represent the means and S.E. of triplicate wells. *B*, log-phase *S. epidermidis* 9 (●) and *S. epidermidis* 9 *gehD*::*ermC* (■) cultures were washed and preincubated with mAb 11H11 before addition to wells coated with type I collagen. Attached cells were detected by staining the cells with crystal violet and measuring their absorbance at 590 nm. Monoclonal Ab 13G12 did not inhibit the attachment (data not shown). Values represent the means and S.E. of triplicate wells.

These data suggest that surface-associated mature GehD may act as a collagen adhesin and mediate the attachment of *S. epidermidis* to collagen-coated surfaces.

DISCUSSION

In contrast to *S. aureus*, the adherence of *S. epidermidis* to extracellular matrix proteins has not been well characterized. It is known that *S. epidermidis* can adhere to fibrinogen, fibronectin, laminin (7), and vitronectin (11). The adherence to fibrinogen is mediated by protein adhesins such as Fbe (9) or SdrG (10), and attachment to vitronectin seems to be promoted by the autolysin AtlE. However, the proteins responsible for the interactions with collagen and fibronectin have not been identified. Thus, to search for additional adhesins, we constructed a genomic expression library from the clinical isolate *S. epidermidis* 9491. To screen our library, we took advantage of a panel of 22 mAbs that were raised against Cna-(151–318), the collagen binding MSCRAMM from *S. aureus.* Two of these monoclonals (11H11 and 1F6) cross-reacted to epitopes present on the surface of *S. epidermidis* cells. Therefore, we used mAbs 11H11, 1F6, and labeled collagen to screen our expression library and isolate a collagen binding clone. Surprisingly, the clone that bound to both mAbs and collagen expressed an N-terminal truncation of the GehD preproenzyme. This *S. epidermidis* extracellular lipase has the same overall organization as the other staphylococcal lipases GehC, Geh, *Sal*II, and Lip (22). These lipases appear to be synthesized as preproenzymes consisting of three major domains: signal peptide, propeptide, and mature lipase. The signal peptide is essential for secretion, and it is removed during export of the protein. The propeptide domain has been found to be important for efficient translocation and proteolytic stability during secretion (23). Previous data (12) suggest that GehD is similarly translated as a preproenzyme and post-translationally processed into mature lipase. The size of this active, extracellular lipase is ~45 kDa.

The mature form of GehD can be found associated to whole cells and in lysostaphin extracts from the cell wall.[2] Interestingly, the typical LP*X*TG motif associated with the cell-wall anchored proteins found in most Gram-positive bacterial surface proteins is not present in the C-terminus of the GehD protein. Recently, several Gram-positive cell-surface adhesins that do not contain a LP*X*TG motif have been described. These include the fibronectin binding adhesins PavA (24) from *Streptococcus pneumoniae*, FBP54 (25) from *Streptococcus pyogenes*, and the plasminogen binding Eno (26) from *S. pneumoniae.* A possible mechanism for cell surface display of these anchorless adhesins has been described for Eno (26) from *S. pneumoniae*. Eno, a glycolytic cytoplasmic enzyme, is secreted by an unknown mechanism and can re-associate by interacting with receptors on pneumococci. Once Eno is surface-associated, it binds to plasminogen and facilitates the invasion of pneumococci into the host cells. Although the nature of the association between GehD and the *S. epidermidis* cell surface is currently not understood, it is tempting to speculate that, similarly to Eno, it remains associated to the bacterial surface after secretion. Additional proteins with adhesive functions located on the surface without LP*X*TG motifs include SEN (27), a surface enolase of *S. pyogenes*, SDH (28), a surface dehydrogenase of group A streptococci, and the *S. epidermidis* autolysin AtlE, which specifically binds to vitronectin (11). Anchorless proteins with other biological functions have also been described (29, 30). Clearly, the number of anchorless adhesins identified in Gram-positive bacteria will increase in the future, but it is not clear if these proteins are virulence factors.

The staphylococcal lipases have been considered virulence factors in localized infections such as absesses (13–15), and *in vitro* expression technology (16) showed that lipase gene expression is induced during infection in a murine abscess model. The contribution of these enzymes to virulence is not clearly understood, although lipases may be important for the colonization and persistence of organisms on the skin. Amino acid sequence analysis has shown that GehC and GehD are 51% identical to each other. GehC is closely related to lipase Sal-2 from *S. aureus* NCTC 8530 (84% identity), whereas GehD has greater homologies to the *S. aureus* PS54 lipase, Geh (58% identity), and the lipase of *Staphylococcus hemolyticus*, Lip (70%) identity (12). Although the staphylococcal lipases are a diverse group of enzymes, the predicted secondary structures contain many conserved elements. It would be of great interest to determine whether any of these staphylococcal lipases have adhesive properties in addition to their lipolytic activities. The ability of this enzyme to be bi-functional may be indicative of its importance to the *S. epidermidis* successful colonization and growth on both skin and artificial surfaces.

Mutants of *S. epidermidis* 9 defective in GehD or GehC were used to examine the role of GehD in bacterial interactions with collagen. GehD can mediate bacterial attachment to immobilized collagen. This interaction was blocked by recombinant, mature GehD. In addition, two monoclonal antibodies raised against Cna and antibodies raised against the mature GehD lipase inhibit the attachment of *S. epidermidis* 9 to collagen. Both the *gehC* and *gehD* mutants show a decreased attachment to collagen, which raises the possibility that GehC might also

[2] M. G. Bowden, unpublished information.

interact with collagen. It is interesting to note that we did not find GehC in our library search for collagen adhesins. There are at least two possibilities that could explain this phenomenon; GehC might have a lower binding affinity for collagen, rendering a GehC-expressing clone very hard to detect. Alternatively, when generating the library, the GehC-coding sequence could have been inserted in a different translation frame to that of the vector, thus impeding its correct expression. The ability of recombinant GehC to bind to collagen has not been explored, but it is of future interest.

Mature GehD was identified as a collagen binding adhesin using mAbs raised against Cna-(151–318). However, amino acid sequence comparisons did not reveal any significant similarities between the linear amino acid sequences of Cna and mature GehD. Furthermore, the CD spectra of mature GehD is very different from that of Cna. Deconvolution of the mature GehD data revealed that the predicted overall secondary structure of mature GehD consists of ~26.5% α-helix, 20.6% β-sheet, and 52.9% coil. This secondary structure composition differs markedly from that of the reported crystal structure of Cna-(151–318): 8% α-helix, 53% β-sheet, and 39% coil (31). These data suggest that these proteins may have radically different structures. In contrast, the mature GehD CD spectra strongly resembles that of a mammalian cell surface collagen binding receptor known as the α_1 integrin I domain. The secondary structure composition of this domain is 33.2% α-helix, 20.7% β-sheet, and 46.1% coil, which is comparable with that of mature GehD. We observed several common features between mature GehD and the integrin α_1 I domain. They both bind to collagens, have similar percentage and spatial distribution of α-helices and β-strands, bind divalent cations for full activity, and have open-close conformations (32–35). Because of these common features, it is tempting to speculate that GehD and the integrin α_1 I domain may bind to collagen, adopting similar mechanisms. Although these highly speculative observations may provide some understanding of the collagen binding behavior of GehD, they also underscore the need for a staphylococcal lipase high resolution x-ray structure.

The data described in this work predict that GehD and Cna have radically different secondary structures. However, mAbs 1F6 and 11H11 recognize both proteins. It has been shown that mAb 11H11 recognizes epitopes located in the central segment of Cna-(151–318), and it has been hypothesized that it inhibits ligand binding by directly interfering with collagen within the binding trench (19). This raises the possibility that the collagen binding conformational epitopes present on Cna-(151–318), recognized by 11H11 and 1F6, may also be found in GehD. Therefore, because they seem to recognize a conformational collagen binding epitope, these mAbs can be used as powerful tools to unveil diverse collagen-binding proteins in many other Gram-positive organisms.

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Is the GehD Lipase from *Staphylococcus epidermidis* a Collagen Binding Adhesin?

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