

Ace Is a Collagen-binding MSCRAMM from *Enterococcus faecalis**

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A putative collagen-binding MSCRAMM, Ace, of *Enterococcus faecalis* was identified by searching bacterial genome data bases for proteins containing domains homologous to the ligand-binding region of Cna, the collagen-binding MSCRAMM from *Staphylococcus aureus*. Ace was predicted to have a molecular mass of 71 kDa and contains features characteristic of cell surface proteins on Gram-positive bacteria, including a LPXTG motif for cross-linking to the cell wall. The N-terminal region of Ace contained a region (residues 174–319) in which 56% of the residues are identical or similar when compared with the minimal ligand-binding region of Cna (Cna 151–318); the remainder of the Ace A domain has 46% similarity with the corresponding region of the Cna A domain. Antibodies raised against recombinant Ace A domain were used to verify the cell surface expression of Ace on *E. faecalis*. These antibodies also effectively inhibited the adhesion of enterococcal cells to a collagen substrate, suggesting that Ace is a functional collagen-binding MSCRAMM. Structural modeling of the conserved region in Ace (residues 174–319) suggested a structure very similar to that reported for residues 151–318 of the Cna collagen-binding domain in which the ligand-binding site was identified as a trench transversing a β -sheet face (Symersky, J., Patti, J. M., Carson, M., House-Pompeo, K., Teale, M., Moore, D., Jin, L., DeLucas, L. J., Höök, M., and Narayana, S. V. L. (1997) *Nat. Struct. Biol.* 10, 833–838). Biochemical analyses of recombinant Ace and Cna A domains supported the modeling data in that the secondary structures were similar as determined by CD spectroscopy and both proteins bound at multiple sites in type I collagen with micromolar affinities, but with different apparent kinetics. We conclude that Ace is a collagen-binding MSCRAMM on enterococci and is structurally and functionally related to the staphylococcal Cna protein.

Enterococcus faecalis is a commensal Gram-positive coccus colonizing the intestines of human and other animal hosts. It has been recognized as a common cause of endocarditis since the early 1900s and in the past two decades as an opportunistic pathogen that can lead to serious nosocomial infections (1). *E. faecalis* has many intrinsic and acquired antibiotic resistances that have long been known to complicate therapy of endocarditis, and during recent years resistances to almost all commercially available antibiotics have appeared (1). As a result, care providers may be left without an effective therapy to treat serious infections caused by the emerging multidrug-resistant enterococci. New and alternative strategies to prevent and treat these infections are clearly needed.

Adherence of pathogenic bacteria to the host tissue, mediated by adhesins, is the first event in a multistep process that may lead to clinically manifested infections. For organisms such as *Staphylococcus aureus* and *E. faecalis*, which are primarily extracellular pathogens, ECM¹ components are the targets for adherence. MSCRAMMs (designation for microbial surface components recognizing adhesive matrix molecules) represent a subfamily of bacterial adhesins that recognize and bind to ECM components. Several MSCRAMMs have been isolated and characterized from staphylococci and streptococci (2, 3), among them the *S. aureus* collagen-binding MSCRAMM, Cna.

Cna is a mosaic protein with a molecular mass of 135 kDa (Fig. 1c) (4–8). An N-terminal signal sequence is followed by a 500-residue-long A domain of unique amino acid sequence and a B domain that contains a 110-residue-long unit repeated tandemly one to four times in Cna isolated from different strains of *S. aureus* (9). The C-terminal region of Cna contains a cell wall-associated domain, which includes the LPXTG motif that is a putative recognition site for the hypothetical enzyme sortase that covalently links Cna to the cell wall (4). A hydrophobic transmembrane region is followed by a short cytoplasmic tail rich in positively charged residues. Earlier work showed that the presence of Cna is necessary and sufficient to allow *S. aureus* cells to adhere to collagenous tissues such as cartilage (10). Furthermore, Cna was shown to be a virulence factor in experimental septic arthritis (11), and vaccination of mice with a recombinant form of the Cna A domain was shown

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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) AF159247 (Ace from *E. faecalis* strain CG110).

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¹ The abbreviations used are: ECM, extracellular matrix; Ace, adhesin of collagen from enterococci; BHI, brain-heart-infusion; CD, circular dichroism; Cna, *S. aureus* collagen adhesin; contig, group of overlapping clones; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; recers, recombinant sites in genes that also serve as flexible spacers in the protein; SPR, surface plasmon resonance spectroscopy; TIGR, The Institute for Genomic Research.

to protect against induced staphylococcal sepsis (6).

Currently, our knowledge of the molecular pathogenesis of enterococcal infections is very limited. We and others have recently shown that clinical isolates can adhere to ECM proteins such as collagen, laminin, and fibrinogen (12, 13), but the MSCRAMMs involved have not been previously identified. We report here the discovery and initial characterization of Ace, an enterococcal collagen-binding MSCRAMM.

EXPERIMENTAL PROCEDURES

Identification of *E. faecalis* Ace in a Microbial Genome Data Base—The amino acid sequence comprising the minimal collagen-binding region (residues 151–318) of the *S. aureus* collagen adhesin, Cna (4, 5), was used to search for homologous sequences in the Microbial Genome Database at the National Center for Biotechnology Information. The BLAST (14) search resulted in the discovery of a novel putative gene sequence from *E. faecalis* of significant homology. The complete open reading frame comprising this sequence was subsequently obtained from The Institute for Genomic Research (TIGR) web site.

Structural Modeling Studies—The 335-amino acid sequence of Ace A domain was sent to the ExPASy SWISS-MODEL Automated Protein Modeling Server and modeled using the Cna 151–318 molecule structure (1AMX.pdb, Genbank accession no. M81736) as a template. A sequence alignment and model of the Ace A domain was returned by the ExPASy server composed of 145 amino acid residues based on the structure of Cna 151–318. Even when the 335-amino acid sequence of Ace A domain was sent to the SWISS-MODEL server without specific instructions to model the sequence on the Cna 151–318 structure, the Cna 151–318 structure file was chosen as a template automatically, as determined by a BLAST P(N) search of known protein structure sequences in the ExPDB modified Protein Data Bank data base. Manipulation of the Ace A domain model and Cna 151–318 (1AMX.pdb) was accomplished using the Swiss-PDB Viewer 3.0 software package available at the ExPASy web site, and images were rastered using the software package Persistence of View Ray-tracer (POV-Ray 3.0) (15–17). Temperature factors for the Ace model were higher in the loop regions, especially in regions where there are gaps in the sequence alignment. The root mean square deviation calculated between the two structures was 0.63 Å for the C α atoms (142 total) and 0.61 Å for the analysis of all backbone atoms (426 total). These values for the root mean square deviation of the backbone atoms suggested a high level of accuracy for the model.

Bacterial Strains and Culture Conditions—Unless otherwise noted, chemicals and reagents were molecular-biology grade from Sigma or U.S. Biochemical Corp. Based on our previous report (12), the *E. faecalis* strains were grown in BHI medium (Difco) overnight at 46 °C. The two *E. faecalis* strains used for the Western blot analysis are designated EF1 (originally described by Caparon and Scott (18)) and EF2 (a clinical *E. faecalis* isolate obtained from University of Alabama at Birmingham), respectively. Strain OG1RF Δ Gel is a gelatinase mutant of strain OG1RF (12, 19) and was grown in BHI supplemented with 2 mg/ml kanamycin. *S. aureus* strain Phillips, a clinical isolate from an osteomyelitis case (11), was grown in tryptic soy broth (Difco) at 37 °C.

Cloning and Construction of Expression Plasmids—The nucleotide sequence encoding the Ace A or A+B domains (Fig. 1a) was obtained by PCR using a thermocycler (Perkin-Elmer Cetus 480) and chromosomal DNA (20) from *E. faecalis* strain EF1 as the template. Primers (USB Life Technologies) were designed to amplify nucleotides 94 (5'-GCAGGATCCGAATTGAGCAAAAAGTTCAATC-3') to 1101 (5'-GCAGTTCGACTCAGTCTGTCTTTTCACTTGTTC-3') of the A domain and nucleotides 94 (5'-GCAGGATCCGAATTGAGCAAAAAGTTCAATC-3') to 1750 (5'-GCAGTTCGACTCATGGCTGTTTTTCTCAGTTGTAG-3') of the A+B domain sequence as determined from the nucleotide information obtained from TIGR. The resulting gene fragments were subcloned into pQE-30 (Qiagen Inc., Chatsworth, CA), transformed into *Escherichia coli* strain JM101 and analyzed by automated DNA sequencing (University of Texas Medical School, Houston, TX). Construction of the Cna plasmids that yield the recombinant proteins in Fig. 1d has been described previously (5).

Expression and Purification of Recombinant Proteins—Recombinant Ace A domain with a His tag at its N terminus was produced by inoculating 1-liter cultures of Luria broth (supplemented with 100 μ g/ml ampicillin) with 40 ml of an overnight culture of the A domain expression construct described above. Following 2.5 h of growth at 37 °C, isopropyl- β -D-thiogalactoside was added to a final concentration of 0.2 mM to induce protein expression and the cultures were allowed to

grow for another 3 h. Bacteria were harvested by centrifugation, the supernatant decanted, and the cell pellets resuspended in PBS before being stored at –80 °C. The suspension was later thawed in an ambient-temperature water bath for 30 min and the cells lysed using a French press. Insoluble cell debris was removed by centrifugation at 28,000 $\times g$ for 20 min, followed by filtration through a 0.45- μ m membrane. Recombinant Ace A domain was then initially purified using metal-chelating chromatography. Bacterial lysates were applied to a 5-ml Ni²⁺-charged HiTrap chelating column (Amersham Pharmacia Biotech) and bound protein eluted with a 200-ml linear gradient of 0–200 mM imidazole in 4 mM Tris-HCl, 100 mM NaCl, pH 7.9, at a flow rate of 5 ml/min. Fractions corresponding to recombinant Ace A domain, as determined by SDS-PAGE, were pooled and dialyzed against 25 mM Tris-HCl, pH 8.0, before further purification by ion-exchange chromatography. Dialyzed protein was applied to a 5-ml HiTrap Q column (Amersham Pharmacia Biotech) and bound protein eluted with a 200-ml linear gradient of 0–0.5 M NaCl in 25 mM Tris-HCl, pH 8.0, at a flow rate of 5 ml/min. Fractions containing purified Ace A domain were identified by SDS-PAGE and estimated to be >90% pure. Production and isolation of recombinant Cna proteins was performed as described previously (5).

Preparation of Ace A Domain Polyclonal Antibodies—Purified Ace A domain was dialyzed against 10 mM Na₂HPO₄, 150 mM NaCl, pH 7.4 (PBS), before being sent to HTI Bio Products (La Jolla, CA) for immunization in rabbits and production of polyclonal antisera. For some experiments, IgGs were purified from both immune and pre-immune serum by chromatography using Protein A-Sepharose (Sigma).

Western Blot Analysis—Mutanolysin surface extracts (21) were prepared from *E. faecalis* strains EF1 and EF2 grown at 46 °C and analyzed by Western blot analysis. The presence of Ace was detected following incubation with anti-Ace A domain polyclonal antiserum, followed by goat anti-rabbit IgG horseradish peroxidase, and development in the presence of 4-chloronaphthol and H₂O₂.

Bacterial Adherence Assays—Enzyme-linked immunosorbent assay plates were coated with 5 μ g of type I collagen in 100 μ l of PBS/well overnight at 4 °C. Wells were then washed three times with PBS and then blocked with 1% bovine serum albumin in PBS for 1 h before the addition of bacteria. Bacteria (*E. faecalis* grown at 46 °C, *S. aureus* at 37 °C) were harvested from liquid cultures and diluted to a concentration having an absorbance of 1.0 at 600 nm in PBS (approximately 5 \times 10⁸ bacteria/ml) before being labeled with FITC (22). 100 μ l of labeled bacteria were added per well, and the plates were incubated at 37 °C for 1 h. The total fluorescence (F_{total}) per well was measured after a 1-h incubation using a Fluoroskan II fluorescence reader (Labsystems, Beverly, MA), with λ_{ex} = 485 nm and λ_{em} = 535 nm. The wells were washed with PBS three times to remove unbound bacteria and the remaining fluorescence (F_{test}) measured. Adherence was calculated as follows: adherence = $F_{\text{test}}/F_{\text{total}}$. For the data shown in Fig. 6, adherence of labeled cells in the absence of antibodies was normalized to 100%. Bovine serum albumin-coated wells were used as negative controls. For inhibition assays, FITC-labeled bacteria were first incubated with anti-Ace A domain IgG for 1 h at 37 °C before addition of the mixture to the collagen-coated wells.

Absorption Spectroscopy—Absorption measurements were taken at ambient temperature (23 \pm 2 °C) on a Beckman DU-70 UV-visible spectrophotometer using a 1.0-cm path length cuvette. All spectra were corrected for background noise. Molar extinction coefficients of each protein were calculated using values of Pace *et al.* (23) for the extinction coefficients of the individual residues.

Circular Dichroism Spectroscopy—Far-UV CD data were collected on a Jasco J720 spectropolarimeter calibrated with *d*-10-camphorsulfonic acid, employing a bandpass of 1 nm and integrated for 4 s at 0.2-nm intervals. All samples were less than 15 μ M in 0.1 mM Na₂HPO₄, 1 mM NaCl, pH 7.0. Spectra were recorded at ambient temperature in cylindrical 0.5-mm path length cuvettes. Twenty scans were averaged for each spectrum, and the contribution from buffer was subtracted. Quantitation of secondary structural components was performed as described in Ref. 8. The validity of these results was confirmed by comparison with the results obtained from x-ray crystallographic data for Cna 151–318; the breakdown of secondary structural components is nearly identical for the solid- and solution-phase structures (Table I).

Surface Plasmon Resonance Spectroscopy—Analyses were performed using the BIAcore 1000 system. Bovine type I collagen predissolved in 0.1 M HCl (Collagen Corp., Fremont, CA) was immobilized on a CM5 sensor chip as described previously (5). Recombinant proteins in 150 mM NaCl, 50 mM HEPES, 0.005% P-20 surfactant, pH 7.4, were flowed over multiple flow cells containing different amounts of immobilized collagen. The slowest flow rate (1 μ l/min) specified for the instrument

was employed. Even at this rate, however, the association and dissociation of the recombinant Ace A domain protein with the collagen-coated surface were too rapid to be quantitated. Specific binding response data were obtained by subtracting the response obtained using a flow cell that was not coated with collagen. Analytical conditions were as described previously (24, 25). No mass transport effects were observed in these measurements.

The data for the construction of the Scatchard plots were obtained from the equilibrium portion of the surface plasmon resonance spectroscopy (SPR) sensorgrams (e.g. the response at approximately 900 s in the Ace sensorgram of Fig. 4a). Values for the collagen-bound protein, ν_{bound} , and concentration of unbound protein, $[P]_{\text{free}}$, were calculated from Equations 1–3.

$$\nu_{\text{bound}} = \frac{R_P m_C}{R_C m_P} \quad (\text{Eq. 1})$$

$$\nu_{\text{total}} = \frac{10^{12} [P]_0 \nu_{\text{flowcell}} m_C}{R_C \text{area}_{\text{flowcell}}} \quad (\text{Eq. 2})$$

$$[P]_{\text{free}} = [P]_0 \frac{\nu_{\text{total}} - \nu_{\text{bound}}}{\nu_{\text{total}}} \quad (\text{Eq. 3})$$

In Equation 1, R is the SPR response, m is the molecular mass, P is the protein, C is collagen; in Equation 2, $[P]_0$ is the concentration of total protein, ν_{flowcell} is the volume of sample in the flow cell, and $\text{area}_{\text{flowcell}}$ is the surface area of the flow cell. Plotting $\nu_{\text{bound}}/[P]_{\text{free}}$ versus ν_{bound} yields the plot shown in Fig. 4b. The negative reciprocal of the slope yields the dissociation constant, K_D , and the x axis intercept is equivalent to the number of sites, n , in collagen at which the MSCRAMM protein binds.

RESULTS

E. faecalis Ace Is a Mosaic Protein Having Critical Sequence Homology with *S. aureus* Cna—In an attempt to identify novel collagen binding proteins, we searched microbial genome data bases for amino acid sequences which have significant similarity to that of Cna 151–318 (previously referred to as Cna M19; Refs. 4–8), the central region of the A domain of the *S. aureus* collagen-binding MSCRAMM (Fig. 1c). A significant match was recorded in the *E. faecalis* genome data base.² The complete sequence of the gene encoding this protein, which has been given the working name Ace (adhesin of collagen from *E. faecalis*), was obtained from TIGR and was present on contig gef 6285.³ Translation of the nucleotide sequence revealed a 74-kDa protein, which has a structural organization very similar to that of MSCRAMMs from other Gram-positive bacteria (Fig. 1a). A possible signal sequence involving the first 31 amino acids is followed by a 335-amino acid-long A domain. The B domain is composed of 4.5 tandemly repeated 47-residue units of >90% identity. The C-terminal region is composed of a putative cell wall-associated domain rich in proline residues and contains the cell wall-anchoring LPXTG consensus sequence (26). An 18-amino acid hydrophobic transmembrane region followed by a short cytoplasmic tail represents the C-terminal end of the protein.

PCR primers were designed to amplify the nucleotide sequence encoding the A or A+B domains of ACE from *E. faecalis* strain EF1. The resulting PCR fragment for the A domain corresponded to the same size fragment encoded by strain V583 (27) in the TIGR sequence. However, the PCR fragment for the A+B domain construct was approximately 300 base pairs smaller than expected. DNA sequence analysis revealed that the *ace* gene from strain EF1 contained only 2.5 B domain repeat units, whereas 4.5 B domain repeat units were present in the sequence of strain V583. With the exception of having two fewer B domain repeat units, the DNA sequence of *ace* from strain EF1 was greater than 95% identical to that of strain

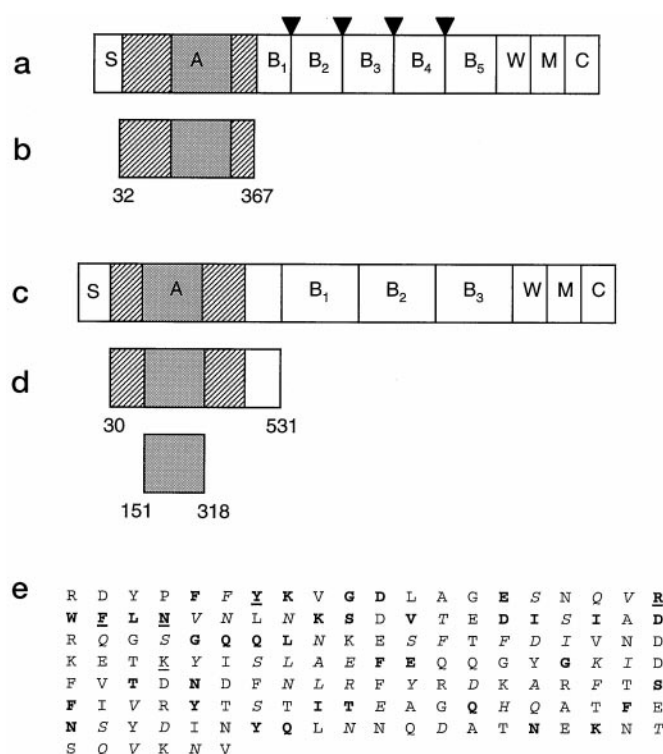


FIG. 1. a, domain organization of *E. faecalis* Ace. The recer sequence present in multiple copies within the B domain is denoted by arrows. b, recombinant protein used in this study that mimics the Ace A domain, with the inclusive residues indicated. c, domain organization of *S. aureus* FDA 574 Cna. d, recombinant proteins used in this study that mimic portions of the Cna MSCRAMM's A domain, with the inclusive residues indicated. The putative signal peptide (S), collagen-binding domain (A), domain of repeat units (B), cell wall domain (W), membrane-spanning domain (M), and charged C-terminal domain (C) are indicated for both MSCRAMMs. The region of homology between Ace and Cna spans the hash-marked blocks, with the shaded blocks depicting the regions modeled in Fig. 2. In these recombinant proteins, MRG-SHHHHHHGS is the amino acid sequence of the unstructured N-terminal His₆ tag required for purification. e, amino acids 174–319 of the *E. faecalis* Ace protein (obtained from the Microbial Genome Database). Ace residues that are identical to the corresponding residues in Cna 151–318 are in bold; those that are similar are in italics. Residues corresponding to those in Cna 151–318 known to be critical for collagen binding are underlined (the sequence of Cna 151–318 is reported in Ref. 5).

V583.

A central region (residues 174–319) in the A domain of *E. faecalis* Ace (from either strain EF1 or V583) has a high degree of sequence similarity to residues 151–318 of the *S. aureus* Cna protein. Within this span of amino acids, 27% of the residues are identical to residues in Cna 151–318 and an additional 29% are similar (Fig. 1e). Significant similarity (46%) continues throughout the A domain of Ace and the corresponding region of the Cna A domain; outside the A domains, however, there is no obvious sequence homology between Ace and Cna.

Structural Models Suggest a Similar Folding Motif for S. aureus Cna 151–318 and *E. faecalis* Ace 174–319—Modeling of Ace 174–319 onto the structure determined for Cna 151–318 gave the structure shown in Fig. 2a. To obtain the best sequence alignment, three one-residue gaps and one three-residue gap were introduced into the sequence of Ace 174–319 and a two-residue gap was introduced into the sequence of Cna 151–318. It is noteworthy that the polypeptide region in Ace covered by residues 174–319 is predicted to fold in a “jellyroll” as Cna 151–318 does, even though a substantial number of the amino acids involved are different as shown in Fig. 2a, where the residues conserved between the two proteins are presented

² Sequence data for *E. faecalis* were obtained from the TIGR web site.

³ K. Ketchum (TIGR), personal communication.

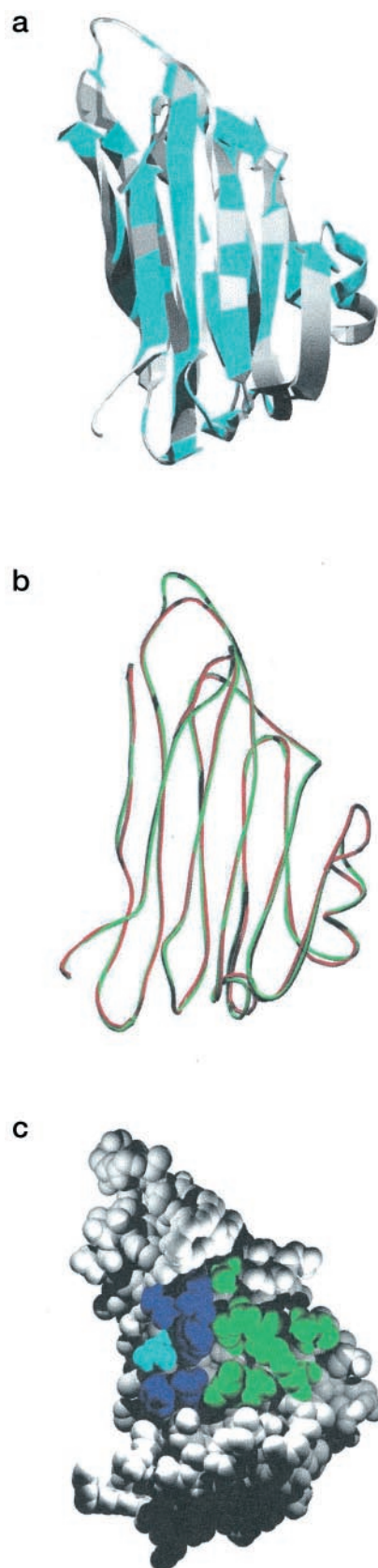


FIG. 2. *a*, ribbon diagram of *E. faecalis* Ace residues 174–319 mapped onto Cna 151–318 x-ray structure, with cyan segments denoting regions of sequence identity or similarity and gray denoting regions lacking homology. *b*, ribbon diagrams of *E. faecalis* Ace A domain residues 174–319 (green) overlaid with Cna 151–318 (red). *c*, space-filled model of *E. faecalis* Ace A domain residues 174–319 mapped onto Cna 151–318 structure. In panel *c*, residues within the putative collagen-binding trench that are conserved in *S. aureus* Cna 151–318 and *E. faecalis* Ace

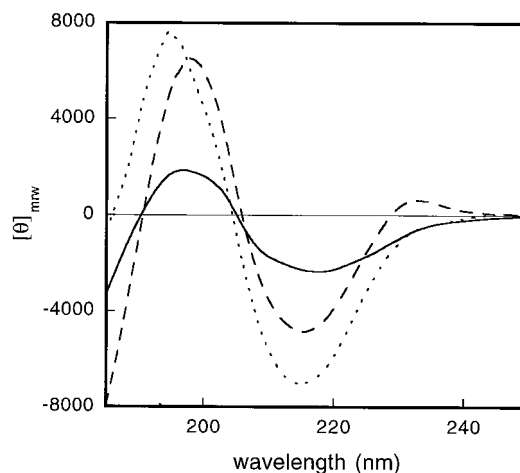


FIG. 3. Far-UV CD spectra of recombinant proteins mimicking the *E. faecalis* EF1 Ace A domain (—) and the *S. aureus* Cna A domain, full-length (---) and residues 151–318 (-.-). Secondary structure compositions are reported in Table I. Mean residue weight ellipticity is reported in degrees \cdot cm²/dmol.

in cyan segments and the residues unique to Ace 174–319 are presented in gray. The more substantial residue differences are located in loops connecting the β -strands. When the polypeptides of Ace 174–319 and Cna 151–318 are overlaid (Fig. 2*b*), the β -strands are almost identical and the most notable folding differences are observed in the loops.

A trench present on the surface of the Cna 151–318 structure has been identified as the collagen-binding site and can accommodate a collagen-like triple-helical peptide (7). The structure predicted for Ace 174–319 contains a trench in the same orientation, as highlighted in Fig. 2*c*. Approximately half of the Cna trench-lining residues are conserved in Ace. Of the conserved putative Ace trench residues shown in blue in Fig. 2*c*, four (Tyr-180, Arg-193, Phe-195, and Asn-197) were shown to be critical for collagen binding in Cna (9). Another residue (Lys-237) known to be critical for collagen binding in Cna 151–318 is not conserved in Ace and is one of the residues shown in green (Fig. 2*c*). These modeling studies, based on the known collagen-binding MSCRAMM, Cna, suggested that Ace: 1) can act as a collagen adhesin and 2) possesses a trench-shaped binding site.

A Recombinant Form of Ace A Domain Has a β -Sheet Structure and Binds Collagen—A recombinant form of the Ace A domain was expressed as a fusion protein with a N-terminal His tag. This protein was soluble and could be purified by chromatography on a Ni²⁺-charged IDA-Sepharose column and an anion-exchange column. Analysis of the protein by CD spectroscopy gave a spectrum with a maximum at 195 nm and a minimum at 217 nm (Fig. 3). This spectrum was qualitatively similar to that of the intact A domain and residues 151–318 of Cna. Deconvolution of the spectra revealed very similar compositions of secondary structure for each of the three proteins dominated by β -sheet structures and with a small α -helical component (Table I).

We used SPR to analyze the predicted collagen-binding activity of Ace. The sensorgrams in Fig. 4*a* show that recombinant Ace A domain and Cna 151–318 both bind to type I collagen immobilized on a BIAcore sensor chip. However, the kinetics of the two interactions were dramatically different.

174–319 are depicted in blue, trench residues that are not conserved are depicted in green, and the one cyan residue is a Thr in Cna 151–318 and a Val in Ace. These structures harbor the introduced gaps described under “Results.”

TABLE I
Summary of secondary structural components

Species	α -Helix	β -Sheet	Other
Ace A	0.07 ± 0.02	0.50 ± 0.13	0.43 ± 0.13
Cna A	0.09 ± 0.04	0.49 ± 0.02	0.42 ± 0.09
Cna 151–318	$0.12 (0.08)^a \pm 0.04$	$0.49 (0.53) \pm 0.04$	$0.39 (0.39) \pm 0.13$

^a Data in parentheses are those obtained from x-ray crystallography (7).

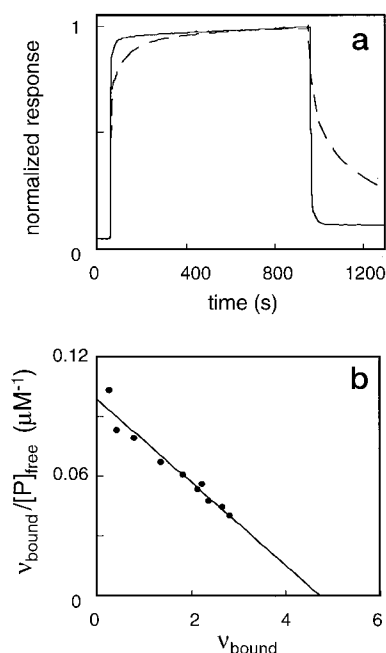


FIG. 4. *a*, representative profiles of the relative SPR responses for the binding of 20 μ M recombinant *E. faecalis* EF1 Ace A domain (—) and *S. aureus* Cna A domain residues 151–318 (---) to immobilized type I collagen. In the analyses shown here, the association occurs from 55 to 960 s and the dissociation begins at 960 s. Both profiles have been corrected for the response of protein over a flow cell containing no collagen. *b*, Scatchard plot of 1–70 μ M *E. faecalis* EF1 Ace A domain binding to 2436 RU immobilized type I collagen as measured by SPR. The analysis was repeated with varying MSCRAMM concentrations and amounts of immobilized collagen. No SPR signal was detected for Ace A domain concentrations of less than 1 μ M. From three measurements, $K_D = 48 \pm 7 \mu$ M; $n = 5.3 \pm 0.3$.

The on and off rates of the Ace/collagen interaction were far too rapid to be determined from these measurements, whereas the association and dissociation rates of the binding of Cna 151–318 to collagen were slower and measurable (24, 25).

Scatchard analysis of SPR equilibrium binding data from increasing concentrations of Ace flowed over immobilized collagen yielded a linear plot (Fig. 4*b*), indicating five copies of a single class of Ace A domain binding sites exist in type I collagen. The calculated dissociation constant (48 μ M) indicated a relatively weak affinity. In contrast, our earlier analyses of the binding of Cna 151–318 and intact Cna A domain to type I or type II collagen yielded a concave upward Scatchard plot, indicating the presence of several classes of Cna binding sites in these collagens (24, 25).⁴

Ace Is a Collagen-binding MSCRAMM—Analyses of the *ace* gene sequence revealed many elements, including the cell wall-anchoring motif characteristic of cell wall-associated surface proteins from Gram-positive bacteria. This raised the question: is Ace a functional collagen-binding MSCRAMM present on the surface of enterococci? We have previously demonstrated that

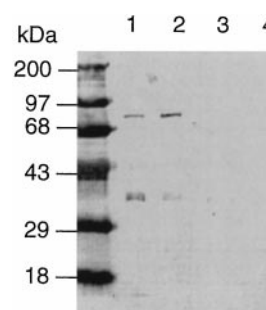


FIG. 5. Western blot analysis of *E. faecalis* surface extracts. *E. faecalis* strains EF1 (lanes 1 and 3) and EF2 (lanes 2 and 4) surface extracts were prepared by mutanolysin digestion and detected using anti-Ace A domain IgG (lanes 1 and 2) or pre-immune IgG (lanes 3 and 4). Prestained molecular mass standards are shown on the left.

most strains of *E. faecalis* can adhere to a collagen substrate after growth at 46 °C, indicating the presence of collagen-binding MSCRAMMs on the bacterial surface (12). Western blot analyses of proteins released from *E. faecalis* strain EF1 and EF2 grown at 46 °C by digestion with mutanolysin demonstrated the presence of two major bands reacting with antibodies raised against the Ace A domain, whereas pre-immune sera did not react with any protein (Fig. 5). The larger band migrated at approximately 80 kDa and most likely represented the full-length Ace protein, whereas the smaller band may represent a proteolytically processed form of the protein. Ace from strain EF1 has an expected molecular mass of only 60 kDa. The difference between this mass and the apparent molecular mass observed in Fig. 5 may be due to the acidic nature ($pI = 4.3$) of the Ace protein.

Antibodies to Ace A domain were tested for their ability to inhibit the adherence of enterococcal cells to a substrate of type I collagen. As shown in Fig. 6*a*, as little as 1 μ g/ml anti-Ace IgG almost completely inhibited bacterial adherence to immobilized collagen, whereas there was no effect of the pre-immune IgG over the range of concentrations examined. Neither immune or pre-immune IgG types had any effect on *S. aureus* strain Phillips adherence to type I collagen, indicating that anti-Ace A domain antibodies did not interfere with the binding of Cna to collagen (Fig. 6*b*). Taken together, these results demonstrate that Ace is present on the surface of *E. faecalis* cells and acts as a collagen adhesin.

DISCUSSION

An earlier study from our laboratories showed that most strains of *E. faecalis* adhered to a substrate of type I collagen when bacteria were grown at elevated temperatures (46 °C), a condition that also retarded growth, but not when grown at 37 °C. We now report the identification of a gene, *ace*, encoding a MSCRAMM, Ace, which may be the agent responsible for the *E. faecalis* adhesion to collagen.

E. faecalis Ace closely resembles the *S. aureus* MSCRAMM, Cna, in its domain organization. Both contain a signal peptide, a nonrepetitive A domain, a B domain composed of multiple repeat units, and cell wall-associated, transmembrane, and cytoplasmic domains (Fig. 1, *a* and *c*). The A domain is present in four of four strains examined: V583, EF1, EF2, and OG1RFΔGel (data not shown). Ace from two strains of *E. faecalis* examined varied in the number of B domain repeat units (V583 has 4.5 B domain repeats; EF1 has 2.5). Similar variation in the number of B domain repeat units has been observed previously for Cna in *S. aureus* (9).

Module shuffling has been observed in *Peptostreptococcus magnus* protein PAB and is presumed to occur at recers (recombinant sites in genes that also serve as flexible spacers in

⁴ Rich, R. L., Narayana, S. V. L., Owens, R. T., Carson, M., Höök, A., Yang, W.-C., Deivanayagam, C. C. S., and Höök, M. (1999) *J. Biol. Chem.*, in press.

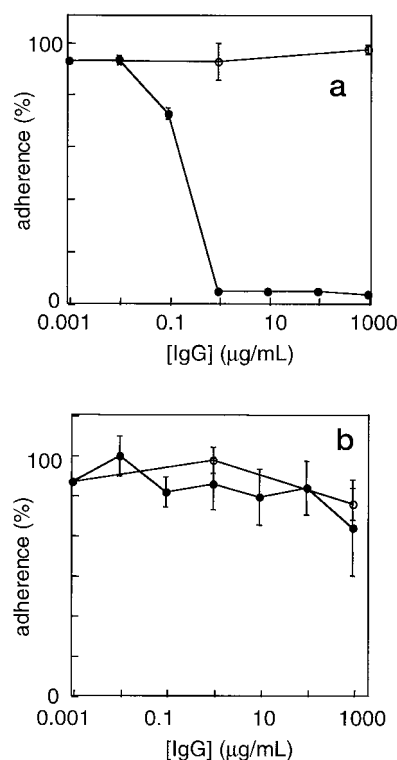


FIG. 6. Inhibition of *E. faecalis* strain OG1RFΔGel (a) and *S. aureus* strain Phillips (b) binding to type I collagen by anti-Ace A domain IgG. FITC-labeled bacteria were preincubated with anti-Ace A domain IgG (●) or pre-immune IgG (○) before addition to wells coated with type I collagen. Values are expressed as the percentage of adherence obtained in the absence of antibody and represent the mean \pm standard deviation of quadruplicate measurements.

the protein) within the nucleotide sequence (29). Employing de Château and Björk's criteria for the identification of recer sequences (GAA AAT CCA GAT GAA, translating into the presumably unstructured ENPDE; Ref. 29), we identified the recer nucleotide consensus sequence at the boundary between each B domain repeat unit in both sequenced *E. faecalis* strains, V583 and EF1 (Fig. 1a). No recer sequences were identified in the Ace A domain or in the entire Cna sequence. Although we have no evidence that recombination occurs at the putative Ace recer sites shown in Fig. 1a, module shuffling of a genetic element may explain why the number of Ace B domain repeat units varies among strains of *E. faecalis*. The role of these B domains is unidentified to date, but it has been shown that the B domain in Cna does not influence the MSCRAMM's collagen-binding capability (8).

Antibodies raised against the Ace A domain effectively inhibited the adhesion of *E. faecalis* grown at 46 °C to collagen (Fig. 6). Although 46 °C is a nonphysiological condition, antibodies to Ace have been isolated from serum from *E. faecalis* endocarditis patients,⁵ indicating that under some physiologic conditions Ace is expressed *in vivo*. The failure of anti-Ace antibodies to prevent collagen adhesion by *S. aureus* was most likely due to the fact that these antibodies did not cross-react well with Cna. This concept is supported by the fact that anti-Ace antibodies failed to react with a recombinant Cna construct in a Western blot (data not shown). In addition, a panel of monoclonal antibodies raised against Cna 151–318 did not cross-react with recombinant Ace A domain.⁶

Not only does the domain organization of Ace resemble that

of Cna, but we suggest that the A domains of the two MSCRAMMs also may fold similarly. The hypothesis that Ace domain residues 174–319 fold as Cna 151–318 does is derived from sequence homologies and molecular modeling studies (Figs. 1e and 2). This is supported by the CD spectra of the Cna and Ace A domains and the deconvolution results from these spectra (Fig. 3 and Table I). Not only are the A domains of both Cna and Ace composed primarily of β -sheets structures, with a minor α -helical component, but the arrangement of the secondary structural elements in the two MSCRAMMs are alike. This secondary structural organization may be an important factor in the MSCRAMMs' ligand-binding capabilities. Based on the molecular modeling, Ace contains a trench similar to the collagen-binding site identified in Cna 151–318. Furthermore, many of the trench residues in Cna 151–318, including most of those known to affect collagen binding, are conserved in Ace 174–319 (six residues highlighted in blue, Fig. 2c).

Although the models and spectra in Figs. 2 and 3 suggest similar structures for the Ace A domain and Cna 151–318, the mechanism of binding collagen is apparently distinct for the two proteins. Not only are their respective on and off rates to collagen of different magnitudes (Fig. 4a), but their specificities for sites within the collagen macromolecule are also different, as demonstrated by the Scatchard plots of Ace and Cna; the Scatchard plot of Ace is linear (Fig. 4b), but that of Cna 151–318 is distinctly nonlinear (5). The Ace A domain associates and dissociates with collagen rapidly, binding at five sites in the type I collagen strand with equal affinity. Under similar analytical conditions, Cna 151–318 and full-length Cna A domain associate and dissociate with collagen much more slowly and interact more promiscuously with collagen, binding at a great number of sites in the ligand and with a range of affinities (5).⁴ We cannot exclude the possibility of lower affinity interactions occurring between Ace and collagen at Ace concentrations greater than 70 μ M, but consider protein concentrations much above 100 μ M to approach the boundary between specific and nonspecific protein-ligand interactions. Therefore, we have chosen to study the collagen binding by ACE over the range of MSCRAMM concentrations that have yielded the multiphasic Scatchard plots for Cna.

Although the collagen-binding regions of Cna and Ace may be so similar in structure, what accounts for their very different interaction mechanisms with type I collagen? Perhaps the residues that are conserved in these proteins (particularly those residues in the binding-site trench) are: 1) responsible for recognition of a common element within the triple-helical collagen or 2) vital for maintaining the MSCRAMM's gross trench structure. In the first scenario, the binding-trench residues that are not conserved may regulate a particular MSCRAMM's specificity for and affinity to collagen. In the second, the nonconservation of residue Lys-237 and other trench residues in the Ace A domain may result in a more rigid and/or "slippery" binding trench, in which collagen may fit with little conformational rearrangement of the binding site or ligand. Under such conditions, only a few sites within collagen may be amenable to MSCRAMM binding and rapid interaction rates would be possible. On the other hand, the trench in Cna 151–318 may be more flexible or contain more residues that form hydrogen bonds or hydrophobic patches with collagen, thereby: 1) exhibiting slower interaction rates as conformational reorganization occurs during the binding event, and 2) providing for suitable contact with a variety of sites in collagen. These results suggest different mechanisms of ligand interactions may exist for MSCRAMMs binding to the same ECM molecule. It is also possible that collagens other than type I contain high affinity Ace-binding sites. Identification of the residues critical for col-

⁵ B. E. Murray, unpublished results.

⁶ P. Speziale, personal communication.

lagen binding in *E. faecalis* ACE and the resolution of the Ace A domain crystal structure would answer many of the unresolved questions concerning this new member of the MSCRAMM family; these studies are under way.

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Ace Is a Collagen-binding MSCRAMM from *Enterococcus faecalis*
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