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Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in *Enterococcus faecium*

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SUMMARY

Attention has recently been drawn to *Enterococcus faecium* because of an increasing number of nosocomial infections caused by this species and its resistance to multiple antibacterial agents. However, relatively little is known about pathogenic determinants of this organism. We have previously identified a cell wall anchored collagen adhesin, Acm, produced by some isolates of *E. faecium*, and a secreted antigen, SagA, exhibiting broad spectrum binding to extracellular matrix proteins. Here, we analyzed the draft genome of strain TX0016 for potential MSCRAMMs (microbial surface component recognizing adhesive matrix molecules). Genome-based bioinformatics identified 22 predicted cell wall anchored *E. faecium* surface proteins (Fms) of which 15 (including Acm) have typical characteristics of MSCRAMMs including predicted folding into a modular architecture with multiple immunoglobulin-like domains. Functional characterization of one (Fms10, redesignated Scm for second collagen adhesin of *E. faecium*) revealed that recombinant Scm_{65} (A- and B-domains) and Scm_{36} (A-domain) bound efficiently to

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collagen type V in a concentration dependent manner, bound considerably less to collagen type I and fibrinogen, and differed from Acm in their binding specificities to collagen types IV and V. Results from far-UV circular dichroism of recombinant Scm_{36} and of Acm_{37} indicated that these proteins are rich in β-sheets, supporting our folding predictions. Whole-cell ELISA and FACS analyses unambiguously demonstrated surface expression of Scm in most *E. faecium* isolates. Strikingly, 11 of the 15 predicted MSCRAMMs clustered in four loci, each with a class C sortase gene; 9 of these showed similarity to *Enterococcus faecalis* Ebp pilus subunits and also contained motifs essential for pilus assembly. Antibodies against one of the predicted major pilus proteins, Fms9 (redesignated as $EbpC_{fm}$), detected a "ladder" pattern of high-molecular weight protein bands in a Western blot analysis of cell surface extracts from *E. faecium*, suggesting that $EbpC_{fm}$ is polymerized into a pilus structure. Further analysis of the transcripts of the corresponding gene cluster indicated that *fms1 (ebpA$_{fm}$), fms5 (ebpB$_{fm}$)* and *ebpC$_{fm}$* are co-transcribed, consistent with pilus-encoding gene clusters of other gram-positive bacteria. All 15 genes occurred frequently in 30 clinically-derived diverse *E. faecium* isolates tested. The common occurrence of MSCRAMM and pilus-encoding genes and the presence of a second collagen-binding protein may have important implications for our understanding of this emerging pathogen.

INTRODUCTION

Enterococcus faecium, a member of the normal commensal flora, has recently emerged as a prominent nosocomial pathogen causing serious infections, including infective endocarditis (Murray, 2000). Nosocomial infections due to *E. faecium* can be a life threatening challenge to physicians because of this organism's multi-drug resistance (Murray, 2000; Rice, 2001). Furthermore, in addition to the selective advantage conferred by antibiotics, strains that emerge in the hospital setting more often carry putative virulence genes such as *esp* encoding enterococcal surface protein (Rice *et al.*, 2003; Willems *et al.*, 2001), *hyl* encoding a putative hyaluronidase (Rice *et al.*, 2003) and a functional copy of the *acm* gene encoding a collagen adhesin (Nallapareddy *et al.*, 2003; Nallapareddy *et al.*, 2007b).

It is known that pathogenic bacteria have evolved a plethora of proteins to adhere to and invade host tissues and to resist host defenses (Pizarro-Cerda & Cossart, 2006). Among these is a family of surface proteins with well-established roles in host-pathogen adherence which have been termed MSCRAMMs (microbial surface component recognizing adhesive matrix molecules) (Patti *et al.*, 1994). MSCRAMMs share several characteristics including i) an N-terminal signal peptide, ii) a non-repeated A-domain consisting of Ig-like fold(s), iii) a B-domain with a variable number of repeats among different strains, and iv) a C-terminal CWA (cell wall anchor) domain. Some of these MSCRAMMs were recently shown to be tethered to each other by a designated sortase to make up multimeric cell surface structures, named pili (Mora *et al.*, 2005; Nallapareddy *et al.*, 2006). Sortases, encoded by the *srtA* to *srtD* classes of genes (Dramsi *et al.*, 2005), were originally described as membrane-bound transpeptidases that cleave the LPXTG-like motif in the CWA domain and covalently link them to the peptidoglycan (Marraffini *et al.*, 2006). While Class A sortases appear to be ubiquitous and involved in cell surface anchoring of a large number of LPXTG containing proteins (Marraffini *et al.*, 2006), the class C (subfamily 3) sortase enzymes, which have a more limited substrate specificity, have recently been shown to be involved in pilus

biogenesis, in addition to their role in surface anchoring (Kemp *et al.*, 2007; Scott & Zahner, 2006; Telford *et al.*, 2006).

Studies that have characterized the binding interactions of staphylococcal and enterococcal MSCRAMMs identified that the ligand-binding A-domains consist of two to three subdomains (N1-N2/N3) each adopting an Ig-like fold (Liu *et al.*, 2007; Nallapareddy *et al.*, 2007a; Ponnuraj *et al.*, 2003; Zong *et al.*, 2005). Based on the crystal structures of prototype MSCRAMMs, two slightly different models have been proposed to explain their binding mechanisms to linear peptides of fibrinogen and to triple-helical collagen. In the "dock, lock and latch" binding model, a fibrinogen chain is inserted into a cleft between two Ig-folded subdomains and is then secured by a C-terminal N3 extension (latch) that is reoriented upon ligand binding and complements a β-sheet on the N2 subdomain (Ponnuraj *et al.*, 2003). A variation of this two-subdomain binding model, "the collagen hug", has been proposed for the collagen-binding MSCRAMMs (Liu *et al.*, 2007; Zong *et al.*, 2005).

Previous *in silico* analyses have identified a family of genes encoding MSCRAMM-like proteins in genomes of several gram-positive bacteria including our reports of the *ebp* (endocarditis and biofilm-associated pilus of *Enterococcus faecalis*) operon, Ace (adhesin of collagen from *E. faecalis*) and Acm (adhesin of collagen from *E. faecium*) (Bowden *et al.*, 2005; Nallapareddy *et al.*, 2000; Nallapareddy *et al.*, 2003; Nallapareddy *et al.*, 2006; Roche *et al.*, 2003; Sillanpaa *et al.*, 2004; Xu *et al.*, 2004). Recently, Hendrickx *et al.* (2007) predicted 22 CWA proteins from *E. faecium* TX0016 (formerly, DO (Arduino *et al.*, 1994)) genome, of which five were found to be enriched in isolates of the hospital-adapted clonal complex 17 (CC17). However, with the exception of the prototype MSCRAMM, Acm (Nallapareddy *et al.*, 2003)), there has been no demonstration of other MSCRAMMs in *E. faecium*. Although our previous report identified an essential and secreted broad-spectrum adhesin, SagA, exhibiting binding to fibrinogen, collagen type I (CI), collagen type IV (CIV), fibronectin, and laminin, this protein lacks a CWA domain and other MSCRAMM characteristics (Teng *et al.*, 2003).

In the present study, we identified 14 genes (in addition to *acm*) encoding predicted MSCRAMMs in the genome of *E. faecium* TX0016. Recombinant forms of one of these proteins, designated as Scm (for second collagen adhesin of *E. faecium*), were characterized to confirm the structural predictions and to identify its ligand. Cell surface expression of Scm by selected *E. faecium* strains was quantitated using FACS, and antibodies raised against a recombinant form of one of the major pilus proteins showed a high-molecular weight (HMW) ladder pattern characteristic of gram-positive pili. Co-transcription of one of the pilus-encoding gene clusters was demonstrated by Northern hybridization and RT-PCR. In addition, we determined the distribution of the MSCRAMM encoding genes among 30 diverse *E. faecium* clinical isolates.

METHODS

Strains, plasmids and cultivation of bacteria

Relevant characteristics of bacterial strains, growth conditions, and plasmids used in this study are summarized in Table 1 and in Supplementary Methods. All constructs were given

TX numbers and plasmids from these constructs were assigned respective pTEX numbers (Table 1).

Escherichia coli and *E. faecium* cells were grown in Luria-Bertani (LB) broth/agar and Brain Heart Infusion broth/agar (Difco), respectively. For *E. coli* constructs, antibiotics were used at the following concentrations: 25 μg kanamycin ml^{-1} and 100 μg ampicillin ml^{-1}.

Identification and structural analysis of CWA proteins

Bioinformatics methods used for genome-wide identification of CWA proteins are described in the Supplementary Methods. Domain architecture as well as fold-recognition analyses were carried out by comparing the protein sequences against domain databases as described in Supplementary Methods.

Construction of expression plasmids

Genomic DNA from *E. faecium* strains was isolated as described earlier (Wilson, 1994). DNA regions encoding amino acids 27 to 624 (A-domain and B-repeats) and 27 to 333 (A-domain) of Scm and aa 33 to 590 of $EbpC_{fm}$ (Fms9 was renamed as $EbpC_{fm}$ based on its 74% aa identity (84% similarity) with EbpC of *E. faecalis* over the entire protein) (mature protein without the signal peptide and the CWA domain) were amplified using primers listed in Supplementary Table S1 and cloned into the expression vector pQE30 (Qiagen) to obtain pTEX5432, pTEX5628 and pTEX5630 (Table 1). The corresponding expressed protein segments of Scm were designated as $rScm_{65}$ and $rScm_{36}$, respectively, based on their calculated molecular masses. Similarly, the cloned segment of $EbpC_{fm}$ was designated as $rEbpC_{fm62}$. Constructs were confirmed by sequencing.

Purification of recombinant proteins

Recombinant proteins with N-terminal His_6-tags were expressed and purified using nickel affinity chromatography followed by anion exchange chromatography (Nallapareddy *et al.*, 2007a; Sillanpaa *et al.*, 2004). Protein concentrations were determined by absorption spectroscopy at 280 nm using calculated molar absorption coefficient values. Molecular masses were determined with MALDI-TOF MS for $rScm_{36}$ and $rScm_{65}$.

Circular dichroism (CD) specta

Far-UV CD spectroscopy data were collected as described previously (Sillanpaa *et al.*, 2004). Secondary structure compositions were quantitated with ContinLL, SELCON3 and CDSSTR (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) (Lobley *et al.*, 2002; Whitmore & Wallace, 2004).

ELISA-type solid phase ligand binding assays

Binding of the recombinant His-tag fusion proteins to components of the ECM (extracellular matrix) was tested using a previously described assay with minor modifications (Nallapareddy *et al.*, 2000). In brief, 1 μg of each ECM protein (for source, see Supplementary Methods) was coated in 100 μl PBS in Immulon 2HB (Thermo Scientific) 96-well microplate wells. Wells were incubated with various concentrations of rScm and bound His-tag proteins were detected with anti-His_6 mAb (GE Healthcare) followed by

alkaline phosphatase-conjugated anti-mouse antibody (Bio-Rad). *p*-nitrophenyl phosphate (Sigma) was used for signal detection.

Production of polyclonal antibodies and purification of antigen-specific Igs

Polyclonal goat antibodies against $rScm_{36}$ and $rEbpC_{fm62}$ were raised at Bethyl Laboratories. Scm_{36}- and $EbpC_{fm62}$-specific Igs were eluted from CnBr-activated Sepharose 4B coupled with the corresponding antigen, according to the manufacturer's protocol (GE Healthcare). 0.1 M glycine, pH 2.8, was used for elution of bound antibodies, which were immediately neutralized by 1 M Tris/HCl, pH 8.0, and dialyzed extensively against PBS. Antibody concentrations were determined using an estimated IgG molar absorption coefficient value of 210 000 $M^{-1}cm^{-1}$ and a molecular mass of 150 000 Da.

Whole-cell ELISA and FACS

Surface expression of Scm on *E. faecium* cells was detected by a whole-cell ELISA assay (Nallapareddy *et al.*, 2003) using affinity purified $rScm_{36}$-specific Igs. Control antiserum against formalin-killed TX0016-whole-cells (Rakita *et al.*, 2000) was used as a positive control.

To quantitate surface expression of Scm by FACS analysis, bacteria grown in BHI 14 h from an inoculum of OD_{600nm} = 0.01, were labeled with preimmune or affinity-purified anti-Scm-specific antibodies followed by donkey anti-goat IgG conjugated with $F(ab')_2$-fragment-specific *R*-phycoerythrin, as described earlier (Kemp *et al.*, 2007). Cells were then fixed in 1 % paraformaldehyde in PBS and analyzed with a Coulter EPICSXL AB6064 flow cytometer (Beckman Coulter) and System II software.

Extraction of CWA proteins and Western blot analysis

CWA proteins were extracted from *E. faecium* strains grown for 8 h in BHI broth to late-exponential phase (starting inoculum, ~0.01 OD_{600nm}) with mutanolysin as described earlier (Nallapareddy *et al.*, 2006). Equal amounts of concentrated mutanolysin extracts were separated using 4%-15% gradient SDS-PAGE gels (Bio-Rad) under reducing conditions and transferred to PVDF membranes according to the manufacturer's protocol. Membranes were probed with affinity-purified anti-Scm_{36} and anti-$EbpC_{fm62}$ antibodies (see above) followed by HRP-conjugated anti-goat IgG antibodies and, as a control, total IgG antibodies purified from preimmune goat sera were used. Signal was detected using SuperSignal West Pico Chemilumiscent detection reagents (Thermo Scientific).

Northern hybridization

Total RNA was isolated from TX0082 grown in BHI to mid-exponential phase using the RNAprotect Bacteria Reagent and RNeasy Mini Kit (Qiagen). Thirty micrograms of total RNA were separated using a formaldehyde-containing agarose gel under denaturing conditions and transferred to a Hybond-N+ membrane as described by the manufacturer (GE Healthcare). DNA probes obtained with primers listed in Supplementary Table S1 were radiolabeled by using the RadPrime DNA labeling system (Invitrogen). Hybridization was performed under high stringency conditions as detailed earlier for Southern blots (Murray *et al.*, 1993).

RT-PCR

Total RNA (isolated as above for Northern hybridization) was treated twice with 20 U RQ1 DNase (Promega) for 30 minutes at 37°C. DNase was removed using the RNeasy Mini Kit and purification protocol (Qiagen). Total RNA was then reverse transcribed with specific primers (Supplementary Table S1) using the SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen) according to the manufacturer's instructions. DNA sequencing verified that the primer regions of TX0082 are 100 % identical to the corresponding sequences in TX0016. As an internal control, a 509-bp fragment of *gyrA* (encoding gyrase A) was amplified using the FmGyrF and FmGyrR primers (Supplementary Table S1). Reactions without RT were used as controls to verify lack of DNA contamination in the total RNA preparation.

Colony hybridization

Preparation of colony lysate membranes and hybridization under high stringency conditions were performed as previously described (Coque *et al.*, 1995; Singh *et al.*, 1998). DNA probes were generated and radiolabeled as described above for Northern hybridization.

RESULTS AND DISCUSSION

Identification of putative CWA proteins with Ig-like folds

Our search of the nearly completed genome sequence of *E. faecium* endocarditis-derived strain TX0016 (GW, BEM, *et al.*, unpublished data, http://www.hgsc.bcm.tmc.edu/) yielded a total of 22 ORFs encoding putative CWA proteins (designated Fms, for *E. faecium* surface proteins) with a tripartite pattern near the C-terminus (an LPXTG motif or variant, followed by a membrane-spanning hydrophobic region and a positively charged tail). This number is in the range of predicted CWA proteins of related gram-positive bacteria (Ponnuraj *et al.*, 2003) which vary from 8 (*Streptococcus mutans)* to 41 (*E. faecalis*). While the number of CWA proteins identified here is the same as the number reported in a recent study (Hendrickx *et al.*, 2007), which identified CWA proteins from a partial TX0016 genome sequence (available at NCBI), the published report did not include one of the ORFs here (Fms6) and classified a pseudogene (Fms15) as two ORFs (Supplementary Results).

Among these 22 putative CWA proteins, 18 contained a predicted N-terminal signal peptide sequence required for Sec-dependent secretion. Further analysis of the 5′-regions of the remaining four ORFs (Fms14, Fms15, Fms16, and Fms19) lacking a signal peptide revealed the presence of N-terminal signal peptides in the ORFs immediately upstream to Fms15, Fms16, and Fms19. Careful examination of the junction regions showed the presence of a premature stop codon due to a point mutation or frame-shift in *fms15, fms16*, and *fms19* (Supplementary Results). Sequencing of *fms16* and *fms19* regions from additional isolates identified intact *fms16* and *fms19* genes in two of 10 *E. faecium* clinical isolates tested (Supplementary Results). However, we did not find an *E. faecium* strain with an intact *fms15* gene (Supplementary Results).

Subsequently, using the fold recognition servers 3D-PSSM and PHYRE, we identified 15 of the predicted Fms proteins (Table 2) as containing one or more Ig-like folds enriched with β-

sheets and only a small quantity of α-helices; similar secondary structure compositions have been found in the ligand binding A-regions of MSCRAMMs (Deivanayagam *et al.*, 2002). One of these, Fms8, was previously identified in our laboratory as Acm (Nallapareddy *et al.*, 2003). The percentage of *E. faecium* LPXTG-proteins containing Ig-like folds is greater than the percentages of other gram-positive bacteria (22 % - 45 %) (Ponnuraj *et al.*, 2003). The Ig-like fold containing modules of the A-domain of a number of well characterized *S. aureus* MSCRAMMs (e.g., the fibronectin-, fibrinogen-, and elastin-binding protein, FnbpA; the fibrinogen-binding proteins, ClfA and ClfB; the collagen-binding protein, Cna) and the prototype MSCRAMM of *E. faecium*, Acm, have been shown to play a direct role in interactions with their respective ECM protein ligands (Nallapareddy *et al.*, 2007a; Ponnuraj *et al.*, 2003; Zong *et al.*, 2005).

Structural organization of the *E. faecium* putative MSCRAMMs

Because of our interest in surface adhesins, we next performed additional analyses on the subset of predicted Ig-like fold-containing proteins and identified that most possess a multi-domain architecture similar to the characterized MSCRAMMs of *S. aureus* and *E. faecalis* (Deivanayagam *et al.*, 2002; Perkins *et al.*, 2001; Sillanpaa *et al.*, 2004) (Supplementary Results). A schematic of the predicted primary sequence organization of these proteins is depicted in Fig. 1. The N-terminal signal peptide sequences, found in all but Fms14, ranged from 24-56 aa in length. The majority had perfectly conserved canonical LPXTG motifs, while six had anchor motifs with a broader consensus (Table 2).

Individually organized genes encoding putative MSCRAMMs

Fms10, Fms15, and Fms18, like Acm, are encoded by individual genes and are not clustered with other MSCRAMM-encoding genes. Among these, Fms18 has 94 % similarity (Table 2) to the *E. faecalis* MSCRAMM EF1896 which is predicted to be mostly Ig-folded and shows binding to a serum protein (J. S, S.R.N., B.E.M., and M. Hook, unpublished data). Scm (Fms10) of TX0016 has thirteen B-repeats of 19 aa in length and a partial repeat of 10 aa, while pseudogene *fms15* predicts 8 B-repeats of 91 to 93 aa in length. The primary sequences of Scm and Fms15 each predict a single subdomain with an Ig-like fold in the A-domain, unlike the well-established MSCRAMMs which have two to three subdomains. Hence, we hypothesize that, if these proteins are adhesins, they may adopt a different binding mechanism, and we chose Scm for further characterization.

Characteristics of recombinant Scm protein segments

Total yields of the recombinant protein $rScm_{65}$ (full mature protein consisting of the A-domain and B-repeats, aa 27-624) were low. Although we estimated that this protein was over 95 % pure immediately after eluting from the chromatographic columns as shown in Supplementary Fig. S1, it was susceptible to partial degradation even after short storage. $rScm_{65}$ migrated as a larger band than expected from the calculated molecular weight of 64.5 kDa, likely due to the highly acidic nature (pI = 3.9) of the protein. To obtain a stable construct with higher yields, we next expressed $rScm_{36}$ encompassing the complete A-domain (aa 27-333) (Supplementary Fig. S1). A MALDI-TOF mass spectrometry analysis determined the molecular mass of $rScm_{36}$ as 35 711 Da, which is in good agreement with the calculated mass of 35 691 Da, and confirmed the absence of posttranslational

modifications and homogeneity of the purified protein. As an additional confirmation of the identities of the purified protein segments and evidence of their intact N-termini, a monoclonal anti-His_6 antibody detected the N-terminal His_6- fusion in both purified protein segments in a Western blot assay (data not shown).

Recombinant Scm binds to collagen in a dose-dependent manner

Since the adhesive functions of previously studied MSCRAMM proteins have mostly been ascribed to binding to protein components of the host ECM, we examined the potential ECM-adhesive functions of Scm by testing its binding to a panel of individual ECM proteins in an ELISA-type solid-phase ligand binding assay. As seen in Fig. 2(a), both $rScm_{65}$ (containing the full mature protein) and Scm_{36} (containing only the A-domain) exhibited significant binding to CV (35-fold and 82-fold, respectively, higher than the background level seen with immobilized BSA). Interestingly, binding of these rScm proteins to fibrinogen and CI (especially $rScm_{36}$) was also observed compared to their binding to CIV, laminin, and fibronectin (Fig. 2a).

Further analysis of the binding interactions of increasing concentrations of $rScm_{36}$ with CI and CV as well as fibrinogen by dose response assays showed concentration dependent binding of $rScm_{36}$ to CV which approached saturation with apparent half-maximal binding (K_D) reached at 30.8 ± 1.8 μM (Fig. 2b). The apparent affinity of $rScm_{36}$ for fibrinogen and CI was low and did not reach reliable saturation levels. In spite of partial degradation, Scm_{65} showed dose dependent binding with similar affinity for CV as Scm_{36} (data not shown). Although K_D's from steady-state assays are estimations, our affinity determination for the Scm A-domain is in the same range as previously shown for the minimal ligand binding domain of Ace to CI (48 ± 7 μM) (Rich *et al.*, 1999), but lower than the A-domain of Acm to CI (3.8 μM) or CIV (12.8 μM) (Nallapareddy *et al.*, 2003). Interestingly, Acm did not show appreciable binding to CV (data not shown) and bound efficiently to CI and, to a lesser degree to CIV, in agreement with our previous report (Nallapareddy *et al.*, 2003). Taken together, our results suggest that CV is a binding ligand for Scm and this binding is mediated by the A-domain.

The three collagen types included in our assays have distinct structures and tissue distributions. CI is the most abundant form and the main component of collagen fibers that are widely distributed in human tissues, while CIV forms structurally different cross-linked networks and is found nearly exclusively in basement membranes. CV, although quantitatively less abundant, has a critical role in formation of the fibrillar collagen matrix and connecting interstitial collagen fibrils with membranous collagen networks (Nicholls *et al.*, 1996; Wenstrup *et al.*, 2004). Considering the diverse tissue localizations and structural differences of the various collagen types, possessing two collagen adhesins with different binding specificities to various collagen types could give *E. faecium* the ability to fine-tune its adherence phenotype to suit a given tissue. Since collagens, including CV, are also present in the intestinal submucosa (Liang *et al.*, 2006), Scm could alternatively be involved in colonization and persistence in the intestinal tract, a major natural reservoir of both commensal and infection-associated strains of *E. faecium* in humans, or in facilitating translocation through damaged intestinal epithelium.

Structural analysis of recombinant Scm protein

To investigate the structural predictions made above, we analyzed the full A-domain of Scm with far-UV CD spectroscopy. The obtained spectra showed a maximum at 197 nm and another at 191 nm, and a minimum at 217 nm (Fig. 3a). A similar overall pattern was observed with the collagen-binding A-domain of the *E. faecium* prototype MSCRAMM Acm, which we have previously characterized (Nallapareddy *et al.*, 2003; Nallapareddy *et al.*, 2007a), and also with the minimal collagen binding region of Ace from *E. faecalis*, for which crystal structure studies have recently revealed that it folds into a similar DEv variant of the Ig-fold as previously found in the ligand binding A-domains of staphylococcal MSCRAMMs (Liu *et al.*, 2007).

Deconvolution of the collected data showed a secondary structure composition of 0.15 ± 0.03 of α-helix and 0.34 ± 0.01 of β-sheet for $rScm_{36}$ (Fig. 3b). Although the helical content of this protein is slightly higher than in the two control proteins, these results generally resemble the overall secondary structure compositions of the ligand binding regions of Acm and Ace and are in good agreement with earlier CD analyses and crystal structure data of other MSCRAMM A-regions (Rich *et al.*, 1999). While fold analyses and multiple alignments predicted a single Ig-folded subunit from aa 125 to 325 in the A-domain of Scm, our CD measurements indicate a high β-sheet composition for the entire Scm A-domain, thus suggesting that an Ig-folded or similar β-sheet-rich structure may extend over the whole A-domain (aa 27 to 333).

Cell surface expression of Scm

Using Scm-specific antibodies affinity purified (with $rScm_{36}$) from goat anti-serum, we assessed the surface expression of Scm by 8 *E. faecium* clinical isolates (including the sequenced strain TX0016) using whole-cell ELISA. Two *E. faecium* community derived strains which lacked *scm* (identified below) were used as negative controls. Among 8 scm^+ isolates, four (TX0074, TX2535, TX2400 and TX2589) were strongly positive, two (TX0068 and TX2081) were positive, one (TX2416) was weakly positive and one (TX0016) was negative in this assay (Supplementary Fig. S2), suggesting that Scm is produced on the cell surface of many *E. faecium* isolates during *in vitro* growth. Subsequent quantitation of Scm surface expression in these isolates by FACS (Fig. 4) showed variable levels of surface expression, with the percentage of cells expressing Scm ranging from 2.3 % to 96.9 %. In strains TX0074, TX2535, TX2400 and TX2589, Scm was detected on the surface of 90 to 97 % of cells and with relatively high fluorescence intensities, thus confirming our observations with whole-cell ELISA. Furthermore, these surface detection studies suggest that the majority of cells of scm^+ isolates are actively producing Scm on the cell surface during *in vitro* growth. Consistent with our whole-cell ELISA and FACS results, a Western blot assay using the anti-Scm_{36} antibody identified a protein band corresponding to the expected size of mature Scm (~ 63 kDa) in mutanolysin cell wall extracts from TX0074 and TX2535, but not from TX0016 (data not shown).

Eleven of 15 putative MSCRAMM-encoding genes are clustered at four different genomic loci and nine are predicted to produce four distinct types of pili

Unlike Acm, Scm (Fms10), Fms15, and Fms18, the remaining 11 putative MSCRAMM genes were found clustered in four genomic loci. Nine of the clustered genes show significant similarity to pilus-associated proteins from other species (Table 2). Analysis of the nucleotide region of the locus spanning *fms1, fms5* and $ebpB_{fm}$ (*fms9)* predicted the presence of four ORFs, all oriented in the same direction, including an *srtC1* gene encoding a class C (subfamily 3) sortase (Fig. 1b). We subsequently renamed Fms1, Fms5, and SrtC1 as $EbpA_{fm}$, $EbpB_{fm}$, and Bps_{fm}, respectively, based on their high sequence identity with the *E. faecalis* Ebp cluster proteins (Nallapareddy *et al.*, 2006) (Table 2). A similar arrangement of predicted pilus-associated proteins with an adjacent class C sortase was found in the *fms14-17-13* and the *fms11-19-16* clusters. Besides our prediction of $ebpABC_{fm}$, *fms14-17-13* and *fms11-19-16* co-transcription, due to short or overlapping intergenic regions, we were unable to identify transcriptional terminator-like sequences in these gene clusters; thus, it is likely that these genes are in three operons (see also below). The genes encoding Fms20 and Fms21 were found to be located close to each other in the genome, separated by two ORFs. One of these ORFs encodes a predicted class C sortase (SrtC4). In addition to *srtC4*, this locus also contains one class A sortase-encoding gene (*srtA*) immediately upstream of *fms21* (Fig. 1b). Another ORF located between SrtC4 and Fms20 shows 27 % similarity to the EbpB pilus protein and has a signal peptide sequence but no CWA motif. Of note, the *E. faecium* TX0016 genome also contains a sixth sortase gene (*srtC5*) predicting a class C sortase. However, none of the CWA protein genes were associated with the *srtC5* locus.

Subsequent *in silico* predictions identified conserved pilin motifs and E-boxes of gram-positive pilus proteins in all four predicted major pilus protein homologues ($EbpC_{fm}$, Fms13, Fms16, and Fms21) and all five of the predicted accessory protein homologues ($EbpA_{fm}$, $EbpB_{fm}$, Fms14, Fms17, and Fms19) associated with the four gene clusters described above (Supplementary Fig. S3). The lysine (K) residue of the pilin motif and the glutamic acid (E) of the E-box that were demonstrated to be essential in polymerization of *Corynebacterium diphtheriae* pilus (Ton-That & Schneewind, 2003; Ton-That *et al.*, 2004) were found to be 100 % conserved. Two accessory proteins, namely $EbpA_{fm}$ and Fms14, have a von Willebrand factor type A-domain with a MIDAS-motif which is frequently found in pili-associated accessory proteins. Recently, crystal structure analyses of the minor pilin protein GBS52 of *Streptococcus agalactiae* and the major pilin Spy0128 of *S. pneumoniae* demonstrated that these proteins contain two Ig-like domains (Kang *et al.*, 2007; Krishnan *et al.*, 2007), consistent with our prediction of Ig-like folds in *E. faecium* pilus proteins. These features, together with the presence of an independent class C sortase (Class C sortases are used in the assembly of pili of related gram-positive bacteria) in each of the four clusters, indicate that *E. faecium* may harbor genes for multiple pilus-like structures. Recent studies on pili of *C. diphtheriae, E. faecalis*, group A and B streptococci, and pneumococci have demonstrated their role in bacterial adherence and biofilm formation, and their contribution to bacterial pathogenesis and modulation of the host immune system (Barocchi *et al.*, 2006; Dramsi *et al.*, 2006; Mandlik *et al.*, 2007; Nallapareddy *et al.*, 2006; Singh *et al.*, 2007; Telford *et al.*, 2006).

EbpC$_{fm}$ is present as a HMW polymeric protein complex at the cell surface of *E. faecium*

To confirm the predicted location of EbpC$_{fm}$ on the surface of *E. faecium* as part of polymeric pili, we probed mutanolysin cell wall extracts of endocarditis-derived *E. faecium* strains TX0082 and TX0016 with affinity-purified anti-rEbpC$_{fm62}$ antibodies. A ladder-like pattern of HMW bands with sizes greater than 200 kDa was detected from strain TX0082 (Fig. 5). In contrast, only a single band was seen with Acm in mutanolysin extracts of TX0082 (Nallapareddy *et al.*, 2003). The HMW banding pattern is consistent with observations of a large number of pilus proteins from other gram-positive bacteria and suggests that EbpC$_{fm}$ is similarly assembled into multimeric pilus structures on the *E. faecium* cell surface. In contrast to TX0082, no signal was detected from the cell wall extract of TX0016, which is similar to our whole-cell ELISA results (data not shown) and indicates that the EbpC$_{fm}$-containing pilus is not expressed in this strain under the growth conditions used.

The *ebpABC$_{fm}$* genes are transcribed as a single polycistronic mRNA

As stated above, bioinformatics analyses indicated that each of the four pilus-encoding gene clusters is likely to be organized as an operon. For the *ebpA$_{fm}$* to *bps$_{fm}$* cluster, a 12-bp inverted repeat that could form a stem-loop structure (free energy of -22.55 kcal mol^{-1}) located in the intergenic region between *efbC$_{fm}$* and *bps$_{fm}$*, is likely to function as a ρ-independent transcriptional terminator, thus, predicting an *efbA$_{fm}$* to *efbC$_{fm}$* transcript and an independent *bps$_{fm}$* transcript. To validate this, we have carried out transcriptional analyses of the *ebpA$_{fm}$* to *bps$_{fm}$* locus using Northern hybridization. The *ebpA$_{fm}$*, *ebpB$_{fm}$*, and *ebpA$_{fm}$* probes all hybridized to a single RNA band of ~ 7 kb (Fig. 6a) which corresponds to the expected size of a polycistronic *ebpABC$_{fm}$* mRNA transcript, demonstrating that these three genes are co-transcribed. Probing with *bps$_{fm}$* detected a very low intensity band (~ 1 kb), consistent with the expected size of a monocistronic *bps$_{fm}$* mRNA transcript, suggesting that this transcript may either be produced in low levels at this time point or has a relatively short half-life. RT-PCR using three independent sets of internal *bps$_{fm}$* primer pairs amplified fragments of the expected sizes (lane 4 of Fig. 6b) (data not shown for two additional primer pairs), thus confirming the expression of *bps$_{fm}$*. Another faint band of ~8 kb was also observed with the *bps$_{fm}$* -probed Northern blots; while its identity is currently unclear, it might represent a related mRNA sequence such as one transcribed from the other predicted pilus operons.

RT-PCR experiments of the *ebp$_{fm}$* operon with primers designed to amplify intergenic regions of co-transcribed genes, confirmed the expected amplification between *ebpA$_{fm}$* and *ebpB$_{fm}$* as well as *ebpB$_{fm}$* and *ebpC$_{fm}$*, but not between *ebpC$_{fm}$* and *bps$_{fm}$* (Fig. 6b). Taken together, our data unambiguously demonstrate that the *E. faecium* Ebp$_{fm}$ pilus-encoding gene cluster represents a three-gene operon, unlike the *E. faecalis* locus which produces a four-gene *ebp-bps* transcript in addition to an independent *bps* transcript (Nallapareddy *et al.*, 2006).

Genes encoding MSCRAMM and pili proteins are commonly found in *E. faecium* isolates of clinical origin

We next examined 30 diverse (source/year/geographical location) human clinical isolates by colony hybridization using PCR generated DNA probes. *E. faecium* strain TX0016 and the *acm* gene served as controls for the hybridization studies. As shown in Table 2, the percentage of isolates showing hybridization to the individual gene probes varied from 73 % (*fms20*) to 100 %. The range in the number of probes hybridizing per isolate varied from 11 genes in one isolate (that lacked *fms15, fms18, fms20*, and *fms21*) to all 15 genes in 16 isolates (53 %). One isolate lacked the *ebpABC*$_{fm}$ (*fms1-5-9*) operon and another lacked the *fms11-19-16* operon. The *scm* (*fms10*), *fms14, fms17*, and *fms13* genes were present in all isolates tested, while *fms15* was present in all but one. Among the endocarditis isolates included in this analysis, the multilocus sequence type 18 (ST18) isolate TX0068, belonging to the global epidemic hospital associated clonal complex CC17 (Leavis *et al.*, 2003), had all 15 genes as did the sequenced ST18 isolate, TX0016. Another endocarditis isolate TX2535 (ST17) that also belongs to CC17, lacked *fms18*. A fourth endocarditis isolate, TX0074 (non-CC17, ST337), had 14 genes and lacked *fms20*. Thus, our results indicate broad but variable distribution of MSCRAMM-encoding genes in clinical isolates, consistent with a recent study of 22 *E. faecium* CWA protein-encoding genes in which four of the genes, *fms11-19-16* (*orf903-905-907*) and *fms18* (*orf2430*) were shown to be specifically enriched in CC17 isolates, while the remaining 9 genes were considered widespread in both CC17 and non-CC17 nosocomial isolates (Hendrickx *et al.*, 2007). Of note, our preliminary data from screening for *scm* from several hundred of *E. faecium* isolates belonging to clinical and non-clinical groups indicate that *scm* is absent only rarely from clinical isolates but is frequently absent from stool isolates.

In summary, this study identified genes for 14 new *E. faecium* CWA proteins with MSCRAMM-like features. We characterized the function of one of these proteins, Scm, and demonstrated that it is a second collagen binding protein for *E. faecium* with specificity to collagen type V. We further showed that the identified minimal ligand-binding region has a β-sheet-rich secondary structure composition characteristic of the A-domains of MSCRAMMs. Scm was shown to be expressed on the surface of many *E. faecium* clinical isolates during *in vitro* growth. Eleven of the 14 MSCRAMM-like protein encoding genes were clustered in four loci and 9 are predicted to encode the components needed to form multiple, distinct pilus-like structures. We characterized one of these clusters (*ebpABC*$_{fm}$-*bps*$_{fm}$) and showed that the four genes of this cluster are expressed in TX0082 as two transcripts, *ebpABC*$_{fm}$ and *bps*$_{fm}$. Subsequent detection of a HMW ladder pattern with anti-EbpC$_{fm}$ in Western blots of cell surface extracts from TX0082 provides experimental evidence for the assembly of EbpC$_{fm}$ into polymeric structures, such as pili. Our hybridization results for all 14 genes indicate a broad distribution of MSCRAMM and pilus-encoding genes among clinical isolates; a future study will assess their distribution in natural *E. faecium* populations from a variety of sources. The ability of *E. faecium* to produce two surface-anchored collagen adhesins with different collagen type specificities and the common occurrence of the *scm* gene as well as the 13 other newly identified MSCRAMM- and pilus-encoding genes among clinical *E. faecium* isolates may have important implications for colonization and infection by this opportunistic pathogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CWA	cell wall anchor
ECM	extracellular matrix
MSCRAMMs	microbial surface component recognizing adhesive matrix molecules
CI	collagen type I
CIV	collagen type IV
CV	collagen type V
CD	circular dichroism

REFERENCES

Arduino RC, Murray BE, Rakita RM. Roles of antibodies and complement in phagocytic killing of enterococci. Infect Immun. 1994; 62:987–993. [PubMed: 8112874]

Barocchi MA, Ries J, Zogaj X. A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A. 2006; 103:2857–2862. [PubMed: 16481624]

Bowden MG, Chen W, Singvall J, Xu Y, Peacock SJ, Valtulina V, Speziale P, Hook M. Identification and preliminary characterization of cell-wall-anchored proteins of *Staphylococcus epidermidis*. Microbiology. 2005; 151:1453–1464. [PubMed: 15870455]

Coque TM, Patterson JE, Steckelberg JM, Murray BE. Incidence of hemolysin, gelatinase, and aggregation substance among enterococci isolated from patients with endocarditis and other infections and from feces of hospitalized and community-based persons. J Infect Dis. 1995; 171:1223–1229. [PubMed: 7751697]

Deivanayagam CC, Wann ER, Chen W, Carson M, Rajashankar KR, Hook M, Narayana SV. A novel variant of the immunoglobulin fold in surface adhesins of *Staphylococcus aureus*: crystal structure of the fibrinogen-binding MSCRAMM, clumping factor A. Embo J. 2002; 21:6660–6672. [PubMed: 12485987]

Dramsi S, Trieu-Cuot P, Bierne H. Sorting sortases: a nomenclature proposal for the various sortases of Gram-positive bacteria. Res Microbiol. 2005; 156:289–297. [PubMed: 15808931]

Dramsi S, Caliot E, Bonne I, Guadagnini S, Prevost MC, Kojadinovic M, Lalioui L, Poyart C, Trieu-Cuot P. Assembly and role of pili in group B streptococci. Mol Microbiol. 2006; 60:1401–1413. [PubMed: 16796677]

Hendrickx AP, van Wamel WJ, Posthuma G, Bonten MJ, Willems RJ. Five genes encoding surface-exposed LPXTG proteins are enriched in hospital-adapted *Enterococcus faecium* clonal complex 17 isolates. J Bacteriol. 2007; 189:8321–8332. [PubMed: 17873043]

Kang HJ, Coulibaly F, Clow F, Proft T, Baker EN. Stabilizing isopeptide bonds revealed in gram-positive bacterial pilus structure. Science. 2007; 318:1625–1628. [PubMed: 18063798]

Kemp KD, Singh KV, Nallapareddy SR, Murray BE. Relative contributions of *Enterococcus faecalis* OG1RF sortase-encoding genes, *srtA* and *bps* (*srtC*), to biofilm formation and a murine model of urinary tract infection. Infect Immun. 2007; 75:5399–5404. [PubMed: 17785477]

Krishnan V, Gaspar AH, Ye N, Mandlik A, Ton-That H, Narayana SV. An IgG-like domain in the minor pilin GBS52 of *Streptococcus agalactiae* mediates lung epithelial cell adhesion. Structure. 2007; 15:893–903. [PubMed: 17697995]

Leavis HL, Willems RJ, Top J, Spalburg E, Mascini EM, Fluit AC, Hoepelman A, de Neeling AJ, Bonten MJ. Epidemic and nonepidemic multidrug-resistant *Enterococcus faecium*. Emerg Infect Dis. 2003; 9:1108–1115. [PubMed: 14519248]

Liang R, Woo SL, Takakura Y, Moon DK, Jia F, Abramowitch SD. Long-term effects of porcine small intestine submucosa on the healing of medial collateral ligament: a functional tissue engineering study. J Orthop Res. 2006; 24:811–819. [PubMed: 16514641]

Liu Q, Ponnuraj K, Xu Y, Ganesh VK, Sillanpaa J, Murray BE, Narayana SV, Hook M. The *Enterococcus faecalis* MSCRAMM ACE binds its ligand by the Collagen Hug model. J Biol Chem. 2007; 282:19629–19637. [PubMed: 17392280]

Lobley A, Whitmore L, Wallace BA. DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra. Bioinformatics. 2002; 18:211–212. [PubMed: 11836237]

Mandlik A, Swierczynski A, Das A, Ton-That H. *Corynebacterium diphtheriae* employs specific minor pilins to target human pharyngeal epithelial cells. Mol Microbiol. 2007; 64:111–124. [PubMed: 17376076]

Marraffini LA, Dedent AC, Schneewind O. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. Microbiol Mol Biol Rev. 2006; 70:192–221. [PubMed: 16524923]

Mora M, Bensi G, Capo S, Falugi F, Zingaretti C, Manetti AG, Maggi T, Taddei AR, Grandi G, Telford JL. Group A *Streptococcus* produce pilus-like structures containing protective antigens and Lancefield T antigens. Proc Natl Acad Sci U S A. 2005; 102:15641–15646. [PubMed: 16223875]

Murray BE, Singh KV, Ross RP, Heath JD, Dunny GM, Weinstock GM. Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. J Bacteriol. 1993; 175:5216–5223. [PubMed: 8349561]

Murray BE, Weinstock GM. Enterococci: new aspects of an old organism. Proc Assoc Am Physicians. 1999; 111:328–334. [PubMed: 10417741]

Murray BE. Vancomycin-resistant enterococcal infections. N Engl J Med. 2000; 342:710–721. [PubMed: 10706902]

Nallapareddy SR, Qin X, Weinstock GM, Hook M, Murray BE. *Enterococcus faecalis* adhesin, Ace, mediates attachment to extracellular matrix proteins collagen type IV and laminin as well as collagen type I. Infect Immun. 2000; 68:5218–5224. [PubMed: 10948147]

Nallapareddy SR, Weinstock GM, Murray BE. Clinical isolates of *Enterococcus faecium* exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family. Mol Microbiol. 2003; 47:1733–1747. [PubMed: 12622825]

Nallapareddy SR, Singh KV, Sillanpaa J, Garsin DA, Hook M, Erlandsen SL, Murray BE. Endocarditis and biofilm-associated pili of *Enterococcus faecalis*. J Clin Invest. 2006; 116:2799–2807. [PubMed: 17016560]

Nallapareddy SR, Sillanpaa J, Ganesh VK, Hook M, Murray BE. Inhibition of *Enterococcus faecium* adherence to collagen by antibodies against high-affinity binding subdomains of Acm. Infect Immun. 2007a; 75:3192–3196. [PubMed: 17438036]

Nallapareddy, SR; Singh, KV; Okhuysen, PC; Murray, BE. A Functional *acm* gene contributes to *Enterococcus faecium* infective endocarditis and correlates with the recent success of this emerging nosocomial pathogen; 47th Interscience Conference on Antimicrobial Agents and Chemotherapy (Abstract number B-1239b); Chicago, USA. 2007b;

Nicholls AC, Oliver JE, McCarron S, Harrison JB, Greenspan DS, Pope FM. An exon skipping mutation of a type V collagen gene (COL5A1) in Ehlers-Danlos syndrome. J Med Genet. 1996; 33:940–946. [PubMed: 8950675]

Patti JM, Allen BL, McGavin MJ, Hook M. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol. 1994; 48:585–617. [PubMed: 7826020]

Perkins S, Walsh EJ, Deivanayagam CC, Narayana SV, Foster TJ, Hook M. Structural organization of the fibrinogen-binding region of the clumping factor B MSCRAMM of *Staphylococcus aureus*. J Biol Chem. 2001; 276:44721–44728. [PubMed: 11568183]

Pizarro-Cerda J, Cossart P. Bacterial adhesion and entry into host cells. Cell. 2006; 124:715–727. [PubMed: 16497583]

Ponnuraj K, Bowden MG, Davis S, Gurusiddappa S, Moore D, Choe D, Xu Y, Hook M, Narayana SV. A “dock, lock, and latch” structural model for a staphylococcal adhesin binding to fibrinogen. Cell. 2003; 115:217–228. [PubMed: 14567919]

Rakita RM, Quan VC, Jacques-Palaz K, Singh KV, Arduino RC, Mee M, Murray BE. Specific antibody promotes opsonization and PMN-mediated killing of phagocytosis-resistant *Enterococcus faecium*. FEMS Immunol Med Microbiol. 2000; 28:291–299. [PubMed: 10891652]

Rice LB. Emergence of vancomycin-resistant enterococci. Emerg Infect Dis. 2001; 7:183–187. [PubMed: 11294702]

Rice LB, Carias L, Rudin S, Vael C, Goossens H, Konstabel C, Klare I, Nallapareddy SR, Huang W, Murray BE. A potential virulence gene, hyl_{Efm}, predominates in *Enterococcus faecium* of clinical origin. J Infect Dis. 2003; 187:508–512. [PubMed: 12552437]

Rich RL, Kreikemeyer B, Owens RT, LaBrenz S, Narayana SV, Weinstock GM, Murray BE, Hook M. Ace is a collagen-binding MSCRAMM from *Enterococcus faecalis*. J Biol Chem. 1999; 274:26939–26945. [PubMed: 10480905]

Roche FM, Massey R, Peacock SJ, Day NP, Visai L, Speziale P, Lam A, Pallen M, Foster TJ. Characterization of novel LPXTG-containing proteins of *Staphylococcus aureus* identified from genome sequences. Microbiology. 2003; 149:643–654. [PubMed: 12634333]

Scott JR, Zahner D. Pili with strong attachments: Gram-positive bacteria do it differently. Mol Microbiol. 2006; 62:320–330. [PubMed: 16978260]

Sillanpaa J, Xu Y, Nallapareddy SR, Murray BE, Hook M. A family of putative MSCRAMMs from *Enterococcus faecalis*. Microbiology. 2004; 150:2069–2078. [PubMed: 15256550]

Singh KV, Coque TM, Weinstock GM, Murray BE. In vivo testing of an *Enterococcus faecalis efaA* mutant and use of *efaA* homologs for species identification. FEMS Immunol Med Microbiol. 1998; 21:323–331. [PubMed: 9753005]

Singh KV, Nallapareddy SR, Murray BE. Importance of the *ebp* (endocarditis- and biofilm-associated pilus) locus in the pathogenesis of *Enterococcus faecalis* ascending urinary tract infection. J Infect Dis. 2007; 195:1671–1677. [PubMed: 17471437]

Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in gram-positive pathogens. Nat Rev Microbiol. 2006; 4:509–519. [PubMed: 16778837]

Teng F, Kawalec M, Weinstock GM, Hryniewicz W, Murray BE. An *Enterococcus faecium* secreted antigen, SagA, exhibits broad-spectrum binding to extracellular matrix proteins and appears essential for *E. faecium* growth. Infect Immun. 2003; 71:5033–5041. [PubMed: 12933846]

Ton-That H, Schneewind O. Assembly of pili on the surface of *Corynebacterium diphtheriae*. Mol Microbiol. 2003; 50:1429–1438. [PubMed: 14622427]

Ton-That H, Marraffini LA, Schneewind O. Sortases and pilin elements involved in pilus assembly of *Corynebacterium diphtheriae*. Mol Microbiol. 2004; 53:251–261. [PubMed: 15225319]

Wenstrup RJ, Florer JB, Brunskill EW, Bell SM, Chervoneva I, Birk DE. Type V collagen controls the initiation of collagen fibril assembly. J Biol Chem. 2004; 279:53331–53337. [PubMed: 15383546]

Whitmore L, Wallace BA. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res. 2004; 32:W668–673. [PubMed: 15215473]

Willems RJ, Homan W, Top J. Variant *esp* gene as a marker of a distinct genetic lineage of vancomycin-resistant *Enterococcus faecium* spreading in hospitals. Lancet. 2001; 357:853–855. [PubMed: 11265956]

Wilson, K. Preparation of genomic DNA from bacteria. In: Ausubel, FM, Brent, R, Kingston, RE, David, DM, Scidman, JG, Smith, JA, Struhl, K, editors. Current Protocols in Molecular Biology. Green Publishing Associates; Brooklyn, NY: 1994. 2.4.1–2.4.2.

Xu Y, Liang X, Chen Y, Koehler TM, Hook M. Identification and biochemical characterization of two novel collagen binding MSCRAMMs of *Bacillus anthracis*. J Biol Chem. 2004; 279:51760–51768. [PubMed: 15456768]

Zong Y, Xu Y, Liang X, Keene DR, Hook A, Gurusiddappa S, Hook M, Narayana SV. A 'Collagen Hug' model for *Staphylococcus aureus* CNA binding to collagen. Embo J. 2005; 24:4224–4236. [PubMed: 16362049]

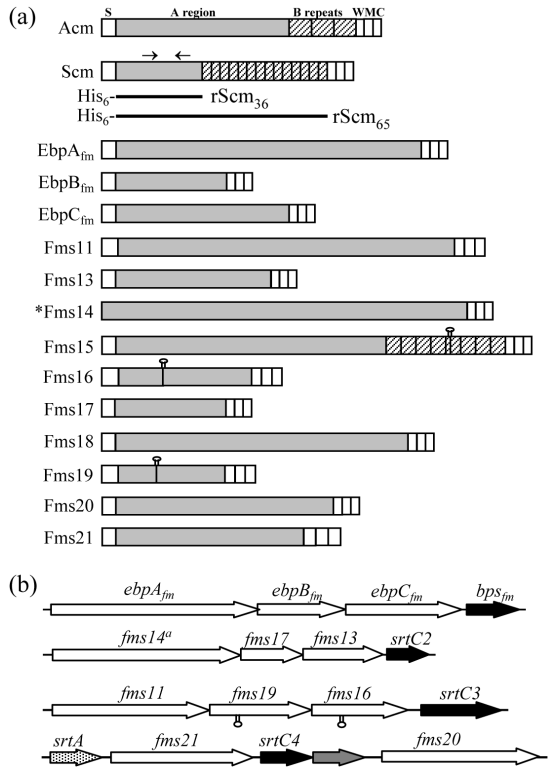


Fig. 1.

Domain organization of *E. faecium* CWA proteins with Ig-like folds. (a) S, signal peptide; W, cell-wall-spanning region; M, membrane anchor region; C, cytoplasmic tail with charged residues; Shaded A region, non-repetitive sequences; hatched B region, repetitive sequences; lollypop denotes presence of a stop codon due to a nucleotide substitution/deletion in Fms15, Fms16 and Fms19 of TX0016; solid black bars, His_6-tag expressed regions of Scm (Fms10). Arrows above Scm show regions with predicted Ig-like folding. (b) Contiguously arranged ORFs encoding CWA proteins in *E. faecium* genome; *srt*, sortase. *Fms14 lacks an identifiable signal peptide, but has all the other characteristics of CWA MSCRAMMs.

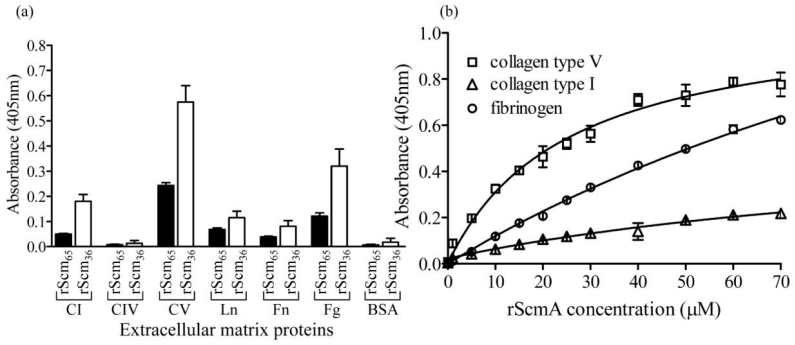
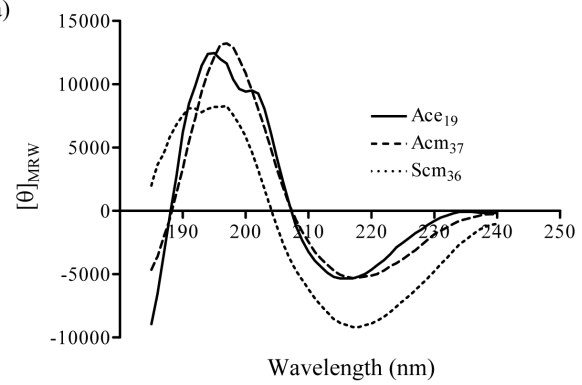


Fig. 2.

Binding of recombinant Scm proteins to ECM proteins in a solid phase ELISA-type ligand binding assay. (a) Binding of 20 μM $rScm_{65}$ (aa 27-624) and $rScm_{36}$ (aa 27-333) to a collection of immobilized ECM proteins. CI, type I collagen, Ln, laminin; Fn, fibronectin; Fg, fibrinogen; BSA, bovine serum albumin. Data points for $rScm_{36}$ represent the means of A_{405nm} values of 18 wells representing six independent experiments and including proteins from two different purifications, and for $rScm_{65}$, 15 wells from five independent assays. (b) Binding of $rScm_{36}$ to CI, CV and fibrinogen with increasing $rScm_{36}$ concentrations. Data points represent the means of $A_{405\ nm}$ values from three wells. The experiment was repeated up to six times using protein from two purifications. The reported K_D value is the average ± standard error from the six assays. BSA values were subtracted from the respective collagen-binding values after which affinity calculations were performed with the one-ligand binding site model. The resulting curves are depicted in the figure.

(a)



(b)

Protein*	Secondary structure composition			
	α-helix	β-sheet	turn	unordered
Scm_{36}	0.15 ± 0.03	0.34 ± 0.01	0.22 ± 0.02	0.30 ± 0.03
Ace_{19}	0.04 ± 0.03	0.47 ± 0.02	0.25 ± 0.02	0.27 ± 0.03
Acm_{37}	0.06 ± 0.01	0.43 ± 0.02	0.23 ± 0.02	0.28 ± 0.01

Fig. 3.

Secondary structure analysis by far-UV circular dichroism spectroscopy. (a) Collected CD-spectra of Scm_{36}, Ace_{19} and Acm_{37}. Mean residue weight ellipticity is reported in deg cm^2 $dmol^{-1}$. (b) Summary of secondary structure compositions. *Scm_{36} contains the predicted A-domain of Scm (aa 27-333). Ace_{19} and Acm_{37} contain the corresponding minimal ligand binding regions of the collagen binding MSCRAMMs Ace (*E. faecalis)* and Acm (*E. faecium)*, respectively.

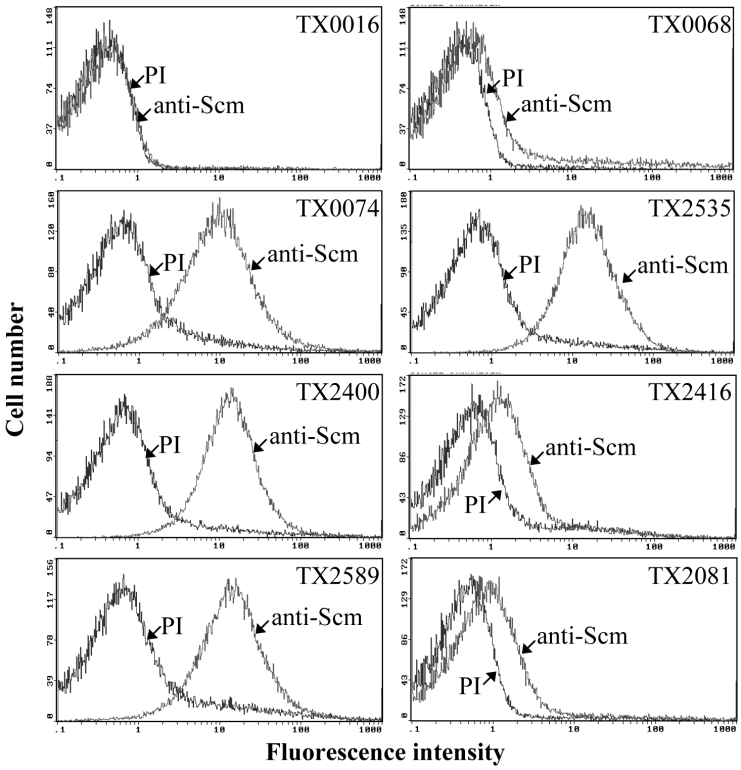


Fig. 4.

Quantitation of Scm surface expression by FACS analysis. Reactivity of both preimmune Igs (PI) and anti-Scm Igs for each strain was shown. Although some *E. faecium* isolates exhibit slight binding to goat PI, significant difference was observed between PI and anti-Scm Igs. Bacteria were analyzed using side scatter as the threshold for detection. Specific binding by anti-Scm antibodies is indicated as log fluorescence intensity on the X-axis. Each histogram represents 50 000 events of bacterium-sized particles.

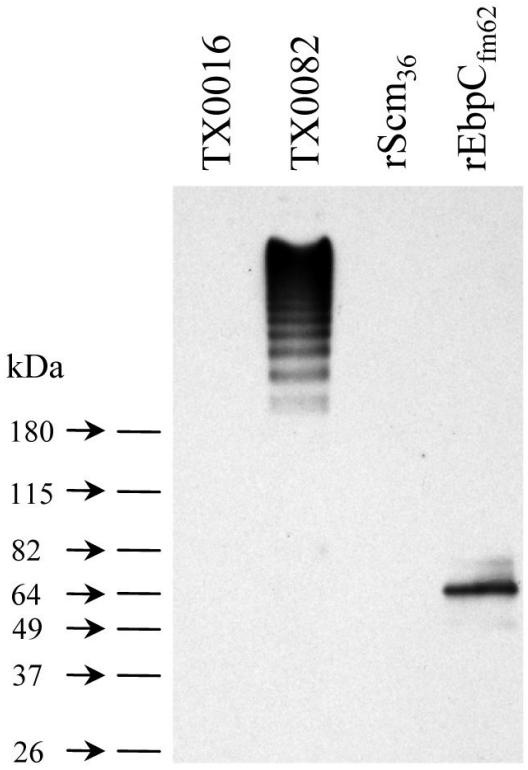


Fig. 5.

Western blot analysis of cell wall anchored proteins from *E. faecium* strains TX0082 and TX0016. The mutanolysin cell wall extracts (10 μg total protein/lane) were separated on 4-15% SDS-PAGE gels, transferred to PVDF membranes, and probed using affinity-purified anti-rEbpC$_{fm62}$ antibodies. Purified rScm$_{36}$ and rEbpC$_{fm62}$ proteins (20 ng/lane) were included as controls.

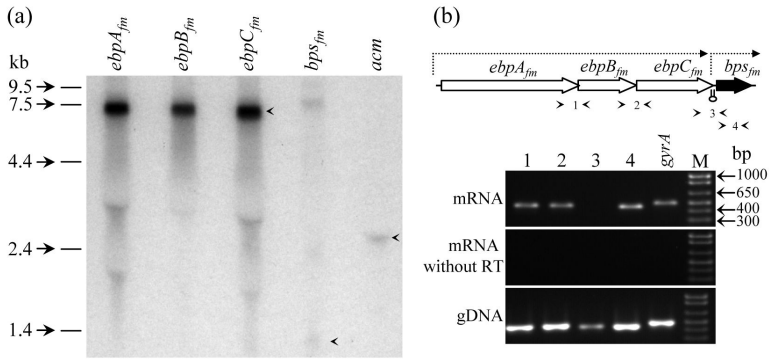


Fig. 6.

Transcriptional analysis of the *ebpA*$_{fm}$ to *bps*$_{fm}$ gene cluster. (a) Northern hybridization of total RNA (30 μg/lane) from TX0082 probed with intragenic fragments of *ebpA*$_{fm}$, *ebpB*$_{fm}$, *ebpC*$_{fm}$, and *bps*$_{fm}$. The *acm* probe was used as a control. RNA bands with expected sizes are indicated with arrows. (b) RT-PCR analysis of the *ebpA*$_{fm}$ to *bps*$_{fm}$ gene cluster. Location of each primer pair is shown with arrows in the schematic representation of the gene cluster. A lollypop between *ebpC*$_{fm}$ and *bps* depicts a predicted transcriptional terminator. Top gel, RT-PCR with DNase-treated total RNA (10 ng) as template; middle gel, control reaction of the same total RNA (100ng) preparation amplified without reverse transcriptase; bottom gel, control reaction amplified with genomic TX0082 DNA as template. An intragenic region of gyrase A was used as a control. M, molecular weight marker.

Table 1

Bacterial strains and plasmids used in this study

Strains/Plasmids	Relevant characteristics*; Origin; Yr of isolation	Reference or source
Strains		
E. faecium		
TX0016 (DO)	Endocarditis isolate; Van^S; Amp^R; Houston, Texas; 1992. (http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-detail.xsp?project_id=118)	(Arduino *et al.*, 1994)
TX2535	Endocarditis isolate; Van^R; Amp^R; Houston, Texas; 1995	(Nallapareddy *et al.*, 2003)
TX0068	Endocarditis isolate, Van^R, Amp^R; Worcester, Massachusetts; 1994	This study
TX0074	Endocarditis isolate, Van^R, Amp^R; Valhalla, New York; 1995	This study
E. coli		
M15(pREP4)	*E coli* strain for expression of recombinant proteins	Qiagen
TX5432	M15 (pTEX5432), Amp^r, Kan^r	This study
TX5628	M15 (pTEX5628), Amp^r, Kan^r	This study
Plasmids		
pQE30	Expression vector	Qiagen
pTEX5432	1794 bp fragment from TX0016 *scm* (coding for complete A and B domains) cloned into pQE30 expression vector	This study
pTEX5628	921 bp fragment from TX2535 *scm* (coding for complete A domain) cloned into pQE30 expression vector†	This study
pTEX5630	1674 bp fragment from TX0016 *ebpC*$_{fm}$ (coding for mature $EbpC_{fm}$ without signal peptide or CWA domain) cloned into pQE30 expression vector	This study

*Amp^r: ampicillin resistant; Van^r: vancomycin resistant.

†The DNA sequence of *scm* cloned into pTEX5628 from strain TX2535 is 100 % identical with that of TX0016.

Table 2

Summary of features of the predicted MSCRAMM proteins of *E. faecium* and the distribution of the corresponding genes in 30 *E. faecium* isolates associated with clinical infections

Gene name	Accession no.	Protein length (aa)	Cell-wall anchoring motif	Best match to other CWA proteins (% similarity)	No. of isolates $+^{ve}$ (%) n = 30
Genes Not Clustered					
acm[*] *(fms8)*	ZP_00604683	721	LPKTS	*Staphylococcus aureus* Cna (62 %)[*]	30 (100 %)
scm (*fms10*)	ZP_00604835	660	LPKTG	*Lactobacillus brevis* hypothetical protein (54 %)	30 (100 %)
fms15 [†]	ZP_00602631	1412	LPKTG	*Bacillus anthracis* collagen binding protein, BA0871 (49 %)	29 (97 %)
fms18	ZP_00603098	1075	FPQTG	*Enterococcus faecalis* EF1896 (91 %)	24 (80 %)
Cluster 1					
ebpA$_{fm}$ *(fms1)*	ZP_00602689	1129	LPETG	*E. faecalis* EbpA (75 %)	29 (97 %)
ebpB$_{fm}$ *(fms5)*	ZP_00602688	473	LPKTN	*E. faecalis* EbpB (65 %)	29 (97 %)
ebpC$_{fm}$ *(fms9)*	ZP_00602687	625	LPSTG	*E. faecalis* EbpC (84 %)	29 (97 %)
Cluster 2					
fms11	ZP_00604460	773	LPSTG	*Lactobacillus johnsonii* hypothetical protein (46 %)	29 (97 %)
fms19 [‡]	ZP_00604462	499	FPKTS	*E. faecalis* EbpB (54 %)	29 (97 %)
fms16 [‡]	ZP_00604464	470	LPSTG	*E. faecalis* EbpC (46 %)	29 (97 %)
Cluster 3					
fms14 [§]	ZP_00603043	1313	LPATG	*E. faecalis* EbpA (41 %)	30 (100 %)
fms13	ZP_00603041	565	LPATG	*E. faecalis* EbpC (25 %)	30 (100 %)
fms17	ZP_00603042	437	FPQTN	*E. faecalis* EbpB (47 %)	30 (100 %)
Cluster 4					
fms21	ZP_00603529	658	VPMTG	*E. faecalis* EbpC (41 %)	28 (93 %)
fms20	ZP_00603526	696	VPKTG	*E. faecalis* EF2525 (41 %)	22 (73 %)

[*] Identified in our previous studies (Nallapareddy *et al.*, 2003).

[†] *In silico* analysis predicts a one-nucleotide deletion in the B-repeat region.

[‡] These genes had a nucleotide substitution or deletion in *E. faecium* strain TX0016 vs. TX0068 and TX0074, leading to a pseudogene.

[§] Fms14 lacks an identifiable signal peptide, but has the other characteristics of a CWA protein.