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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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 the pathogenic determinants of this organism. We have previously identified a cell-wall-anchored collagen adhesin, Acn, produced by some isolates of *E. faecium*, and a secreted antigen, SagA, exhibiting broad-spectrum binding to extracellular matrix proteins. Here, we analysed the draft genome of strain TX0016 for potential microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). Genome-based bioinformatics identified 22 predicted cell-wall-anchored *E. faecium* surface proteins (Fms), of which 15 (including Acn) had characteristics typical of MSCRAMMs, including predicted folding into a modular architecture with multiple immunoglobulin-like domains. Functional characterization of one [Fms10; redesignated second collagen adhesin of *E. faecium* (Scm)] revealed that recombinant Scm₆₅ (A- and B-domains) and Scm₃₆ (A-domain) bound to collagen type V efficiently in a concentration-dependent manner, bound considerably less to collagen type I and fibrinogen, and differed from Acn in their binding specificities to collagen types IV and V. Results from far-UV circular dichroism measurements of recombinant Scm₃₆ and of Acn₃₇ indicated that these proteins were 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; nine 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 EbpC_{fm}), detected a 'ladder' pattern of high-molecular-mass 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, a result consistent with those for 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.

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INTRODUCTION

Enterococcus faecium, a member of the normal commensal flora, has recently emerged as a prominent nosocomial pathogen that causes 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, 2008a, b).

It is known that pathogenic bacteria have evolved a plethora of proteins to adhere to and invade host tissues and to resist host defences (Pizarro-Cerda & Cossart, 2006). Among these is a family of surface proteins with well-established roles in host-pathogen adherence which have been termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs; Patti *et al.*, 1994). MSCRAMMs share several characteristics, including (i) an N-terminal signal peptide, (ii) a non-repeated A-domain consisting of immunoglobulin (Ig)-like fold(s), (iii) a B-domain with a variable number of repeats among different strains, and (iv) a C-terminal cell wall anchor (CWA) domain. Some of these MSCRAMMs have been shown recently 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 CWA proteins 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 have 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.*, 2007; 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 that encode MSCRAMM-like proteins in the 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, 2003, 2006; Roche *et al.*, 2003; Sillanpää *et al.*, 2004; Xu *et al.*, 2004). Recently, Hendrickx *et al.* (2007) predicted 22 CWA proteins from the *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*, that exhibits 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*) that encode predicted MSCRAMMs in the genome of *E. faecium* TX0016. Recombinant forms of one of these proteins, designated *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 quantified using FACS, and antibodies raised against a recombinant form of one of the major pilus proteins showed a high-molecular mass (HMM) ladder pattern characteristic of Gram-positive pili. Co-transcription of one of the pilus-encoding gene clusters was demonstrated by Northern

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Abbreviations: CI, collagen type I; CIV, collagen type IV; CV, collagen type V; CD, circular dichroism; CWA, cell wall anchor; ECM, extracellular matrix; HMM, high molecular mass; Ig, immunoglobulin; MSCRAMM, microbial surface components recognizing adhesive matrix molecule.

Supplementary methods, describing bacterial isolates, identification and structural analysis of CWA proteins, and sources of extracellular matrix proteins; supplementary results, describing sequence variation in *fms15*, *fms16* and *fms19*, and structural motifs of the putative *E. faecium* MSCRAMMs; supplementary references; three supplementary figures, showing Coomassie-stained SDS-PAGE results for purified recombinant His6-*Scm* proteins, analysis of *Scm* expression on the cell surface of *E. faecium* isolates, and conserved lysine and glutamic acid residues in the pilin motif and E-box of predicted *E. faecium* major pilus proteins; and a supplementary table, listing oligonucleotides used in this study, are available with the online version of this paper.

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 the Supplementary Methods. All constructs were given TX numbers and plasmids from these constructs were assigned corresponding pTEX numbers (Table 1).

Escherichia coli and *E. faecium* cells were grown in Luria–Bertani (LB) broth/agar and Brain Heart Infusion (BHI) broth/agar (Difco), respectively. For *Escherichia 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 27–624 (A-domain and B-repeats) and 27–333 (A-domain) of Scm, and amino acids 33–590 of EbpC_{fm} [Fms9 was renamed EbpC_{fm} based on its 74% amino acid 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 rScm₆₅ and rScm₃₆, based on their calculated molecular masses. Similarly, the cloned segment of EbpC_{fm} was designated rEbpC_{fm62}. Constructs were confirmed by sequencing.

Purification of recombinant proteins. Recombinant proteins with N-terminal His₆-tags were expressed and purified using nickel affinity chromatography followed by anion-exchange chromatography (Nallapareddy *et al.*, 2007; Sillanpää *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 rScm₃₆ and rScm₆₅.

Circular dichroism (CD) spectra. Far-UV CD spectroscopy data were collected as described previously (Sillanpää *et al.*, 2004). Secondary structure compositions were quantified 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 extracellular matrix (ECM) was tested using an assay described previously, with minor modifications (Nallapareddy *et al.*, 2000). In brief, 1 µg of each ECM protein (for sources, see Supplementary Methods) was coated in 100 µl PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4) 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-His₆ 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 rScm₃₆ and rEbpC_{fm62} were raised at Bethyl Laboratories. Scm₃₆- 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). Glycine (0.1 M, pH 2.8) was used for elution of bound antibodies, which were neutralized immediately by 1 M Tris/HCl, pH 8.0, and dialysed 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 rScm₃₆-specific Igs. Antiserum against formalin-killed TX0016 whole cells (Rakita *et al.*, 2000) was used as a positive control.

To quantify surface expression of Scm by FACS analysis, bacteria grown in BHI for 14 h from an inoculum of OD₆₀₀=0.01 were labelled 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 previously (Kemp *et al.*, 2007). Cells were then fixed in 1% paraformaldehyde in PBS and analysed 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, OD₆₀₀ ~0.01) with mutanolysin as described previously (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-Scm₃₆ 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 chemiluminescent detection reagents (Thermo Scientific).

Northern hybridization. Using the RNeasy protect bacteria reagent and RNeasy Mini kit (Qiagen), total RNA was isolated from TX0082 grown in BHI to mid-exponential phase. Samples (30 µg) 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 radiolabelled by using the RadPrime DNA labelling system (Invitrogen). Hybridization was performed under high-stringency conditions, as detailed previously 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 min 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 were 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 reverse transcriptase were used as controls to verify lack of DNA contamination in the total RNA preparations.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*; origin; year of isolation	Reference or source
<i>E. faecium</i> strains		
TX0016 (DO)	Endocarditis isolate, Van ^S , Amp ^R ; Houston, TX; 1992 (http://www.hgsc.bcm.tmc.edu/projects/microbial/microbial-detail.xsp?project_id=118)	Arduino <i>et al.</i> (1994)
TX2535	Endocarditis isolate, Van ^R , Amp ^R ; Houston, TX; 1995	Nallapareddy <i>et al.</i> (2003)
TX0068	Endocarditis isolate, Van ^R , Amp ^R ; Worcester, MA; 1994	This study
TX0074	Endocarditis isolate, Van ^R , Amp ^R ; Valhalla, NY; 1995	This study
<i>Escherichia coli</i> strains		
M15(pREP4)	<i>Escherichia coli</i> 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 <i>scm</i> (encoding complete A- and B-domains) cloned into pQE30 expression vector	This study
pTEX5628	921 bp fragment from TX2535 <i>scm</i> (encoding complete A-domain) cloned into pQE30 expression vector†	This study
pTEX5630	1674 bp fragment from TX0016 <i>ebpC_{fm}</i> (encoding mature EbpC _{fm} without signal peptide or CWA domain) cloned into pQE30 expression vector	This study

*Amp^R, ampicillin resistant; Kan^R, kanamycin resistant; Van^R, vancomycin resistant.

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

Colony hybridization. Preparation of colony lysate membranes and hybridization under high-stringency conditions were performed as described previously (Coque *et al.*, 1995; Singh *et al.*, 1998). DNA probes were generated and radiolabelled 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 (G. M. Weinstock & B. E. Murray, 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 eight (*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; accession no. NZ_AAAK000000000), the published report did not include one of the ORFs reported 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) that lack a signal peptide revealed the presence of N-terminal signal peptides in the ORFs immediately upstream of 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, had been previously identified in our laboratory as Acn (Nallapareddy *et al.*, 2003). The percentage of *E. faecium* LPXTG proteins containing Ig-like folds is greater than the percentages for 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 *Staphylococcus aureus* MSCRAMMs (e.g. the fibronectin-, fibrinogen- and elastin-binding protein FnbpA, the fibrinogen-binding proteins ClfA and ClfB, and the collagen-binding protein Cna) and the prototype MSCRAMM of *E. faecium*, Acn, have been shown to play

a direct role in interactions with their respective ECM protein ligands (Nallapareddy *et al.*, 2007; 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 found that most were predicted to have a multi-domain architecture similar to the characterized MSCRAMMs of *S. aureus* and *E. faecalis* (Deivanayagam *et al.*, 2002; Perkins *et al.*, 2001; Sillanpää *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, were 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. Sillanpää, S. R. Nallapareddy, B.E. Murray and M. Hook, unpublished data). Scm (Fms10) of TX0016 has 13 B-repeats of 19 aa in length and a partial repeat of 10 aa, while pseudogene *fms15* is predicted to encode eight B-repeats of 91–93 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 rScm₆₅ (fully mature protein consisting of the A-domain and B-repeats; amino acids 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 brief storage. rScm₆₅ migrated as a larger band than expected from the calculated molecular mass 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 rScm₃₆ encompassing the complete A-domain (amino acids 27–333; Supplementary Fig. S1). A MALDI-TOF MS analysis determined the

molecular mass of rScm₃₆ to be 35 711 Da, which is in good agreement with the calculated mass of 35 691 Da, and confirmed the absence of post-translational modifications and the 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-His₆ antibody detected the N-terminal His₆ 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 rScm₆₅ (containing the full mature protein) and Scm₃₆ (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, stronger binding of these rScm proteins (especially rScm₃₆) to fibrinogen and CI was also observed compared with their binding to CIV, laminin, and fibronectin (Fig. 2a).

Further analysis of the binding interactions of increasing concentrations of rScm₃₆ with CI and CV, as well as fibrinogen, by dose-response assays showed concentration-dependent binding of rScm₃₆ to CV that approached saturation, with apparent half-maximal binding (K_D) reached at $30.8 \pm 1.8 \mu\text{M}$ (Fig. 2b). The apparent affinity of rScm₃₆ for fibrinogen and CI was low and did not reach reliable saturation levels. In spite of partial degradation, Scm₆₅ showed dose-dependent binding, with an affinity for CV similar to that for Scm₃₆ (data not shown). Although K_D values from steady-state assays are estimates, our affinity determination for the Scm A-domain is in the same range as that shown previously for the minimal ligand-binding domain of Ace to CI ($48 \pm 7 \mu\text{M}$) (Rich *et al.*, 1999), but lower than that for 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 that 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 the collagen fibres 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

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 number	Protein length (aa)	Cell wall anchoring motif	Best match to other CWA proteins (similarity, %)	Number of isolates positive (%) (n=30)
Genes not clustered					
<i>acm*</i> (<i>fms8</i>)	ZP_00604683	721	LPKTS	<i>Staph. aureus</i> Cna (62 %)*	30 (100 %)
<i>scm</i> (<i>fms10</i>)	ZP_00604835	660	LPKTG	<i>Lactobacillus brevis</i> hypothetical protein (54 %)	30 (100 %)
<i>fms15</i> †	ZP_00602631	1412	LPKTG	<i>Bacillus anthracis</i> collagen-binding protein BA0871 (49 %)	29 (97 %)
<i>fms18</i>	ZP_00603098	1075	FPQTG	<i>E. faecalis</i> EF1896 (91 %)	24 (80 %)
Cluster 1					
<i>ebpA_{fms}</i> (<i>fms1</i>)	ZP_00602689	1129	LPETG	<i>E. faecalis</i> EbpA (75 %)	29 (97 %)
<i>ebpB_{fms}</i> (<i>fms5</i>)	ZP_00602688	473	LPKTN	<i>E. faecalis</i> EbpB (65 %)	29 (97 %)
<i>ebpC_{fms}</i> (<i>fms9</i>)	ZP_00602687	625	LPSTG	<i>E. faecalis</i> EbpC (84 %)	29 (97 %)
Cluster 2					
<i>fms11</i>	ZP_00604460	773	LPSTG	<i>Lactobacillus johnsonii</i> hypothetical protein (46 %)	29 (97 %)
<i>fms19</i> ‡	ZP_00604462	499	FPKTS	<i>E. faecalis</i> EbpB (54 %)	29 (97 %)
<i>fms16</i> ‡	ZP_00604464	470	LPSTG	<i>E. faecalis</i> EbpC (46 %)	29 (97 %)
Cluster 3					
<i>fms14</i> §	ZP_00603043	1313	LPATG	<i>E. faecalis</i> EbpA (41 %)	30 (100 %)
<i>fms17</i>	ZP_00603042	437	FPQTN	<i>E. faecalis</i> EbpB (47 %)	30 (100 %)
<i>fms13</i>	ZP_00603041	565	LPATG	<i>E. faecalis</i> EbpC (25 %)	30 (100 %)
Cluster 4					
<i>fms21</i>	ZP_00603529	658	VPMTG	<i>E. faecalis</i> EbpC (41 %)	28 (93 %)
<i>fms20</i>	ZP_00603526	696	VPKTG	<i>E. faecalis</i> 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 versus TX0068 and TX0074, resulting in pseudogenes.

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

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, the possession of 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 analysed the full A-domain of Scm with far-UV CD spectroscopy. The spectra obtained showed a maximum at 197 nm, 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 Acn, which we have characterized previously (Nallapareddy *et al.*, 2003, 2007), and also with the minimal collagen-binding region of Ace from *E. faecalis*, which crystal

structure studies have recently revealed folds into a similar DEv variant of the Ig-fold as that found previously 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 α -helix and 0.34 ± 0.01 β -sheet for rScm₃₆ (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 Acn 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 amino acids 125–325 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 (amino acids 27–333).

Cell surface expression of Scm

Using Scm-specific antibodies affinity-purified (with rScm₃₆) from goat antiserum, we assessed the surface expression of Scm by eight *E. faecium* clinical isolates (including the sequenced strain TX0016) using whole-cell

Fig. 1. Domain organization of *E. faecium* CWA proteins with Ig-like folds. (a) S, signal peptide; W, cell wall anchor region; M, membrane-spanning region; C, cytoplasmic tail with charged residues; shaded A region, non-repetitive sequences; hatched B region, repetitive sequences; lollypops denote the presence of a stop codon due to a nucleotide substitution/deletion in Fms15, Fms16 and Fms19 of TX0016; solid black bars, His₆-tag-expressed regions of Scm (Fms10). Arrows above Scm show regions with predicted Ig-like folding. (b) Contiguously arranged ORFs encoding CWA proteins in the *E. faecium* genome; *srt*_C gene encoding sortase. *Fms14 lacks an identifiable signal peptide, but has all the other characteristics of CWA MSCRAMMs.

ELISA. Two *E. faecium* community-derived strains that lacked *scm* (identified below) were used as negative controls. Among eight *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 (see Supplementary Fig. S2), suggesting that Scm is produced on the cell surface of many *E. faecium* isolates during *in vitro* growth. Subsequent quantification 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 90–97% of cells and with relatively high fluorescence intensities, thus confirming our observations with whole-cell ELISA. Furthermore, these surface detection studies suggested that the majority of cells of *scm*⁺ isolates were 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-Scm₃₆ 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 to be clustered in four genomic loci. Nine of the clustered genes showed significant similarity to pilus-associated proteins from other species (Table 2). Analysis of the nucleotide sequence of the locus spanning *fms1*, *fms5* and *ebpC_{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*) predicted to encode a class C sortase. However, none of the CWA protein genes was 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 have been demonstrated to be essential in the

Fig. 2. Binding of recombinant Scm proteins to ECM proteins in a solid-phase ELISA-type ligand-binding assay. (a) Binding of 20 µM rScm₆₅ (amino acids 27–624) and rScm₃₆ (amino acids 27–333) to a collection of immobilized ECM proteins. Ln, laminin; Fn, fibronectin; Fg, fibrinogen. Data points for rScm₃₆ represent the means of A_{405} values of 18 wells representing six independent experiments and including proteins from two different purifications, and for rScm₆₅, 15 wells from five independent assays. (b) Binding of rScm₃₆ to CI, CV and fibrinogen with increasing rScm₃₆ concentrations. Data points represent the means of A_{405} values from three wells. The experiment was repeated up to six times using protein from two purifications. The reported K_D value is the mean $\pm$ SE from the six assays. BSA-binding 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.

polymerization of the *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 *Streptococcus pneumoniae* have demonstrated that these proteins contain two Ig-like domains (Kang *et al.*, 2007;

(b)

Protein*	Secondary structure composition			
	α -Helix	β -Sheet	Turn	Unordered
Scm ₃₆	0.15 $\pm$ 0.03	0.34 $\pm$ 0.01	0.22 $\pm$ 0.02	0.30 $\pm$ 0.03
Ace ₁₉	0.04 $\pm$ 0.03	0.47 $\pm$ 0.02	0.25 $\pm$ 0.02	0.27 $\pm$ 0.03
Acm ₃₇	0.06 $\pm$ 0.01	0.43 $\pm$ 0.02	0.23 $\pm$ 0.02	0.28 $\pm$ 0.01

Fig. 3. Secondary structure analysis by far-UV CD spectroscopy. (a) Collected CD spectra of Scm₃₆, Ace₁₉ and Acm₃₇. Mean residue weight ellipticity is reported in degrees cm⁻² dmol⁻¹. (b) Summary of secondary structure compositions. *Scm₃₆ contains the predicted A-domain of Scm (amino acids 27–333). Ace₁₉ and Acm₃₇ contain the corresponding minimal and high-affinity ligand-binding regions of the collagen-binding MSCRAMMs Ace (*E. faecalis*) and Acm (*E. faecium*), respectively.

Fig. 4. Quantification of Scm surface expression by FACS analysis. Reactivity of both preimmune Igs (PI) and anti-Scm Igs for each strain is shown. Although some *E. faecium* isolates exhibited slight binding to goat PI, significant differences were observed between PI and anti-Scm Igs. Bacteria were analysed using side scatter as the threshold for detection. Specific binding by anti-Scm antibodies is indicated as fluorescence intensity on the x axis using a log scale. Each histogram represents 50 000 events of bacterium-sized particles.

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 harbour genes for multiple pilus-like structures. Recent studies on the 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 an HMM 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 HMM bands with sizes greater than 200 kDa was detected from strain TX0082 (Fig. 5). In contrast, only a single band has been observed with Acm in mutanolysin extracts of TX0082 (Nallapareddy *et al.*, 2003). The HMM banding pattern is consistent with observations of a large number of pilus proteins from other Gram-positive bacteria and suggests that EbpC_{fm} is assembled similarly 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

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 per lane) were separated on 4–15 % SDS-PAGE gels, transferred to PVDF membranes, and probed using affinity-purified anti-rEbpC_{fm62} antibodies. Purified rScm₃₆ and rEbpC_{fm62} proteins (20 ng per lane) were included as controls.

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 $-22.55 \text{ kcal mol}^{-1}$ ($94.34 \text{ kJ mol}^{-1}$)] located in the intergenic region between *ebpC_{fm}* and *bps_{fm}* is likely to function as a ρ -independent

transcriptional terminator, thus predicting an *ebpA_{fm}* to *ebpC_{fm}* transcript and an independent *bps_{fm}* transcript. To validate this, we carried out transcriptional analyses of the *ebpA_{fm}* to *bps_{fm}* locus using Northern hybridization. The *ebpA_{fm}*, *ebpB_{fm}* and *ebpC_{fm}* probes all hybridized to a single RNA band of $\sim 7 \text{ kb}$ (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 ($\sim 1 \text{ kb}$), consistent with the expected size of a monocistronic *bps_{fm}* mRNA transcript, suggesting that this transcript either is produced at 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 $\sim 8 \text{ kb}$ 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 on 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 demonstrate unambiguously 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).

Fig. 6. Transcriptional analysis of the *ebpA_{fm}* to *bps_{fm}* gene cluster. (a) Northern hybridization of total RNA (30 µg per 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 of the expected sizes are indicated with arrows. (b) RT-PCR analysis of the *ebpA_{fm}* to *bps_{fm}* gene cluster. The location of each primer pair is indicated by arrows in the schematic representation of the gene cluster. A lollypop between *ebpC_{fm}* and *bps_{fm}* 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 (100 ng) 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 mass marker.

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), which 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 nine genes were considered to be widespread in both CC17 and non-CC17 nosocomial isolates (Hendrickx *et al.*, 2007). Notably, our preliminary data from screening for *scm* in several hundred *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 (S. R. Nallapareddy, J. Sillanpää and B. E. Murray, unpublished data).

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 for CV. 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 nine 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 an HMM 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.

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