

## A family of fibrinogen-binding MSCRAMMs from *Enterococcus faecalis*

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We report that three (EF0089, EF2505 and EF1896, renamed here Fss1, Fss2 and Fss3, respectively, for *Enterococcus faecalis* surface protein) of the recently predicted MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) in *E. faecalis* strain V583 bind fibrinogen (Fg). Despite an absence of extensive primary sequence homology, the three proteins appear to be related structurally. Within the N-terminal regions of the three enterococcal proteins, we identified pairs of putative IgG-like modules with a high degree of predicted structural similarity to the Fg-binding N2 and N3 domains of the staphylococcal MSCRAMMs ClfA and SdrG. A second N2N3-like segment was predicted in Fss1. Far-UV circular dichroism spectroscopy revealed that all four predicted N2N3-like regions are composed mainly of  $\beta$ -sheets with only a minor proportion of  $\alpha$ -helices, which is characteristic of Ig-like folded domains. Three of the four identified enterococcal N2N3-like regions showed potent dose-dependent binding to Fg. However, the specificity of the Fg-binding MSCRAMMs differs, as indicated by far-Western blots, which showed that recombinant segments of the MSCRAMMs bound different Fg polypeptide chains. Enterococci grown in serum-supplemented broth adhere to Fg-coated surfaces, and inactivation in strain OG1RF of the gene encoding Fss2 resulted in reduced adherence, whilst complementation of the mutant restored full Fg adherence. Thus, *E. faecalis* contains a family of MSCRAMMs that structurally and functionally resemble the Fg-binding MSCRAMMs of staphylococci.

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## INTRODUCTION

*Enterococcus faecalis* is a component of the human commensal flora, but is also emerging as an opportunistic pathogen and has become one of the leading causes of nosocomial infections in developed countries (Murray & Weinstock, 1999). *E. faecalis* can cause a variety of infections, of which endocarditis, wound and bloodstream infections are the most serious. Treatment of enterococcal infections is complicated by the increased presence of multiple antibiotic-resistance genes in infection-associated enterococcal strains (Malathum & Murray, 1999; Murray,

2000). These resistance determinants are frequently carried on mobile DNA elements (Paulsen *et al.*, 2003) and can easily be exchanged between different strains, leading to rapid dissemination of antibiotic resistance among enterococci.

During infection, enterococci are thought to be located primarily extracellularly in tissues and thus are likely to adhere to the extracellular matrix (ECM) of the host. The attachment of different *E. faecalis* strains to several different ECM proteins has also been reported (Rozdzinski *et al.*, 2001; Styriak *et al.*, 1999, 2002; Tomita & Ike, 2004; Xiao *et al.*, 1998; Zareba *et al.*, 1997). However, the MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) responsible for these interactions have generally not been identified and

Abbreviations: CD, circular dichroism; ECM, extracellular matrix; Fg, fibrinogen; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules; RBS, ribosome-binding site.

characterized. An exception is the collagen adhesin Ace, which was identified based on its sequence similarity to the collagen-binding MSCRAMM Cna from *Staphylococcus aureus* (Nallapareddy *et al.*, 2000; Rich *et al.*, 1999). Staphylococci express a family of structurally related, cell wall-anchored proteins that act as MSCRAMMs and bind to fibrinogen (Fg), collagen, fibronectin, laminin, cytokeratin 10 and elastin (Davis *et al.*, 2001; Jönsson *et al.*, 1991; McDevitt *et al.*, 1994; Nallapareddy *et al.*, 2000, 2003; Ni Eidhin *et al.*, 1998; O'Brien *et al.*, 2002; Patti *et al.*, 1992; Rich *et al.*, 1999; Roche *et al.*, 2004; Signäs *et al.*, 1989; Wann *et al.*, 2000). The recognized staphylococcal MSCRAMMs contain an N-terminal A-region composed of three domains, of which at least two adopt a characteristic IgG-like fold. The A-region is followed by a variable region that often includes tandemly repeated sequences that, in some cases, can also adopt IgG-like folds (Deivanayagam *et al.*, 2000). At their C termini, these bacterial surface proteins contain a set of conserved motifs required for cell-wall anchoring, including an LPXTG-like motif followed by a hydrophobic membrane-spanning region and a short stretch of positively charged amino acid residues. A transpeptidase called sortase recognizes the LPXTG motif and catalyses the covalent attachment of the threonine residue to the peptidoglycan of the cell wall (Mazmanian *et al.*, 2001; Navarre & Schneewind, 1999; Schneewind *et al.*, 1992).

The A-region contains the primary ligand-binding domains in the staphylococcal MSCRAMMs, and biochemical studies have shown that two IgG-folded domains are sufficient for ligand binding. The crystal structure of the ligand-binding domain of the Fg-binding MSCRAMM SdrG from *Staphylococcus epidermidis* has been solved, both as an apoprotein and in complex with an Fg-based ligand peptide (Deivanayagam *et al.*, 2000; Ponnuraj *et al.*, 2003). These structures reveal that the ligand peptide can dock at the interphase between domains 2 and 3 (called N2 and N3). As the ligand peptide docks, the C-terminal extension of N3 is redirected in the complex to cover and lock the bound peptide in the ligand-binding pocket. Furthermore, the N3 extension is inserted into a trench on the surface of the N2 module, where it stabilizes the complex by serving as a latch. The bottom of the latching trench contains a characteristic sequence motif, TYTFTDYVD, which is conserved in a similar location in other staphylococcal MSCRAMMs, as well as the enterococcal MSCRAMMs Ace (*E. faecalis*) and Acm (*Enterococcus faecium*) (McCrea *et al.*, 2000; Nallapareddy *et al.*, 2003; Ponnuraj *et al.*, 2003).

In an effort to identify other putative enterococcal MSCRAMMs, we previously searched the published genome of *E. faecalis* V583 for cell wall-anchored proteins with MSCRAMM-like characteristics (Sillanpää *et al.*, 2004). We have identified 17 putative cell wall-anchored proteins containing predicted repeated IgG-folded modules. However, in enterococci, the putative A-regions vary in length from two modules (Ace) to seven to ten modules (EF0089, EF2505 and EF1896, renamed here Fss1, Fss2 and

Fss3, respectively), compared with the consistent number of three IgG-folded domains found in the A-regions of staphylococcal MSCRAMMs. In the current study, we have investigated the binding of 11 of the identified MSCRAMM-like enterococcal proteins to a selection of host proteins. Recombinant segments of three MSCRAMMs bound Fg with reasonable affinity. Furthermore, the minimal ligand-binding sites in these three enterococcal proteins were identified by comparison of predicted structural similarities to solved crystal structures of ClfA and SdrG. The predicted binding domains were expressed and characterized further.

## METHODS

**Bacterial strains, plasmids and growth conditions.** *E. faecalis* and *Escherichia coli* strains and plasmids used for *E. faecalis* gene disruption and complementation studies are listed in Table 1. Enterococci were grown routinely in brain–heart infusion (BHI) or Todd–Hewitt (TH) broth/agar (Difco) and *E. coli* in Luria–Bertani (Difco) media at 37 °C. The antibiotics used with enterococci were erythromycin (5 µg ml<sup>-1</sup>) and kanamycin (2000 µg ml<sup>-1</sup>), and with *E. coli*, ampicillin (100 µg ml<sup>-1</sup>), kanamycin (50 µg ml<sup>-1</sup>) and erythromycin (200 µg ml<sup>-1</sup>).

**Construction of expression plasmids.** The sequenced *E. faecalis* strain V583 (Sahm *et al.*, 1989) was used as a template for the construction of protein expression plasmids. Primers for cloning PCR-generated DNA fragments into the expression vector pQE30 (Qiagen) are listed in Table 2 and the cloned regions are shown in Fig. 2(a). Isolation of genomic DNA from strain V583 and other cloning procedures were performed as described previously (Sillanpää *et al.*, 2004).

**Expression and purification of His<sub>6</sub>-tagged proteins.** Expression cultures were induced with IPTG and the N-terminally His<sub>6</sub>-tagged proteins were purified by using nickel-affinity chromatography and anion-exchange chromatography as described previously (Sillanpää *et al.*, 2004). Purified proteins were dialysed extensively against PBS, pH 7.4, and stored at -20 °C. Protein concentrations were determined by measuring A<sub>280</sub> by spectroscopy, using calculated molar absorption coefficient values (Pace *et al.*, 1995). Molecular masses of the expressed proteins were confirmed with MALDI-TOF mass spectrometry (Tufts University mass spectrometry facility, MA, USA) from protein samples in H<sub>2</sub>O.

**ELISA-type ligand-binding assays.** Binding of recombinant enterococcal proteins to components of the ECM {mouse laminin [Roche Diagnostics], human fibronectin [purified from plasma in our laboratory as described by Wann *et al.* (2000)], human Fg [plasminogen-, von Willebrand factor- and fibronectin-depleted (Enzyme Research Laboratories)], collagen type I [bovine (Vitrogen 100; Collagen Biomaterials)] and collagen type III and IV [human placenta (Sigma)]} was tested in ELISA-type assays. Microplate (4HBX; Thermo Scientific) wells were coated with 1 µg of each ECM protein in 100 µl TBS [0.05 M Tris–HCl, 0.9% (w/v) NaCl, pH 7.5] or 3% acetic acid for collagens, overnight at 4 °C. The plates were washed once with TBS and the remaining protein-binding sites were blocked by 1 h incubation with 2% BSA, 0.1% Tween 20 in TBS (blocking buffer). Purified His<sub>6</sub>-tagged proteins (50 µl of 10 µM or increasing concentrations) in blocking buffer were added and incubated at ambient temperature for 2 h. Plates were washed three times with 0.1% Tween 20 in TBS and incubated for 1 h with 100 µl of a 1:3000 dilution of monoclonal anti-His<sub>6</sub> antibody (GE

**Table 1.** Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Reference or source
<b>Strains</b>		
<i>Enterococcus faecalis</i>		
OG1RF	Wild-type; Kan <sup>s</sup>	Murray <i>et al.</i> (1993)
V583	Sequenced strain; blood isolate; Van <sup>r</sup> , Gen <sup>r</sup> , Kan <sup>r</sup> , Ery <sup>r</sup> , Str <sup>r</sup>	Paulsen <i>et al.</i> (2003); Sahm <i>et al.</i> (1989)
TX5450	OG1RF <i>fss2::pTEX5446</i> ; <i>fss2</i> insertion disruption mutant of OG1RF; Kan <sup>r</sup>	This study
TX5487	TX5450 harbouring plasmid pMSP3535; Ery <sup>r</sup> , Kan <sup>r</sup> (control for complementation)	This study
TX5488	TX5450 harbouring plasmid pTEX5486; Ery <sup>r</sup> , Kan <sup>r</sup> (complementation with the <i>fss2</i> gene)	This study
<i>Escherichia coli</i>		
M15(pREP4)	<i>E. coli</i> cloning and expression host; Amp <sup>r</sup> , Kan <sup>r</sup>	Qiagen
DH5 $\alpha$	<i>E. coli</i> cloning host	Stratagene
TG1	<i>E. coli</i> cloning host	Sambrook <i>et al.</i> (1989)
TX5446	DH5 $\alpha$ (pTEX5446); Kan <sup>r</sup>	This study
TX5486	TG1 (pTEX5486); Ery <sup>r</sup>	This study
<b>Plasmids</b>		
pTEX4577	Derived from pBluescript SK <sup>-</sup> , used for insertion disruption mutagenesis in enterococci; Kan <sup>r</sup>	Singh <i>et al.</i> (1998)
pMSP3535	Shuttle plasmid used for complementation, with nisin-inducible promoter	Bryan <i>et al.</i> (2000)
pTEX5446	Intragenic <i>fss2</i> fragment cloned into pTEX4577	This study
pTEX5486	Construct for complementation; a 4956 bp fragment containing <i>fss2</i> amplified from strain V583 cloned into pMSP3535; Ery <sup>r</sup>	This study

\*Amp, Ampicillin; Ery, erythromycin; Gen, gentamicin; Kan, kanamycin; Van, vancomycin; Str, streptomycin. For enterococci, Kan<sup>r</sup> indicates MIC >2000 and Kan<sup>s</sup>, MIC ≤2000.

**Table 2.** Primers used in this study

Function*	Primer name	Sequence (5'→3')†
Fss1 protein expression	Fss1 36F	CCGAGCTCGAAGAGGTAAACAGCGATGG
	Fss1 220F	GCGGGATCCGAAACAACGATTATCGATAAATTT
	Fss1 757F	GCGGCATGCAACAATCCTGCGTTGGACCAT
	Fss1 550R	GCGGTCGAC <u>TTA</u> ATTATCCCCATTGTGTCTG
	Fss1 1090R	CGCCTGCAG <u>TTA</u> CCCCGCACTGTGATCAATGT
	Fss1 1143R	CCCTGCAG <u>TTA</u> CCCCACAAATGTGATAACCC
Fss2 protein expression	Fss2 28F	GCGGGATCCGTAACAACGACAGAAGCACAA
	Fss2 111F	GCGGGATCCCAAGCGCCAATTACAGATGA
	Fss2 429R	GCGCTGCAG <u>TTA</u> AATTGTTACATAATATTTCCCTT
	Fss2 449R	GCGCTGCAG <u>TTA</u> ACCGTTTGTACTCTCTAATAC
	Fss3 267F	GCGGGATCCCAACACTTACACGGTCAAAATTG
Fss3 protein expression	Fss3 273F	GCGGGATCCATTGATGTAGAAACCGAGGGT
	Fss3 586R	GCGGTCGAC <u>TTA</u> GCCTAATTGCTCATTATAAGCT
	Fss3 681R	GCGGTCGAC <u>TTA</u> TCTTCTGGCGCTTCTACTT
	Fss3 971R	GCGGTCGAC <u>TTA</u> TCTACTTTTTCCGCAAGCATA
	Fss2 F	CCGGAATTCAGTTATCGAACTCAACACCATCTTTAC
Disruption mutant, intragenic <i>fss2</i>	Fss2 R	CGGGGTACC <u>ACT</u> TGAGGTTCCAAGTTATCTTGTTTT
	Fss2ComF1	GGATCCGAATTTTATTGAAAGGGGATT
	Fss2ComR1	GCACCGTTTGTAGTCTTCTC
	Fss2ComF2	CAACGATTGAATTAACACCA
Complementation, complete <i>fss2</i> gene including RBS	Fss2ComR2	CTGCAGAAATAGTTCGCTGGTTCATC

\*See Table 1 and Fig. 2 for cloned and expressed regions.

†Introduced restriction sites are underlined; the stop codon is italicized.

Healthcare) in blocking buffer. After three washes, 100  $\mu\text{l}$  of a 1 : 3000 dilution of alkaline phosphatase-conjugated anti-mouse antibody (Bio-Rad) in blocking buffer was added to the wells and incubated for 1 h. Finally, the plate was developed with 1 mg *p*-nitrophenyl phosphate  $\text{ml}^{-1}$  (Sigma) in 0.1 M diethanolamine buffer, pH 9.8, containing 1 mM  $\text{MgCl}_2$ .  $A_{405}$  was measured with a microplate reader (ThermoMax; Molecular Devices).

**Far-Western blot analysis.** To test binding of His<sub>6</sub>-tagged recombinant proteins to the individual  $\alpha$ ,  $\beta$  and  $\gamma$  polypeptide chains of Fg, 1  $\mu\text{g}$  Fg per lane was fractionated by SDS-PAGE and transferred to a nitrocellulose membrane (0.45  $\mu\text{m}$ ) with a semi-dry transfer cell (Bio-Rad). The membranes were blocked with 2 % BSA, 0.1 % Tween 20 in PBS at 4 °C overnight. After three washes with PBS containing 0.05 % Tween 20, the membranes were incubated with 100  $\mu\text{g}$  His<sub>6</sub>-tagged recombinant proteins  $\text{ml}^{-1}$  in 1 % BSA, 0.05 % Tween 20 in PBS for 2 h at room temperature. Bound protein was detected with a 1 : 3000 dilution of monoclonal anti-His<sub>6</sub> antibody (GE Healthcare), followed by a 1 : 5000 dilution of alkaline phosphatase-conjugated anti-mouse antibody (Bio-Rad). The phosphatase substrates nitro blue tetrazolium and BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (Bio-Rad) in 0.1 M  $\text{NaHCO}_3$ , 1 mM  $\text{MgCl}_2$ , pH 9.8, were used for signal detection.

**Analysis of secondary-structure components.** Far-UV circular dichroism (CD) spectroscopy data were collected from protein samples in 10 mM potassium phosphate buffer, pH 7.4, as described previously (Sillanpää *et al.*, 2004). Twenty scans were averaged for each spectrum and the contribution of buffer was subtracted. Quantification of secondary-structure components was performed by analysing the spectra with a combination of three deconvolution algorithms, SELCON3, ContinLL and K2D, using the DICHROWEB server (<http://www.cryst.bbk.ac.uk/cdweb/html/home.html>) (Lobley *et al.*, 2002; Whitmore & Wallace, 2004).

**Construction of disruption mutants and complementation.** Gene disruptions were achieved by the method described previously (Qin *et al.*, 1998; Singh *et al.*, 1998). An internal fragment of *fss2* was amplified from *E. faecalis* OG1RF genomic DNA using primers listed in Table 2 and cloned into pTEX4577 (Singh *et al.*, 1998), according to standard methods (Sambrook *et al.*, 1989). The resulting construct has the plasmid integrated between the codons encoding aa 301 and 302, located within the N2N3-like region. This construct was electroporated (Li *et al.*, 1995) into *E. faecalis* OG1RF, followed by selection on TH agar plates with 2000  $\mu\text{g}$  kanamycin  $\text{ml}^{-1}$  to generate TX5450. Correct insertion was confirmed by PCR, PFGE and Southern blot analysis (Nallapareddy *et al.*, 2000; Qin *et al.*, 1998). Growth curves, as well as stability of single-crossover disruptions in mutants, were determined as described previously (Nallapareddy *et al.*, 2000).

A construct for complementation was generated with the shuttle vector pMSP3535, which contains a nisin-inducible promoter (Bryan *et al.*, 2000). Briefly, two DNA fragments containing the complete *fss2* gene and its ribosome-binding site (RBS) were amplified (the first fragment with primers Fss2ComF1 and Fss2ComR1 and the second with Fss2ComF2 and Fss2ComR2; see Table 2) from genomic DNA of the sequenced strain *E. faecalis* V583 (Paulsen *et al.*, 2003) and cloned into the pGEM-T Easy vector (Promega). The first and second fragments were released by using *Bam*HI–*Eco*RI and *Eco*RI–*Pst*I restriction enzymes, respectively, and then ligated with *Bam*HI–*Pst*I-digested pMSP3535. This *in vitro*-ligated construct for complementation (designated pTEX5486) was transformed into *E. coli* TG1 to obtain TX5486 and was then introduced into electrocompetent cells of TX5450.

**Production and purification of Fss2-specific antibodies.** Polyclonal antibodies against rFss2<sub>(28–449)</sub> were raised in rabbit and

purified from rabbit serum by affinity chromatography with rFss2<sub>(28–449)</sub> coupled to cyanogen bromide-activated Sepharose 4B, as described previously (Sillanpää *et al.*, 2008).

**Flow cytometry.** To determine surface expression of Fss2 by flow cytometry, bacteria were grown for 10 h in BHI broth supplemented with 40 % horse serum from an inoculum with an OD<sub>600</sub> of 0.01. Collected and washed cells were labelled with purified preimmune or Fss2-specific antibodies followed by R-phycoerythrin-conjugated F(ab')<sub>2</sub> fragment goat anti-rabbit IgG, as described previously (Kemp *et al.*, 2007; Sillanpää *et al.*, 2008). Cells were fixed with 1 % paraformaldehyde in PBS and analysed with a Coulter EPICSXL AB6064 flow cytometer (Beckman Coulter) and System II software.

**Adherence of radiolabelled *E. faecalis* to immobilized Fg.** Bacteria grown for 10 h in 5 ml BHI broth with 40 % horse serum (Nallapareddy & Murray, 2008) and Tran<sup>35</sup>S label [10  $\mu\text{Ci}$  (370 kBq)  $\text{ml}^{-1}$ ; MP Biochemicals] were harvested, washed three times in PBS and resuspended in 0.5 % Tween 80, 0.1 % BSA in PBS. For the complemented strain (TX5488) and the vector control (TX5487), nisin was added to the growth medium at a final concentration of 25 ng  $\text{ml}^{-1}$ . Immulon 1 Removawells (Dynatech Laboratories), coated overnight with Fg at 4 °C (at a concentration of 1  $\mu\text{g}$  per well), were blocked with 200  $\mu\text{l}$  0.2 % BSA for 2 h with gentle shaking. Labelled bacteria (50  $\mu\text{l}$ , adjusted to an OD<sub>600</sub> of 0.2) were added to wells and allowed to incubate at room temperature for 2 h with gentle shaking. Wells were then washed with 0.5 % Tween 80, 0.1 % BSA in PBS three times. The radioactivity of bound cells in each detachable well was measured in a liquid scintillation counter. To determine the total amount of radioactivity added to each well, the radioactivity in a sample of labelled bacteria (50  $\mu\text{l}$ , adjusted to an OD<sub>600</sub> of 0.2) was measured. Percentage adherence was calculated with the following equation: adherent bacteria (%) = (radioactivity of bound cells / radioactivity of total cells added)  $\times$  100 (Nallapareddy *et al.*, 2000). BSA-coated wells were used as negative controls.

**Statistical analysis.** To compare sample means, an unpaired *t*-test was used.  $P < 0.05$  was considered statistically significant. Statistical tests were performed by using GraphPad Prism v4.

## RESULTS

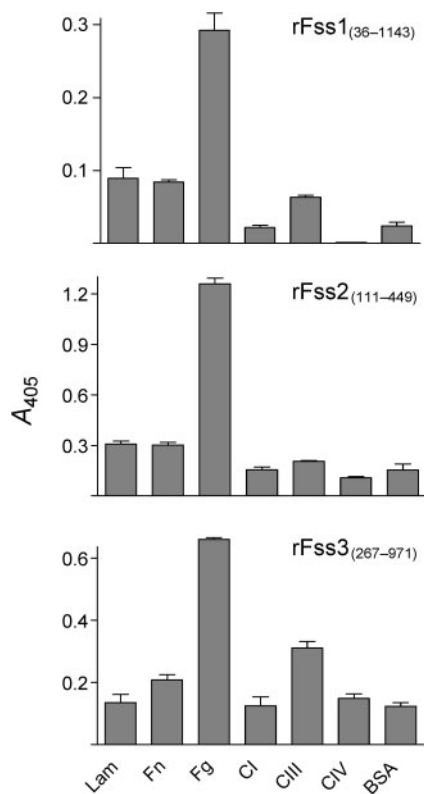
### Recombinant segments of Fss1, Fss2 and Fss3 bind to immobilized human Fg

We have initiated a search for ligands of the orphan *E. faecalis* MSCRAMMs identified in the V583 genome. Initially, the non-repeated regions were cloned and expressed in *E. coli* as N-terminally His<sub>6</sub>-tagged fusion proteins from eight of the 17 cell wall-anchored proteins, Fss1 (EF0089), EF1091, EF1092, EF1093, EF1099, EF1269, EF1824 and EF3023, that we have described previously (Sillanpää *et al.*, 2004). We recently reported that three of these proteins, EF1091, EF1092 and EF1093 (subsequently renamed EbpA, EbpB and EbpC, respectively), are pilus subunit proteins that are important for biofilm formation, as well as experimental endocarditis and urinary-tract infections (Kemp *et al.*, 2007; Nallapareddy *et al.*, 2006; Singh *et al.*, 2007). We also expressed segments from three additional proteins: Fss2 (EF2505; aa 111–449), Fss3 (EF1896; aa 267–971) and EF2347 (aa 272–680), which we subsequently identified as putative cell wall-anchored

proteins with predicted IgG-like folded modules, but with atypical cell wall-anchoring motifs. Purified fragments of the 11 proteins were then tested for binding to a selection of ECM proteins, including Fg, fibronectin, laminin and type I, III and IV collagens, in an ELISA-type ligand-binding assay. Three of the 11 protein segments, rFss1<sub>(36–1143)</sub>, rFss2<sub>(111–449)</sub> and rFss3<sub>(267–971)</sub>, showed binding to human Fg, compared with BSA and the other ECM proteins included in this assay (Fig. 1), whilst the remaining eight proteins showed no significant binding to any of the other ECM proteins tested (data not shown). These three proteins were selected for further structural and functional analysis.

### Domain organization of the Fg-binding proteins Fss1, Fss2 and Fss3

As shown in Fig. 2(a), Fss1, Fss2 and Fss3 are predicted to have a similar overall structural organization to the previously described staphylococcal MSCRAMMs. The putative N-terminal signal-peptide sequences of Fss1, Fss2 and Fss3, which range from 27 to 35 aa in length,

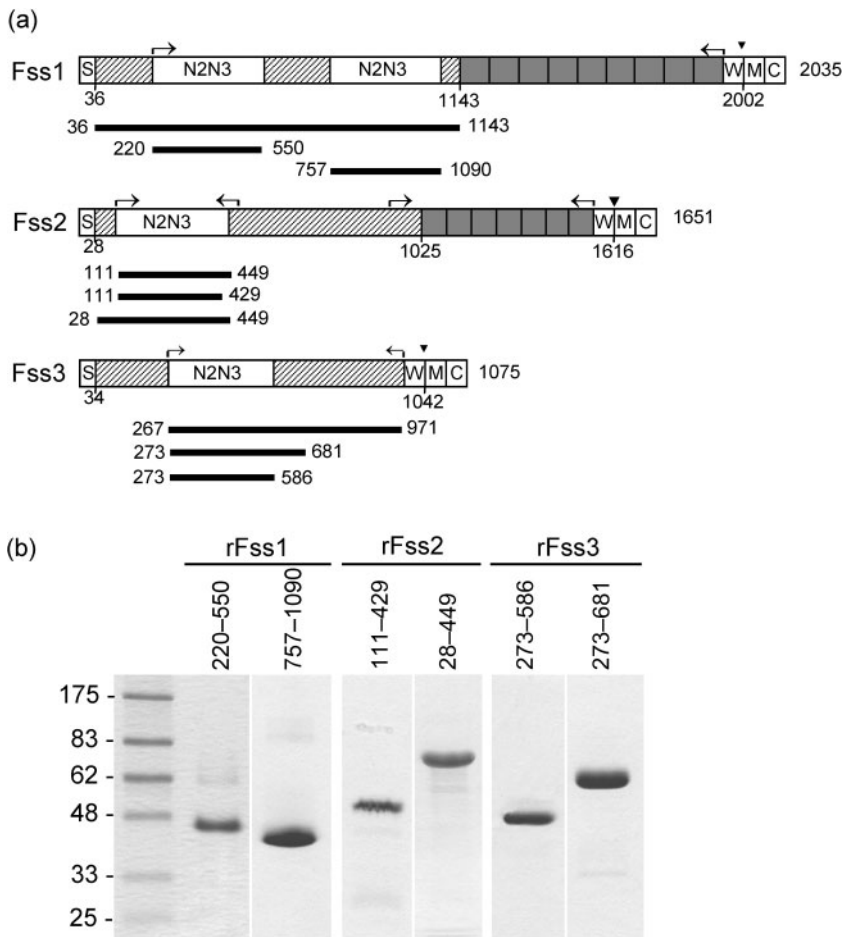


**Fig. 1.** Binding of the non-repeated A-region segments of Fss1, Fss2 and Fss3 to immobilized ECM proteins in an ELISA-type assay. The concentration of each protein assayed was 10  $\mu$ M. The assay was performed in triplicate and the values represent means  $\pm$  SD. Lam, Laminin; Fn, fibronectin; Fg, fibrinogen; Cl, CIII and CIV, collagen types I, III and IV, respectively.

are followed by non-repeated A-regions that vary in length from 740 to 1107 aa. Analyses of their structures with two complementing fold-recognition servers, 3D-PSSM (<http://www.sbg.bio.ic.ac.uk/~3dpssm/index2.html>) and PHYRE (<http://www.sbg.bio.ic.ac.uk/~phyre/index.cgi>) (Kelley *et al.*, 2000), showed that the A-regions of these proteins are predicted to contain tandem repeats of IgG-like modules. At the C-terminal end, Fss1 contains nine sequence repeats of 73–102 aa (Sillanpää *et al.*, 2004) that show amino acid sequence and predicted structural similarity to the IgG-like folded B-repeats of the collagen-binding *S. aureus* MSCRAMM Cna. Similarly, Fss2 contains seven C-terminal tandem repeats ranging from 66 to 85 aa in length that are similar to the B-repeats of Cna and are predicted to be IgG-folded. No conserved sequence repeats were identified in the C-terminal region of Fss3. All three proteins have putative cell wall-anchoring motifs at their C termini.

### The three Fg-binding proteins contain potential MSCRAMM N2N3-like regions

Amino acid sequence comparisons between the A-regions of the *E. faecalis* proteins Fss1, Fss2 and Fss3 and the staphylococcal Fg-binding MSCRAMMs ClfA, ClfB, FnbpA and SdrG did not reveal extensive sequence similarities. We therefore decided to search for structural similarities between the determined crystal structures of the minimal Fg-binding N2N3-segments of ClfA (Deivanayagam *et al.*, 2002) and SdrG (Ponnuraj *et al.*, 2003) and the predicted structures of the *E. faecalis* proteins. A segment of the A-region of Fss1 corresponding to aa 220–559 gave a strong match to the Fg-binding N2N3 domains of ClfA ( $e=1.7 \times 10^{-8}$ ) using the 3D-PSSM server (Fig. 2a) (Sillanpää *et al.*, 2004). Using the PHYRE server, a corresponding region (Fss1 aa 204–491) was predicted to have structural similarity to ClfA N2N3 ( $e=2.8 \times 10^{-14}$ ). Surprisingly, we found a second segment in the large Fss1 A-region that shows structural similarity to the N2N3 domains of ClfA (Fss1 aa 770–1074;  $e=5.0 \times 10^{-9}$ , PHYRE) and SdrG (Fss1 aa 767–1090;  $e=8.0 \times 10^{-3}$ , 3D-PSSM). Fss2 was predicted to contain a ClfA N2N3-like segment from aa 111 to 449 using the 3D-PSSM program ( $e=1.7 \times 10^{-8}$ ) and from aa 105 to 386 using PHYRE ( $e=9.5 \times 10^{-14}$ ). Similar to the N2N3 domains of staphylococcal MSCRAMMs, the identified first N2N3-like segment in Fss1 and the N2N3-like segment in Fss2 are both located near the N terminus. Furthermore, each contains a TYTFTDYVD-like ‘latching-cleft’ motif (Ponnuraj *et al.*, 2003), which aligns with the corresponding motifs of known MSCRAMM N2 domains in multiple sequence alignments (Sillanpää *et al.*, 2004). Fold predictions also identified an N2N3-like region in the N-terminal half of Fss3, with a weaker but significant match to SdrG N2N3 (Fss3 aa 267–586;  $e=1.2 \times 10^{-2}$ , 3D-PSSM). Neither the second N2N3-like segment in Fss1 nor the N2N3-like region in Fss3 contains an obvious latching-cleft sequence.



**Fig. 2.** Primary amino acid sequence characteristics of the three Fg-binding *E. faecalis* MSCRAMMs and analysis of purified recombinant segments encompassing their predicted ligand-binding regions. (a) Domain organization of Fss1, Fss2 and Fss3. Hatched areas represent non-repeated A-regions; shaded areas represent B-repeat regions. N2N3 within the A-regions depicts predicted structural similarity to the N2N3 domains of the Fg-binding staphylococcal MSCRAMMs ClfA and/or SdrG. Arrows above the proteins define regions with predicted Ig-type folding ( $\epsilon$  probability value certainty >90%). S, Signal peptide; W, cell wall-spanning domain; M, hydrophobic membrane-spanning region; C, cytosolic tail with positively charged residues;  $\blacktriangledown$ , putative sortase-cleavage motif LPXTG. (b) SDS-PAGE analysis of the *E. coli*-expressed segments of Fss1, Fss2 and Fss3. The recombinant proteins with N-terminal His<sub>6</sub> tags were purified by nickel-affinity chromatography followed by anion-exchange chromatography and separated by using SDS-PAGE (12% gel) under reducing conditions. Bands were visualized by Coomassie brilliant blue R250 staining.

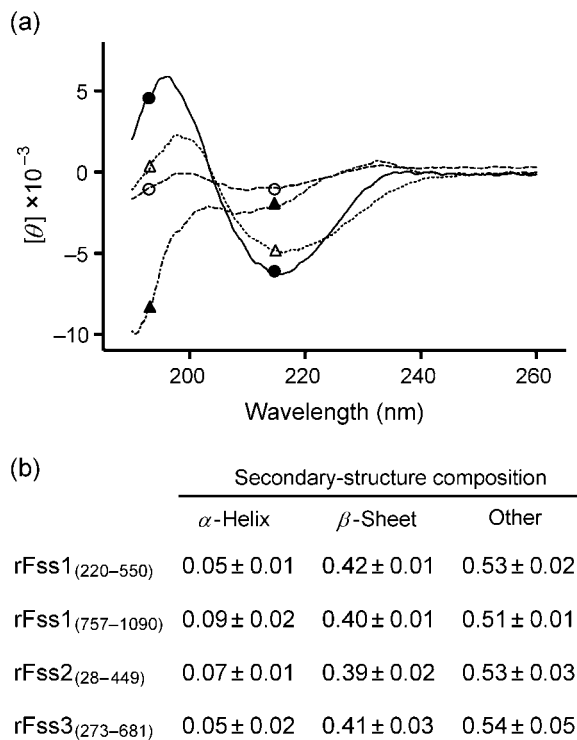
### Expression and purification of the MSCRAMM N2N3-like regions of Fss1, Fss2 and Fss3 as recombinant proteins

To characterize the N2N3-like regions in the enterococcal Fg-binding MSCRAMMs further, we subcloned and expressed segments containing these regions as recombinant proteins with N-terminal His<sub>6</sub>-tag fusions. Often, the initial recombinant segments were partially degraded upon storage, but produced stable degradation products with a slightly smaller apparent molecular size. We interpreted these results to indicate that the smaller stable constructs, and not the original larger ones, represent relatively well-defined and folded domains. We determined, by using a combination of anti-His<sub>6</sub> Western blot, N-terminal sequencing and MALDI-TOF mass spectrometry, the likely proteolytic-cleavage sites in the truncated segments (not shown). We then expressed and purified the corresponding segments: rFss1<sub>(220–550)</sub>, rFss1<sub>(757–1090)</sub> and rFss2<sub>(111–429)</sub> (Fig. 2b). We also created a second construct from Fss2, rFss2<sub>(28–449)</sub>, that included the preceding N-terminal region. From Fss3, two segments were produced and characterized further: rFss3<sub>(273–586)</sub>, which is predicted to correspond to an N2N3-like region, and rFss3<sub>(273–681)</sub>, which also included a C-terminally extended segment

(Fig. 2b). The purified proteins were analysed by MALDI-TOF mass spectrometry, which showed major peaks corresponding closely to the molecular masses calculated from amino acid sequences for all protein segments, thus demonstrating that the expected proteins were expressed (not shown).

### Analysis of secondary-structure components of the predicted N2N3-like regions of Fss1, Fss2 and Fss3

The far-UV CD spectra for recombinant protein segments that encompass the N2N3-like regions in Fss1 (aa 220–550 and 757–1090), Fss2 (aa 28–449) and Fss3 (aa 273–681) share a similar pattern, with a maximum between 194 and 203 nm and a minimum between 214 and 217 nm, resembling the previously determined CD spectra for the ligand-binding N2N3 domains of staphylococcal Fg-binding MSCRAMMs (Fig. 3a). Deconvolution of the collected data revealed a  $\beta$ -sheet content of 39–42% and an  $\alpha$ -helix content of 5–9% in the four N2N3-like segments from the enterococcal MSCRAMMs (Fig. 3b). These values are typical for IgG-like modules and are in good agreement with the secondary-structure compositions of the ligand-



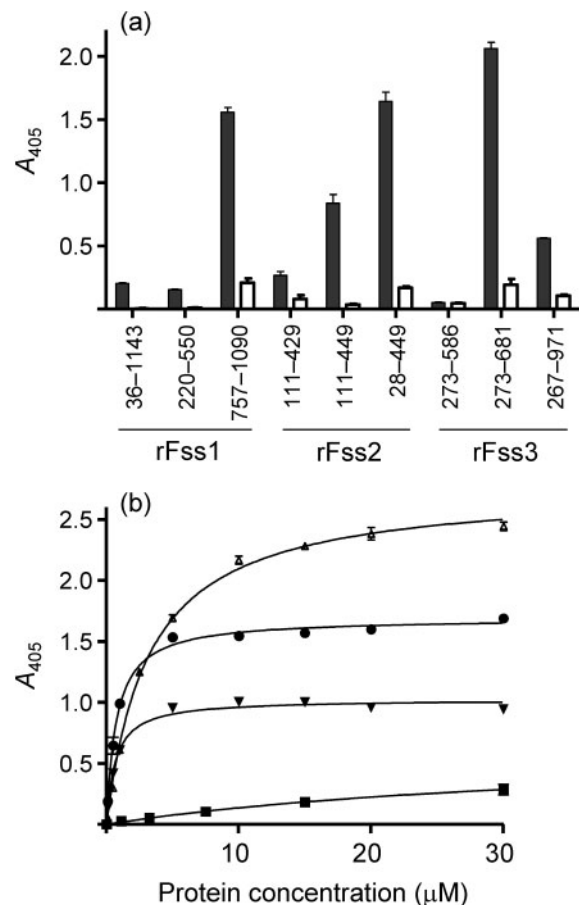
**Fig. 3.** Analysis of secondary-structure compositions by far-UV CD spectroscopy. (a) Recorded spectra of four recombinant segments from the three *E. faecalis* Fg-binding MSCRAMMs.  $\circ$ , rFss1<sub>(220–550)</sub>;  $\bullet$ , rFss1<sub>(757–1090)</sub>;  $\triangle$ , rFss2<sub>(28–449)</sub>;  $\blacktriangle$ , rFss3<sub>(273–681)</sub>. Molar ellipticity ( $\theta$ ) is shown in  $\text{deg cm}^2 \text{dmol}^{-1}$ . For details, see Methods. (b) Summary of secondary-structure components. Deconvolution of the collected far-UV CD spectra was performed with three algorithms, SELCON3, ContinLL and K2D, using the DICHROWEB server (Lobley *et al.*, 2002; Whitmore & Wallace, 2004). Means  $\pm$  SEM are shown.

binding domains in the solved crystal structures of ClfA, SdrG, Cna and Ace.

### The N2N3-like segments are the primary Fg-binding regions of Fss1, Fss2 and Fss3

To determine whether the N2N3-like segments are responsible for the Fg-binding activity of Fss1, Fss2 and Fss3, we compared the Fg-binding activity of the N2N3 domains with that of the intact A-regions, using an ELISA-type ligand-binding assay. The N-terminal N2N3-like region of rFss1<sub>(220–550)</sub> bound Fg weakly, whereas a significantly higher level of Fg binding was seen with the second N2N3-like segment [rFss1<sub>(757–1090)</sub>] (Fig. 4a). In fact, the level of Fg binding recorded for rFss1<sub>(757–1090)</sub> was significantly higher than that observed for the intact A-region [rFss1<sub>(36–1143)</sub>].

rFss2<sub>(111–429)</sub> bound Fg poorly, whereas rFss2<sub>(111–449)</sub> was a potent Fg binder, indicating that the short C-terminal extension contributed significantly to this activity. The N-terminally extended rFss2<sub>(28–449)</sub> was an even more potent



**Fig. 4.** Binding of the three *E. faecalis* MSCRAMMs to human Fg. (a) A collection of recombinant segments from Fss1, Fss2 and Fss3 were tested for their binding to Fg (filled bars) and BSA (empty bars) by using an ELISA-type assay. The concentration of each protein assayed was 10  $\mu\text{M}$ . The assay was performed in triplicate and the values represent means  $\pm$  SD. (b) Dose-dependent binding of the minimal binding regions to Fg. Values of MSCRAMM binding to wells coated with BSA were subtracted from the respective Fg-binding values. Apparent  $K_D$  values were determined by using a one-site-binding non-linear regression model (GraphPad Prism v4). The resulting curves are depicted in the figure. The assay was performed in triplicate and the values represent means  $\pm$  SD.  $\blacksquare$ , rFss1<sub>(220–550)</sub>;  $\blacktriangledown$ , rFss1<sub>(757–1090)</sub>;  $\bullet$ , rFss2<sub>(28–449)</sub>;  $\nabla$ , rFss3<sub>(273–681)</sub>.

Fg binder, suggesting that the N-terminal extension also contributes to the Fg-binding activity of this protein.

The rFss3<sub>(273–586)</sub> segment, which corresponds to the predicted N2N3 region, did not show significant Fg binding (Fig. 4a). In contrast, the C-terminally extended segment rFss3<sub>(273–681)</sub> showed considerably higher binding to Fg than rFss3<sub>(273–586)</sub>, which exceeded that of the larger aa 267–971 region of Fss3. These observations indicate that the N2N3-like region, defined by structural predictions to encompass residues 273–583, requires additional C-terminal residues for full Fg-binding activity.

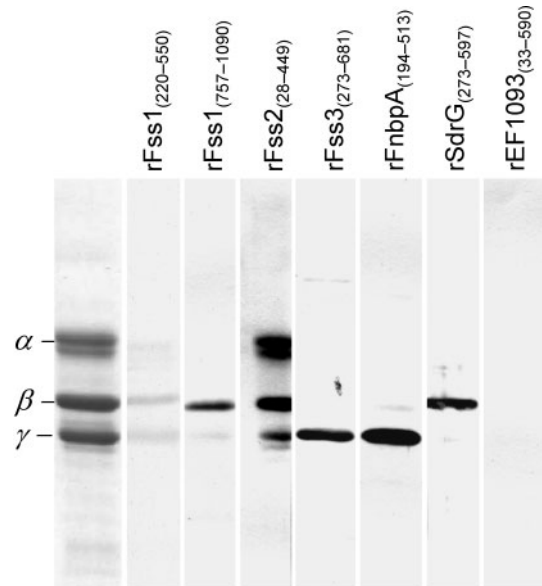
As the purified proteins rFss1<sub>(757–1090)</sub>, rFss2<sub>(28–449)</sub> and rFss3<sub>(273–681)</sub> exhibited potent Fg-binding activity, they were considered to contain the ligand-binding domains of these three enterococcal Fg-binding MSCRAMMs and were selected for further studies. As seen in Fig. 4(b), the three proteins exhibited dose-dependent binding to immobilized Fg with saturation kinetics. rFss1<sub>(757–1090)</sub> and rFss2<sub>(28–449)</sub> showed the highest apparent affinities for Fg, with half-maximal binding (apparent  $K_D$ ) at  $7 \times 10^{-7}$  and  $8 \times 10^{-7}$  M, respectively. The interaction of rFss3<sub>(273–681)</sub> with Fg showed half-maximal binding at a concentration of  $3 \times 10^{-6}$  M. For comparison, we also examined the dose-dependent binding of rFss1<sub>(220–550)</sub>, which corresponds to the N-terminal N2N3-like region. This protein bound Fg weakly, with an estimated apparent  $K_D$  of  $3\text{--}5 \times 10^{-5}$  M. When compared with staphylococcal Fg-binding MSCRAMMs, the apparent  $K_D$  values (concentrations required for half-maximal binding using an ELISA-type assay) were in a range similar to those determined under comparable conditions for ClfB (N2N3,  $2 \times 10^{-8}$  M; N1N2N3,  $>1 \times 10^{-7}$  M) (Perkins *et al.*, 2001), FnbpA (N2N3 region, aa 194–511,  $4.0 \times 10^{-9}$  M) (Keane *et al.*, 2007) and ClfA (N2N3, aa 221–559,  $8 \times 10^{-6}$  M, inhibition ELISA) (Deivanayagam *et al.*, 2002) of *S. aureus* and SdrG (N1N2N3 region, aa 50–597,  $0.9 \times 10^{-7}$  M) (Davis *et al.*, 2001) of *S. epidermidis*.

### The Fg-binding N2N3-like regions of Fss1, Fss2 and Fss3 target different sites in Fg

We next examined whether Fss1, Fss2 and Fss3 showed differences in their binding specificities to Fg by using a far-Western ligand-blot assay. In this assay, the  $\alpha$ ,  $\beta$  and  $\gamma$  chains of Fg were separated by SDS-PAGE, transferred to a supporting membrane and probed with the Fg-binding segments identified above. As seen in Fig. 5, rFss1<sub>(757–1090)</sub> bound preferentially to the  $\beta$  chain, whereas rFss3<sub>(273–681)</sub> recognized the  $\gamma$  chain. rFss2<sub>(28–449)</sub>, on the other hand, detected all three chains, possibly suggesting a more complex binding mechanism for this MSCRAMM. rFss1<sub>(220–550)</sub> bound weakly to the three Fg polypeptide chains. As controls, we used rSdrG<sub>(273–597)</sub>, which binds to a site in the N-terminal region of the  $\beta$  chain (Davis *et al.*, 2001), and rFnbpA<sub>(194–513)</sub>, which recognizes the C terminus of the  $\gamma$  chain (Wann *et al.*, 2000). rEF1093<sub>(33–590)</sub>, which did not show appreciable binding to Fg in the ligand-binding ELISA assays (see above), served as a negative control. In conclusion, these results indicate that Fss1, Fss2 and Fss3 recognize different sites on the Fg molecule.

### Adherence of *E. faecalis* OG1RF to immobilized Fg is reduced by insertional inactivation of the *fss2* gene

To investigate whether the Fg binding observed above with recombinant proteins is responsible for the interaction of *E. faecalis* with Fg, we constructed an isogenic disruption mutation in the genetically manipulatable *E. faecalis* strain



**Fig. 5.** Binding of the minimal ligand-binding regions of Fss1, Fss2 and Fss3 to individual chains of Fg in a far-Western ligand-binding assay. For experimental details, see text.

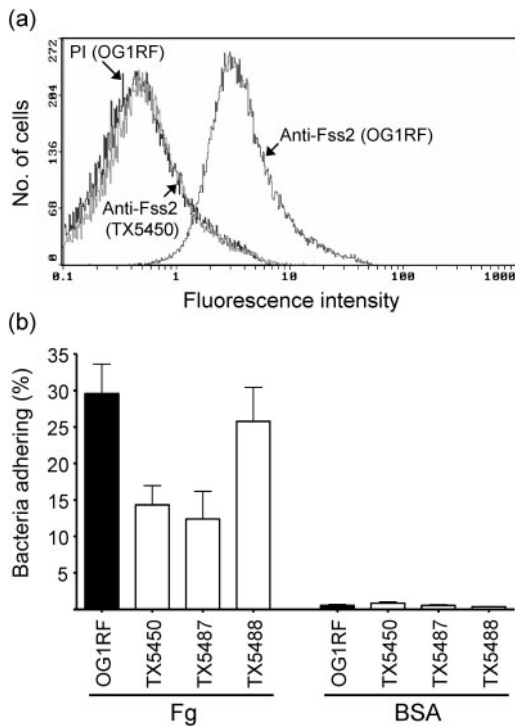
OG1RF (Murray *et al.*, 1993). Lack of Fss2 surface expression by strain TX5450, in which *fss2* is inactivated, and the presence of Fss2 on the surface of the OG1RF parent strain were confirmed by whole-cell ELISA and immunofluorescence microscopy (data not shown), as well as by flow cytometry (Fig. 6a) using affinity-purified Fss2-specific antibodies. TX5450 showed significantly reduced (52% reduction) adherence ( $P < 0.0001$ ) to immobilized Fg compared with the wild-type OG1RF strain (Fig. 6b), yet the two strains had similar doubling times and reached similar cell densities at stationary phase (data not shown). Preliminary experiments with the strain containing the inactivated *fss1* gene showed marginally reduced attachment to Fg-coated surfaces (S. R. Nallapareddy, J. Sillanpää, M. Höök & B. E. Murray, unpublished results), and genome analysis of the sequenced OG1RF strain (Bourgogne *et al.*, 2008) has shown that this strain contains only a remote homologue of *fss3* (60% amino acid identity) as a pseudogene.

To confirm that the *fss2* disruption was responsible for the reduced Fg adherence, we introduced pTEX5486 containing a wild-type copy of the *fss2* gene into TX5450 (*fss2* mutant of OG1RF). Nisin induction (Bryan *et al.*, 2000) resulted in almost-complete restoration of the Fg adherence (Fig. 6b) of TX5488 (TX5450 with pTEX5486), whereas the presence of vector alone in TX5450 (TX5487) showed no increase.

## DISCUSSION

In the present study, we have initiated a search for ligands of putative *E. faecalis* MSCRAMMs. Three of these





**Fig. 6.** Effect of insertional inactivation of *fss2* on surface expression of Fss2 and adherence of *E. faecalis* OG1RF to Fg. (a) Flow-cytometry analysis of Fss2 surface expression. Reactivity of both preimmune (PI) and anti-Fss2 antibodies is shown. OG1RF, Wild-type; TX5450, *fss2* disruption mutant. Bacteria were analysed by using side scatter as the threshold for detection. Specific binding by anti-Fss2 antibodies is indicated on the *x*-axis as fluorescence intensity (exponential scale). For each histogram, 50 000 events of bacterium-sized particles were counted. (b) Adherence of *E. faecalis* OG1RF and its derivatives to immobilized Fg. OG1RF, Wild-type; TX5450, *fss2* disruption mutant; TX5487, TX5450 with vector pMSP3535; TX5488, *fss2*-complemented TX5450. Adherence was tested in wells coated with 1  $\mu$ g Fg (see Methods). Bars represent mean  $\pm$  SD percentages of cells bound for 32 wells (16 wells for complementation experiments). Results are representative of four independent experiments. BSA was used as a negative control.

proteins, Fss1, Fss2 and Fss3, bind human Fg and also resemble previously characterized staphylococcal MSCRAMMs in several ways, although they do not show extensive sequence similarity. First, they share a similar domain organization, with signal-peptide sequences and cell-wall anchor domains flanking a non-repeated A-region that is often followed by tandem repeats. The A-regions of Fss1, Fss2 and Fss3 are much longer than the A-regions of the staphylococcal MSCRAMMs, but are apparently also composed of repeated IgG-like folded modules. Furthermore, we have located the Fg-binding activities to short segments within the A-regions of the enterococcal proteins, which are predicted to adopt structures similar to the ligand-binding N2N3 domains of the staphylococcal

MSCRAMMs. Three of the four N2N3-like regions with the highest scores in the folding-type analyses, rFss1<sub>(220–550)</sub>, rFss2<sub>(111–449)</sub>, and rFss3<sub>(273–681)</sub>, are each located near the N terminus, as are the N2N3 domains of staphylococcal MSCRAMMs. rFss1<sub>(220–550)</sub> and rFss2<sub>(111–449)</sub> also contain sequences homologous to the conserved latching-cleft motif (consensus TYTFTDYVD) of the N2 domains of staphylococcal MSCRAMMs, and align well with them in multiple alignments. Moreover, we located a putative latch sequence at the C-terminal end of rFss1<sub>(220–550)</sub>. This motif represents an extension of the N3 domains of MSCRAMMs and is a critical component of the ‘dock, lock and latch’ ligand-binding model proposed for SdrG (Bowden *et al.*, 2008; Ponnuraj *et al.*, 2003). In this model, the latch sequence secures the bound Fg peptide ligand in the interface between the N2 and N3 domains by complementing a  $\beta$ -strand of the neighbouring N2 domain (Bowden *et al.*, 2008; Ponnuraj *et al.*, 2003). Although rFss1<sub>(220–550)</sub> bound only weakly to Fg, deleting the putative latch sequence to generate rFss1<sub>(220–507)</sub> resulted in even further reduced Fg binding (data not shown). The predicted N2N3-like regions in rFss1<sub>(757–1090)</sub> and rFss3<sub>(273–681)</sub>, although less similar to staphylococcal MSCRAMM N2N3 regions than rFss1<sub>(220–550)</sub> and rFss2<sub>(111–449)</sub>, share the similar predicted fold and secondary-structure composition, and also bind Fg.

The presence of two predicted N2N3-like regions in Fss1 is the first reported such case among MSCRAMMs. The first of these regions (corresponding to residues 220–550) probably recognizes a different protein as its primary ligand, as the affinity for Fg was low. The second of the N2N3 segments (corresponding to residues 757–1090) showed higher affinity for Fg than the complete A-region (aa 36–1143). It is tempting to speculate that the ligand-binding activity of this N2N3 region might be regulated by N-terminal proteolytic processing of the A-region, leading to structural reorganization and activation of the Fg-binding mechanism.

In *S. aureus*, four Fg-binding MSCRAMMs, ClfA (McDevitt & Foster, 1995), ClfB (Ni Eidhin *et al.*, 1998), FnbpA and FnbpB (Wann *et al.*, 2000), are known, and other Fg-binding proteins have also been described. The presence of the three Fg-binding MSCRAMMs described here suggests a similar situation of functional redundancy for *E. faecalis*. Further, our results indicate that the three enterococcal Fg-binding MSCRAMMs bind to different regions of the Fg molecule. The ability to express multiple Fg-binding MSCRAMMs with different binding specificities might increase survival and infectivity of *E. faecalis* within the host, e.g. in endocarditis vegetations where Fg is immobilized in large amounts. The staphylococcal MSCRAMM SdrG is known to bind to the thrombin-cleavage site at the N-terminal end of the  $\beta$  chain of Fg and can thereby interfere with release of the immunoregulating fibrinopeptide B and with fibrin-clot formation in the host (Davis *et al.*, 2001), whilst ClfA specifically recognizes the C-terminal end of the  $\gamma$  chain and competes for the same binding site on Fg as the platelet integrin  $\alpha_{IIb}\beta_3$ , which is involved in platelet adherence and

aggregation. It is an intriguing question whether comparable physiological effects are elicited in the host by the three enterococcal Fg-binding MSCRAMMs described here. Our ongoing studies are aimed at further structural and biochemical characterizations of the N2N3-like regions and their interactions with Fg.

To investigate further the role of these MSCRAMMs in Fg binding with *E. faecalis* as the producing bacterium, we performed mutation analyses using the genetically amenable OG1RF strain, which, like most *E. faecalis* strains, does not express significant adherence to Fg under standard laboratory growth conditions unless supplemented with a biological cue, such as serum (Nallapareddy & Murray, 2008). Inactivation of the expression of Fss2 in *E. faecalis* OG1RF by gene disruption resulted in reduced adherence to Fg, which was restored to near-wild-type levels by complementation, indicating that Fss2 is involved in adherence of *E. faecalis* cells to Fg. The residual Fg binding exhibited by the mutant strain may represent functional redundancy in Fg binding, contributed by other Fg-binding adhesins. This is not likely to include Fss1, however, as we detected no surface expression of Fss1 using whole-cell ELISA with affinity-purified Fss1-specific antibodies when OG1RF was grown in the presence or absence of serum to several time points, representing early, mid-exponential and late-exponential growth stages (data not shown). Consistent with this observation, inactivation of the *fss1* gene in OG1RF did not result in significant reduction in Fg adherence under the conditions tested (S. R. Nallapareddy, J. Sillanpää, M. Höök & B. E. Murray, unpublished results), and a divergent homologue of *fss3* is present as a pseudogene in the *E. faecalis* strain used for genetic manipulations. It is therefore likely that *E. faecalis* contains additional Fg-binding adhesins beyond the MSCRAMMs described here.

In conclusion, we have identified three Fg-binding MSCRAMMs, Fss1, Fss2 and Fss3, from *E. faecalis*. The three proteins are considerably larger than previously described staphylococcal and enterococcal MSCRAMMs, but they share a similar predicted domain organization with predicted IgG-like folded domains. We further identified shorter, Fg-binding regions in these proteins that show structural similarity to the Fg-binding regions in staphylococcal MSCRAMMs and are similarly composed of mostly  $\beta$ -sheets. Finally, disruption of *fss2* gene expression in *E. faecalis* resulted in reduced Fg adherence, which could be restored by complementation.

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