

Carboxypeptidase activity common to viridans group streptococci cleaves angiotensin I to angiotensin II: an activity homologous to angiotensin-converting enzyme (ACE)

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We have found that *Streptococcus gordonii* FSS2, an infective endocarditis (IE) isolate, expresses a dipeptidyl-carboxypeptidase with activity homologous to angiotensin-converting enzyme (ACE). The carboxypeptidase activity was purified to homogeneity as a complex/aggregate from a bacterial surface extract and was also active as a 165 kDa monomer. The specific activity for the carboxypeptidase activity was eightfold higher than that for recombinant human ACE. Selected ACE inhibitors, captopril, lisinopril and enalapril, did not inhibit the ACE activity. The carboxypeptidase also hydrolysed the A α and B β -chains of human fibrinogen, which resulted in impaired fibrin formation by thrombin. The gene encoding ACE carboxypeptidase activity was sequenced and the inferred polypeptide product showed 99% amino acid homology to SGO_0566, *sgc*, 'challisin' of *S. gordonii* CL1 Challis, and had no significant amino acid sequence homology to human ACE. Homologues of challisin ACE activity were commonly detected among the viridans group streptococci most often associated with IE.

INTRODUCTION

Viridans group streptococci remain important aetiological agents of infective endocarditis (IE) associated with native valves (Barrau *et al.*, 2004; Moreillon & Que, 2004). Damage to the endothelial lining of the heart valve is a prerequisite for the deposition of platelets and fibrin that form a nidus for infecting bacteria. Several microbial surface components have been implicated in the initial colonization through interactions with platelets (Petersen *et al.*, 2010; Kerrigan *et al.*, 2007; Bensing *et al.*, 2004; Takahashi *et al.*, 2004) and other mammalian components (Stinson *et al.*, 2003; Jakubovics *et al.*, 2005). Extensive evaluation of *Streptococcus sanguinis* failed to identify cell-wall-anchored proteins that were critical for this initial process (Turner *et al.*, 2009). Streptococcal proteins such as enolase and glyceraldehyde-3-phosphate dehydrogenase, described as cytoplasmic enzymes, have also been identified as bound to the cell wall and secreted as plasminogen-binding proteins (Cork *et al.*, 2009; Kinnby *et al.*, 2008;

Nelson *et al.*, 2001). In lactobacilli, enolase and glyceraldehyde-3-phosphate dehydrogenase are bound to lipoteichoic acid (LTA) when the external pH is acidic and are released under higher-pH conditions (Antikainen *et al.*, 2007). Other studies have shown that genes thought to be important in IE are induced during a shift from acidic conditions to neutral pH (Vriesema *et al.*, 2000). Surface-associated and secreted peptidase activities have also been detected and characterized from an IE isolate of *Streptococcus gordonii*. Amino- and dipeptidyl-peptidases have been shown to significantly cleave important peptides and proteins that may have significance in IE (Goldstein *et al.*, 2001, 2002, 2005), and have also been identified as being multifunctional, acting as binding proteins and peptidases (Chaudhuri *et al.*, 2008).

We have investigated cell wall-associated proteins which can be released by agitation in Tris buffer. We identified, purified and characterized a dipeptidyl-carboxypeptidase with potent activity against the peptide angiotensin (Ang) I. The implications of this novel activity for IE are discussed.

Abbreviations: ACE, angiotensin-converting enzyme; AMC, 7-amido-4-methylcoumarin; Ang, angiotensin; IE, infective endocarditis; PPP, platelet-poor plasma; PRP, platelet-rich plasma; RAS, renin-angiotensin system.

The GenBank/EMBL/DDBJ accession number for the SGO_0566 sequence of *S. gordonii* FSS2 is JF504645.

Three supplementary figures and a supplementary table are available with the online version of this paper.

METHODS

Bacterial strains and growth conditions. All general chemicals were obtained from either Sigma-Aldrich or Merck. *S. gordonii* FSS2 has been described previously (Harty *et al.*, 2000, 2004). All other

streptococcal strains were from the Institute's collection of type strains. All strains were stored as 25 % (v/v) glycerol stocks at -80°C and were plated for purity on Columbia (Oxoid) 5 % horse blood agar plates before being used for inoculation. Streptococcal strains were grown in Brain Heart Infusion (Oxoid) 0.5 % (w/v) yeast extract (Oxoid) broths (BHY), a chemically defined medium (CDM) or in a 100 mM HEPES (Sigma), 100 mM phosphate-buffered defined medium at pH 7.4 (HPCDM) adapted from CDM medium as described previously (Harty *et al.*, 2000). Cultures for enzyme activity determinations (BHY or HPCDM) were grown in 10 ml volumes in a candle jar for 18 h at 37°C and harvested by centrifugation (3000 g, 15 min, 4°C), spent medium was drained off (supernatants filtered to 0.2 μm , Millipore), and the cells were resuspended to OD_{600} 1.0 in the required buffer.

The effect of growth medium (BHY or CDM) with or without additional buffering (100 mM HEPES) was used to examine surface enzyme activity and the extraction of activity from the surface. Bacterial surface protein was extracted by shaking vigorously by hand for 10 min and centrifugation as described above. The efficiency of protein extraction was examined in several buffers (PBS, Tris, HEPES and phosphate) at different pHs (pH 7–8) and molarities (50–100 mM) and with sodium chloride (0–150 mM).

Cell surface protein extraction and purification. Cultures for protein purification (HPCDM, 4×500 ml) were inoculated with the pelleted cells (3000 g, 5 min, room temperature) from 4×10 ml BHY (medium pre-warmed and reduced overnight), grown for 8 h to mid-exponential phase in a candle jar at 37°C and resuspended in 1 ml HPCDM for inoculation. HPCDM cultures were grown for 18 h in an anaerobic cabinet (Don Whitley Scientific) and harvested by centrifugation (13 000 g, 15 min, 4°C). Bacterial pellets were drained of spent medium and resuspended in 200 ml 100 mM Tris, pH 8.0. Extraction of surface protein for enzyme purification was carried out by vigorous shaking by hand for 10 min. This process was repeated twice to give a pooled extract volume of 600 ml at a concentration of $34.3 \mu\text{g ml}^{-1}$, which was filtered to 0.45 μm (Millipore). The extract was concentrated approximately 15-fold, to $408.8 \mu\text{g ml}^{-1}$ on 30 kDa Centricon Plus-70 centrifugal membrane concentrators (Millipore), before being applied to a 4 % native polyacrylamide gel (Protean II, Bio-Rad, 1.5 mm thick, 16 cm wide and 4 cm high). A 20–25 ml volume of the concentrated extract, mixed with $10 \times$ native sample buffer [with 5 % (v/v) mercaptoethanol] was loaded onto the gel and run at 110 V overnight. The top 2 mm of native gel was excised and 5 ml 50 mM Tris, pH 8.0, added and sonicated (Bransonic 12 ultrasound bath) on ice for 15 min. The supernatant was removed, the gel extraction procedure was repeated and the supernatants were pooled. The pooled extracts were applied to a Centricon 100 kDa centrifugal membrane (Millipore), concentrated to no less than 4 ml (to limit further aggregation of the protein) and resuspended thoroughly by pipette several times in 50 mM Tris, pH 8.0, which removed trace low-molecular-mass contaminants. The purified protein was identified by MS (Sydney University) from a band excised from a 7.5 % reducing SDS-PAGE gel. Protein concentration was determined by Bradford assay (Pierce) with BSA as standard.

Protein aggregation. The state of the FSS2 chollisin aggregate/complex was investigated by both performing the cell extraction procedure and incubating extracts in the presence of a series of additives: EDTA, NaCl, KCl, CaCl_2 , ammonium sulphate, sodium sulphate, urea, NP-40, Tween 20, Triton X-100, inositol, sorbitol, glycerol, glucose and ethylene glycol (Bondos & Bicknell, 2003), at several different concentrations, and analysing the aggregation state by native PAGE.

Native PAGE zymography. Analytical zymography was performed on native PAGE gels (4 % stacking and 7.5 % resolving, mini-Protean

III, Bio-Rad) by first washing the gel briefly in water after electrophoresis and then twice in 50 mM Tris, pH 8.0. The surface of the gel was then dried in air slightly before wetting the surface with fluorescently labelled substrate (250 μM in 50 mM Tris, pH 8.0), followed by incubation at 37°C for 5–10 min. Fluorescent bands were observed and digitally photographed under UV illumination at 302 nm. Gels were then briefly washed in water and stained for protein with Gelcode Blue Coomassie stain (Pierce).

SDS-PAGE zymography. Samples for SDS-PAGE gels (4 % stacking and 7.5 % resolving) were boiled for 5 min in SDS reducing sample buffer before loading. After electrophoresis the gels were briefly washed in water and then washed three times for 30 min with 2.5 % (v/v) Triton X-100 before being treated for zymography as described above. Protein standards (Novex Sharp Protein Standard, Invitrogen) were used for SDS-PAGE analysis.

Enzyme assays. Enzyme activity was monitored during analysis and purification by fluorescent substrate, Suc-Ala-Ala-Pro-Phe-AMC (s-AAPF-AMC, Bachem) (stock solution 50 mM in DMSO), in a Perkin Elmer LS50B fluorimeter. Briefly, assays were performed on cells (OD_{600} 1.0), culture supernatant or protein extract in 50 μl 50 mM Tris, pH 8.0, mixed with 50 μl substrate and incubated at 37°C in a water bath for varying times. The reaction was stopped by the addition of 100 μl 0.5 M sodium carbonate buffer, pH 10.5, and the mixture was transferred to a microtitre plate for measurement in the fluorimeter. All assays were performed in triplicate. Activities were determined relative to a standard curve of 7-amido-4-methylcoumarin (AMC; Sigma) in carbonate buffer, pH 10.5. The kinetic parameter K_m for s-AAPF-AMC as substrate was determined in triplicate from Lineweaver–Burk plots of initial velocity for varying concentrations of substrate and 100–200 ng enzyme.

HPLC analysis. Analysis of the activity of streptococcal surface extracts and enzyme preparations against native peptides was performed by HPLC using an AKTA purifier system (GE Healthcare) with a Symmetry C18, 3.5 μm , 2.1×50 mm reverse-phase column (Waters) with UV detection at 254 nm. Of the peptides studied, Ang I, II, III and IV, kallidin and bradykinin were obtained from Bachem, while neurotensin, dynorphin A and insulin beta-chain were obtained from Sigma. Substrate (stock 50 mM in DMSO) at various working concentrations (200–500 μM) in 250 μl PBS, including 0.9 mM calcium chloride and 0.49 mM magnesium chloride, pH 7.4 (PBSC), was added to streptococcal cells (50 μl , OD_{600} 1.0 in PBSC), culture supernatant, protein extract or purified enzyme (100–200 ng) in 50 μl PBSC in a water bath at 37°C for 15 min in duplicate with a peptide control. The enzyme reaction was terminated by the addition of trifluoroacetic acid (TFA; Sigma) to 0.1 % (v/v), then stored on ice until analysed. Cell-based assays were stopped on ice, filtered to 0.2 μm (Millipore) and made up to 0.1 % (v/v) TFA. A 200 μl volume was applied to the HPLC column. Peptides were eluted with a linear gradient of 18 M Ω water, 0.1 % (v/v) TFA to 30 % acetonitrile (with the exception of insulin beta-chain to 35 %), 0.08 % (v/v) TFA in 6 ml at 0.3 ml min^{-1} . Confirmation of the identity of peptide peaks was by comparison with the peak retention times of peptide standards (Ang I and Ang II) and confirmation by N-terminal sequencing of the product peak fractions (Australian Proteome Analysis Facility). Enzyme activity was calculated by measuring the reduction in area under the peak for Ang I relative to the peak area for the control peptide. The kinetic parameter apparent K_m (K_{mapp}) was determined from Lineweaver–Burk plots for varying concentrations of substrate and 100–200 ng enzyme.

Proteinase activity. Human or rat fibrinogen (Sigma) hydrolysis was investigated by reducing and non-reducing 10 % SDS-PAGE. Human or rat fibrinogen, 5 μg , was mixed with 100 ng enzyme and 50 mM Tris, pH 8.0, 5 mM CaCl_2 in a total volume of 25 μl , and incubated for 5, 15, 30 or 60 min. The reaction was stopped by the addition of SDS

reducing sample buffer and boiling for 5 min. Fibrinogen (1 ml at a concentration of 2.5 or 1.0 mg ml⁻¹) was also incubated, in duplicate, with a separate 50 mM Tris, pH 8.0, blank and fibrinogen control, in plastic cuvettes in a six-cell Peltier temperature-controlled cuvette holder (Beckman DU640 spectrophotometer). After reaching temperature, 100 ng enzyme was added and incubated for 1 h at 37 °C. Human thrombin (Sigma, 0.4 U) was then added and fibrin formation observed at OD₄₅₀ for 20 min. Human serum albumin or BSA, transferrin, lactoferrin, fibronectin, α -1 acid glycoprotein and thrombin (1 μ g) were incubated with 100 ng FSS2 challisin for 1 h at 37 °C and analysed by SDS-PAGE as described above.

Platelet aggregation. Platelet aggregation was carried out as previously described (Harty *et al.*, 1993) with platelet-rich plasma (PRP) and platelet-poor plasma (PPP) prepared from a fresh blood sample collected and mixed 9:1 (v/v) with 0.1 M trisodium citrate. Purified FSS2 challisin (300 ng) or an equal volume of PBS as a control was added to 250 μ l PRP in a Chronolog dual-channel aggregometer and incubated for 30 min at 37 °C. ADP (20 μ M final concentration) was added at the end of this time to confirm platelet function. Ang I (50 μ M) was also added to PRP for 5 min before the addition of 300 ng FSS2 challisin and further incubated for 30 min before addition of ADP to confirm platelet function.

DNA sequencing. SGO_0566 and surrounding 5' and 3' regions were amplified by PCR (KOD Hot Start polymerase, Novagen) from *S. gordonii* FSS2 genomic DNA and the PCR product was purified (MoBio PCR Clean-Up kit). Restriction enzyme analysis (*Nde*I and *Nhe*I, New England Biolabs) of the PCR product indicated that there were differences at the DNA level from the published *S. gordonii* CL1 Challis SGO_0566 DNA sequence. Therefore, the entire PCR product was sequenced (Supplementary Table S1).

RESULTS

Identification, extraction and purification of cellular enzyme activity

Initial analysis of peptidase activities from *S. gordonii* FSS2 with fluorescently labelled substrates identified a strong activity against a carboxypeptidase Y and chymotrypsin-like substrate, sAAPF-AMC, from both cells and culture supernatants. The putative substrate cleavage site, Pro-Phe- \downarrow , indicated a possible carboxypeptidase activity against Ang I (Asp-Arg-Val-Tyr-Ile-His-**Pro-Phe**-His-Leu), which would convert it to the important bioactive peptide Ang II (Asp-Arg-Val-Tyr-Ile-His-**Pro-Phe**). The majority of the sAAPF-AMC activity could be detected on the bacterial surface, where it could be released simply by vigorous shaking by hand in 100 mM Tris, pH 8.0. Removal of sAAPF-AMC activity from the bacterial surface was pH-dependent, as buffers (PBS, Tris, HEPES or potassium phosphate) below pH 8.0 and lower buffer molarity (50 mM) reduced the total activity recovered. The pH after surface extraction in 100 mM Tris, pH 8.0, was reduced to pH 7.6. Increasing sodium chloride concentration (5–150 mM) reduced the concentration of protein released from the bacterial surface (20.19 \pm 2.8 to 14.25 \pm 1.3 μ g ml⁻¹). Analytical 4% native PAGE zymography (Fig. 1, lanes 1 and 2) showed that all activity with sAAPF-AMC substrate was concentrated in the first few millimetres of the native gel. Supernatant from HPCDM

cultures also indicated that activity was present in at least two molecular forms (Fig. 1, lane 3). All treatments to prevent complex formation or to disperse putative complexes were unsuccessful (see Methods). Higher-molecular-mass forms increased markedly with increasing protein concentration in both the initial extraction mixture and the purified protein solutions (data not shown). Analysis by SDS-PAGE with a boiled and reduced cell extract or 10 \times concentrated supernatant indicated a 165 kDa protein that could be renatured to an active enzyme by incubation with Triton X-100 (Fig. 1, lanes 4, 5 and 6). Large-format native PAGE gels were used to separate the high-molecular-mass fraction from contaminating proteins. The top 2–3 mm of the native gel was excised and the activity released from the acrylamide gel by mild sonication. Processing of the extracted sample through a 100 kDa centrifugal membrane removed trace low-molecular-mass contaminants, leaving a homogeneous pure protein (Fig. 1, lanes 7 and 8, Table 1). Analysis by MS (25 peptides matched, 16% coverage) against the sequenced *S. gordonii* Challis CH1 genome identified the protein band as SGO_0566, identified before as the serine peptidase challisin (*sgc*) (Wang & Kuramitsu, 2005).

Enzyme activity against human protein and peptide substrates

The effect of purified FSS2 challisin on human peptides was investigated by HPLC. Cells and culture supernatant were able to degrade Ang I (results not shown). The product peptide peaks resulting from purified FSS2

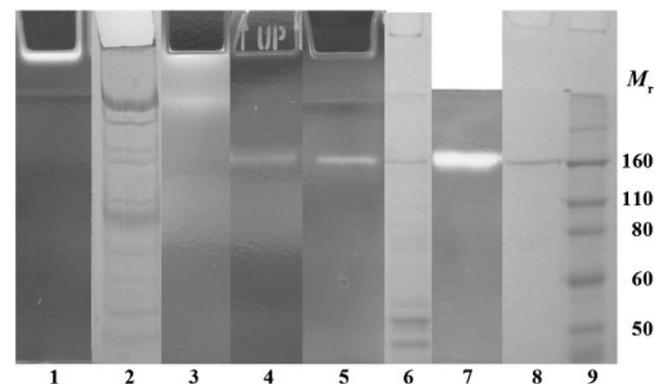


Fig. 1. Native and 7.5% SDS-PAGE purification of *S. gordonii* FSS2 challisin. Lanes: 1, native PAGE of cell extract incubated with sAAPF-AMC; 2, native PAGE of cell extract stained with Coomassie blue; 3, native PAGE of culture supernatant incubated with sAAPF-AMC; 4, SDS-PAGE of culture supernatant incubated with sAAPF-AMC; 5, SDS-PAGE of cell extract incubated with sAAPF-AMC; 6, SDS-PAGE of concentrated cell extract stained with Coomassie blue; 7, purified FSS2 challisin (1 μ g), SDS-PAGE incubated with sAAPF-AMC; 8, purified FSS2 challisin (1 μ g), SDS-PAGE stained with Coomassie blue; 9, Novex Sharp Protein Standards (Invitrogen).

Table 1. Purification of *S. gordonii* FSS2 challisin with sAAPF-AMC substrate

Procedure	Volume (ml)	Total protein (mg)	Total activity (nmol min ⁻¹)	Specific activity (nmol µg ⁻¹ min ⁻¹)	Yield (%)	Purification (fold)
Cell extract	600	20.06	52 333	2.6	100	1
30 kDa membrane	42	17.17	61 667	3.6	117.8	1.4
Native gel extract	9	0.283	7133	25.2	13.6	9.7
100 kDa membrane	4.5	0.177	5117	28.9	9.8	11.2
Ang I as substrate				8.16		

challisin activity were N-terminal-sequenced in their entirety to confirm the removal of the C-terminal His-Leu dipeptide (found as a dipeptide peak) and the formation of Ang II (Fig. 2). No further degradation was observed to Ang III, IV or Ang 1–7 on extended incubation with purified FSS2 challisin, HPCDM-grown cells or culture supernatant. FSS2 challisin was also unable to degrade other important peptides such as kallidin, bradykinin, neurotensin, dynorphin A and the beta-chain of insulin (results not shown). Streptococcal cells and crude extracts could, however, degrade kallidin and bradykinin (results not shown).

The specific activity of purified FSS2 challisin with Ang I as substrate, 8.16 nmol min⁻¹ µg⁻¹, was calculated from the reduction in peak area as compared with the peak area of the Ang I control (Fig. 2). FSS2 challisin activity (150 ng) was not affected by the ACE inhibitors captopril, lisinopril and enalapril, at concentrations up to 1 mM. The protease inhibitors aprotinin, leupeptin and PMSF inhibited activity significantly at 200 µM. EDTA and EGTA did not inhibit activity. The divalent metal ions (1 mM) calcium, zinc, nickel, magnesium and cobalt were stimulatory, copper ions had no effect, while manganese, iron and mercury were all inhibitory (Table 2). We also investigated the

kinetic parameter K_m for both substrates. The kinetic constant K_m determined from Lineweaver–Burk plots of the initial enzyme velocity for sAAPF-AMC was high at 1.09 ± 0.08 mM (Supplementary Fig. S1a), while the K_{mapp} with Ang I as a substrate, estimated from the Ang I peak area, was 176.4 ± 0.8 µM (Supplementary Fig. S1b).

Analysis of the effect of FSS2 challisin activity against fibrinogen disclosed that both the α - and β -chains of human and rat (data not shown) fibrinogen were cleaved within 5 min of incubation when examined by reducing SDS-PAGE (Fig. 3). Under non-reducing conditions, no fibrinogen fragments could be detected by SDS-PAGE (data not shown). Incubation of human fibrinogen with FSS2 challisin for 1 h also significantly reduced fibrin formation, as observed by a change in OD₄₅₀, from fibrinogen by human thrombin (Fig. 4). Incubation of FSS2 challisin (100 ng) with human thrombin (1 µg) for 1 h and analysis by SDS-PAGE indicated that neither enzyme was able to degrade the other. FSS2 challisin was also unable to degrade any of the other proteins examined (see Methods, results not shown).

FSS2 challisin, when incubated with PRP with or without pre-incubation with Ang I, induced no detectable shape change or aggregation response in platelets (data not shown).

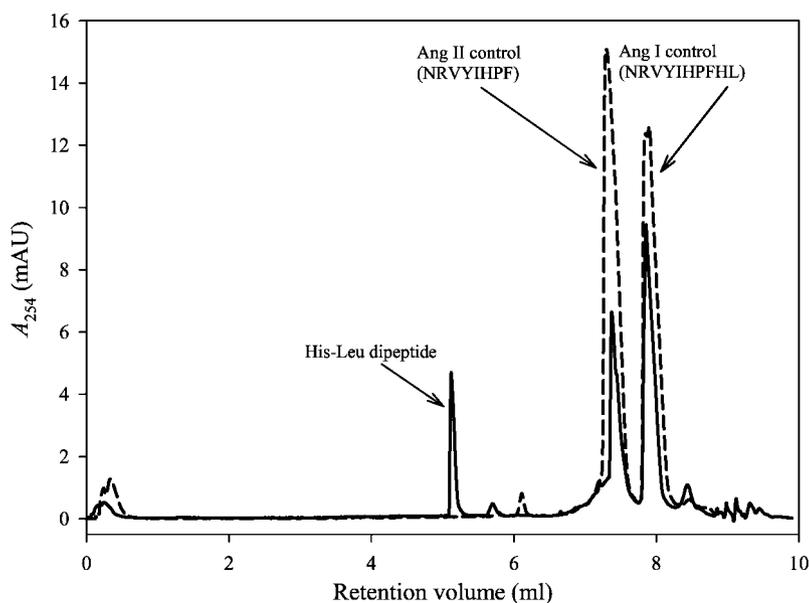


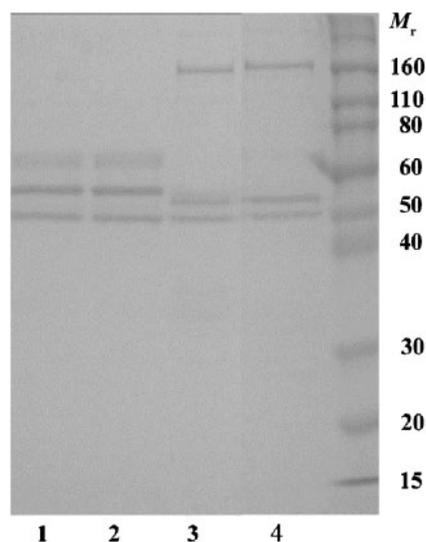
Fig. 2. HPLC analysis of FSS2 challisin activity against Ang I. Ang I (41.7 nmol) was incubated with 125 ng FSS2 challisin at 37 °C for 15 min in PBSC (solid line). Ang I and Ang II controls were incubated as above (dashed line).

Table 2. Effect of additives on the relative activity of purified FSS2 challisin (150 ng) with sAAPF-AMC and Ang I

Inhibitor	Concentration (mmol l ⁻¹)	Relative activity (%)
Control	0	100*
Aprotinin	0.2	25.5
	0.1	42.0
Leupeptin	0.2	54.2
	0.02	79.9
PMSF	0.2	0.0
	0.02	79.7
TLCK†	4.0	96.3
EDTA	10	115.0
EGTA	10	106.8
Ca ²⁺	1.0	243
Zn ²⁺	1.0	213
Ni ²⁺	1.0	229
Mg ²⁺	1.0	218
Co ²⁺	1.0	158
Cu ²⁺	1.0	109
Mn ²⁺	1.0	89.9
Fe ²⁺	1.0	39.6
Hg ²⁺	1.0	27.7
Captopril (Ang I)	1.0	100
Lisinopril (Ang I)	1.0	100
Enalapril (Ang I)	1.0	100

*28.9 nmol µg⁻¹ min⁻¹.

†N-Tosyl-lysyl chloromethyl ketone.

**Fig. 3.** Degradation of human fibrinogen by FSS2 challisin from *S. gordonii* FSS2 as determined by 10% SDS-PAGE. Lanes: 1, human fibrinogen (5 µg); 2, human fibrinogen control, 5 min; 3, human fibrinogen, 100 ng FSS2 challisin, 5 min; 4, human fibrinogen, 100 ng FSS2 challisin, 60 min; 5, Novex Sharp Protein Standards (Invitrogen).

Homologous enzyme activity in streptococcal species

A selection of streptococcal type strains, *S. sanguinis* NCTC7863, *Streptococcus parasanguinis* ATCC15912, *S. gordonii* ATCC10558, *Streptococcus oralis* NCTC11427, *Streptococcus mitis* NCTC12261, *Streptococcus mutans* NCTC10449 and *Streptococcus pyogenes* NCTC8198, were also tested for activity with sAAPF-AMC and Ang I. All streptococci except *S. mutans* NCTC10449 and *S. pyogenes* NCTC8198 had activity against sAAPF-AMC relative to *S. gordonii* FSS2 when cultured in either BHY or HPCDM media. HPCDM cells from all strains had some challisin-like activity against Ang I as substrate; however, *S. oralis* NCTC11427 (13.5%), *S. pyogenes* NCTC8198 (11.5%) and *S. mutans* NCTC10449 (6.8%) had low activity relative to *S. gordonii* FSS2 (100%) (Table 3). A BLAST (GenBank) comparison of the translated amino acid sequence of *S. gordonii* FSS2 (GenBank accession number JF504645) with the sequences for a selection of streptococci showed high homology amongst the oral streptococci. However, no homologous protein was found in *S. mutans*. A Pfam and CLUSTAL W analysis of sequence alignments with the *S. gordonii* FSS2 challisin amino acid sequence identified an N-terminal FSIRK signal sequence, indicating a secreted protein, an S8 subtilisin-like peptidase domain, with conserved D₂₁₀, H₂₇₆ and S₆₀₄ active site residues, a protease-associated (PA) domain, a domain of unknown function, DUF1034, and three found in various architectures (FIVAR) domain and putative carbohydrate-binding domains (Supplementary Figs S2 and S3). While all the species examined shared the same catalytic triad, the number of FIVAR domains detected by Pfam varied among the species, while *S. mitis*, *Streptococcus suis* and *S. pyogenes* all had an LPXTG Gram-positive cell wall anchor motif (Supplementary Fig. S2).

DISCUSSION

We have identified, by N-terminal sequencing, a cell surface and secreted peptidase with ACE-like activity in *S. gordonii* FSS2. Sequencing of the *S. gordonii* FSS2 gene responsible for this ACE-like activity indicates that while there are some differences at the DNA level, there is 99% homology with the *S. gordonii* CL1 Challis challisin amino acid sequence (see Supplementary Fig. S2). Our findings show therefore that *S. gordonii* FSS2, and several other streptococcal species, express a dipeptidyl-carboxypeptidase activity able to degrade Ang I to the peptide hormone Ang II, an activity homologous to angiotensin-converting enzyme (ACE). Only one bacterial genus, *Xanthomonas*, has positively been identified to possess an ACE homologue, while *in silico* analysis has identified bacterial strains within eight genera potentially encoding putative ACE-like enzymes (Rivière *et al.*, 2007). The purified homogeneous FSS2 challisin specific activity (8160 pmol min⁻¹ µg⁻¹) against Ang I is at least eightfold higher than the specific

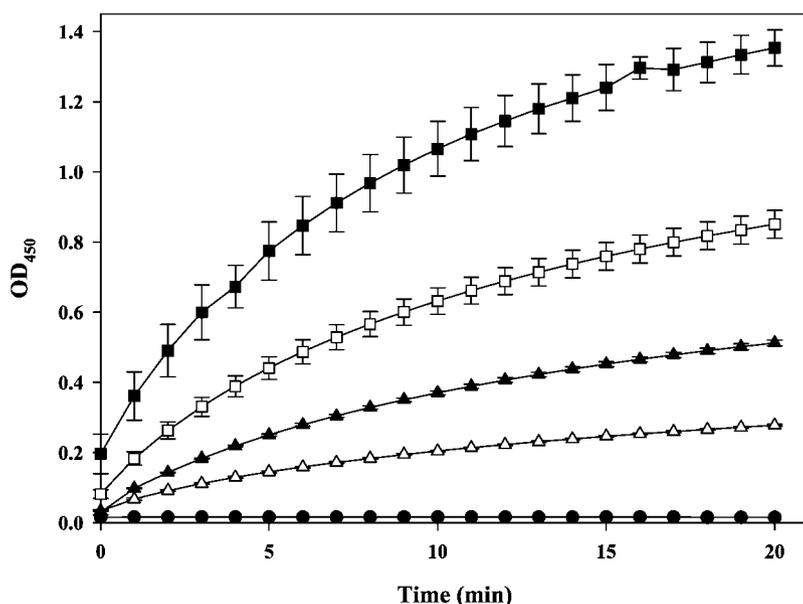


Fig. 4. Fibrin formation by thrombin (0.4 U) from human fibrinogen observed as change in OD_{450} , after incubation with 100 ng FSS2 challisin for 1 h at 37 °C. ●, Fibrinogen control (2.5 mg ml⁻¹); ■, 2.5 mg fibrinogen ml⁻¹, 0.4 U thrombin; □, 2.5 mg fibrinogen ml⁻¹, 100 ng FSS2 challisin, 0.4 U thrombin; ▲, 1.0 mg fibrinogen ml⁻¹, 0.4 U thrombin; △, 1.0 mg fibrinogen ml⁻¹, 100 ng FSS2 challisin, 0.4 U thrombin.

activity of recombinant human ACE [1000 pmol min⁻¹ μg⁻¹ (R&D Systems); 800 pmol min⁻¹ μg⁻¹ (Merck)].

The lack of any observed inhibition with ACE inhibitors indicates a different mode of action. Human recombinant ACE is a zinc-dependent M2-metallo-dipeptidyl-carboxypeptidase (Rivière *et al.*, 2007), while FSS2 challisin also behaves as a dipeptidyl-carboxypeptidase, with no absolute requirement for metal ions, as neither EDTA nor EGTA inhibits activity. Divalent metal ions do, however, promote a more than twofold increase in enzyme activity (Table 2). Human ACE has a wide substrate specificity and can hydrolyse not only Ang I but bradykinin, neurotensin and enkephalins (Bernstein *et al.*, 2011). No activity of FSS2 challisin against bradykinin and neurotensin was detected.

Human ACE has two catalytic domains, N- and C-terminal, and both contain the HEXXH motif indicative of a zinc-binding site which is critical for enzyme activity (Bernstein *et al.*, 2011). FSS2 challisin, as a serine S8 subtilisin-like peptidase, has a very different catalytic triad, D₂₁₀, H₂₇₆ and S₆₀₄ (Supplementary Fig. S2) and no requirement for zinc.

S. gordonii CL1 challisin has previously been identified as a secreted serine peptidase responsible for the inactivation of competence-stimulating peptide (CSP) released from *S. mutans* (Wang & Kuramitsu, 2005). The amino acid sequence of the CSP used in that study (SGSLSTFFR LFNRSFTQALGK) has no similarity to the sequence for Ang I (DRVTIHPFHL), giving no indication as to the

Table 3. Cellular FSS2 challisin activity with sAAPF-AMC or Ang I as substrate: relative fluorescence or total activity as compared with *S. gordonii* FSS2

Organism	Substrate: Growth medium:	Relative fluorescence (%)		Relative total activity (%)
		sAAPF-AMC BHY	sAAPF-AMC HPCDM	Ang I HPCDM
<i>S. gordonii</i> FSS2		100*	100†	100‡
<i>S. gordonii</i> ATCC10558		40.9	45.8	58.8
<i>S. sanguinis</i> NCTC7863		28.0	24.5	21.3
<i>S. parasanguinis</i> ATCC15912		44.7	47.8	80.3
<i>S. mitis</i> NCTC12261		26.8	27.7	55.6
<i>S. oralis</i> NCTC11427		78.3	12.4	13.5
<i>S. mutans</i> NCTC10449		2.9	0	6.8
<i>S. pyogenes</i> NCTC8198		1.8	0	11.6

*194.5 fluorescence units.

†485.8 fluorescence units.

‡18.63 nmol h⁻¹.

mechanism of its activity towards CSP (Wang & Kuramitsu, 2005). Challisin has also been identified both as a high-molecular-mass (165 kDa) single isoform and as lower-molecular-mass (76 kDa) isoelectrically heterogeneous isoforms in 2D-PAGE studies (Davies *et al.*, 2009). The amino acid sequence translated from our sequencing indicates a calculated molecular mass of 164 547 Da and a pI of 5.2. Purified high-molecular-mass FSS2 challisin dissociates during SDS-PAGE analysis to resolve as a single protein band (Fig. 1, lane 8), indicating that other low-molecular-mass isoforms are not present as part of the high-molecular-mass complex. Furthermore, no low-molecular-mass enzymically active isoforms were identified in SDS-PAGE zymograms of cell extracts or culture supernatants (Fig. 1, lanes 4 and 6), indicating that the low-molecular-mass isoforms observed in 2D-PAGE (Davies *et al.*, 2009) are not part of the high-molecular-mass complex and therefore may be degradation products.

Challisin-like homologues are widely found amongst the oral streptococci, particularly those species associated with IE (Table 3, Supplementary Fig. S2). Challisin-like activities were detected, with both fluorescent and peptide substrates, from the streptococcal type strains (Table 3). No sequence homology for FSS2 challisin could be detected in *S. mutans*, confirming the lack of significant activity in enzyme assays (Table 3). A subtilisin-like protease from *S. suis* has been cloned and the recombinant protein characterized as a gelatin- and fibrinogen- (α -chain) degrading protease that also prevents thrombin-mediated fibrin formation and has toxicity to microvascular endothelial cells (Bonifait *et al.*, 2011). FSS2 challisin is also able to degrade the α - and additionally the β -chain of human and rat fibrinogen (Fig. 3). This degradation of fibrinogen significantly reduces fibrin formation by thrombin (Fig. 4). *Finegoldia magna*, a member of the normal flora of the skin, has a cell-surface and secreted subtilisin, SufA, which is also active against fibrinogen and prevents fibrin formation (Karlsson *et al.*, 2009). SufA rapidly cleaves fibrinogen in plasma in the low nanomolar range and cleaves both the α - and β -chains of fibrinogen in solution in a similar manner to FSS2 challisin (Karlsson *et al.*, 2009).

Addition of FSS2 challisin alone or with added Ang I to platelets did not induce a detectable platelet shape change or a significantly changed aggregation response on addition of the agonist ADP. The increased local production of Ang II by FSS2 challisin around platelets should have initiated a response either as platelet shape change (Jagroop & Mikhailidis, 2000) or aggregation (Senchenkova *et al.*, 2010). However, fibrinogen fragments have been shown to significantly inhibit ADP-induced platelet aggregation (Kozek-Langenecker *et al.*, 1999), and it is possible that the formation of Ang II and fibrinogen fragments by FSS2 challisin may be competing with each other in this aggregation assay.

The S8 subtilisin peptidase domain and the active site triad are highly conserved across streptococcal species

(Supplementary Figs S2 and S3). Pfam database analysis reveals some heterogeneity in the FIVAR domains and the presence of LPXTG Gram-positive cell-wall-anchor motifs in *S. mitis*, *S. suis* and *Streptococcus agalactiae*, indicating that in these species challisin-like homologues may be covalently bound to the cell wall (Supplementary Fig. S2).

The bioactive peptides of the renin-angiotensin system (RAS), particularly Ang II, interact with receptors on the surface of endothelial cells and platelets, inducing platelet shape change and aggregation (Jagroop & Mikhailidis, 2000; Senchenkova *et al.*, 2010; Watanabe *et al.*, 2005). Ang II is also a potent regulator of plasminogen activator inhibitor type 1 (PAI-1), which plays a key role in the regulation of thrombosis (Watanabe *et al.*, 2005) and has also been found to mediate the inflammatory response in lung tissue exposed to lipopolysaccharide (Wösten-van Asperen *et al.*, 2010).

It is now accepted that apart from the classical RAS, local tissue RASs are common (Carey & Siragy, 2003). Local RASs have been identified in most organs and tissues and include prorenin/renin receptors for the generation of Ang I from angiotensinogen. These local RASs are abundant in heart, brain and placenta (Fyhrquist & Saijonmaa, 2008). For instance, cardiac interstitial fluid concentrations of Ang I are more than 100-fold those of plasma. Endothelium also expresses an RAS, although there is uncertainty whether the renin component is synthesized or sequestered from plasma sources (Carey & Siragy, 2003). On this basis, there is an indication of local availability of Ang I as a substrate for FSS2 challisin. It is therefore proposed that in endocarditis, the ACE activity of FSS2 challisin functions as an exogenous agonist of the RAS that is not subject to physiological feedback mechanisms.

Cleavage of fibrinogen by FSS2 challisin resulted in cleavage patterns that were discerned only under reducing conditions, implying scission between disulphide bonds linking α - and β -chains to the respective partners. In a preliminary analysis we were unable to detect free peptide fragments equivalent to the fibrinopeptides. Accordingly, the data indicate that reduced formation of fibrin clot following digestion with FSS2 challisin is related to nicks in the C-terminal regions of α - and β -chains. It is probable that a fibrin matrix that incorporates such cleaved fibrinogen molecules will have altered properties, including increased porosity, reduced elasticity and tensile strength, and altered susceptibility to lysis (Weisel, 2004). In this regard the characteristics of the fibrin matrix are critical determinants of the properties of a thrombus (Lord, 2011). For instance, a thrombus formed in the presence of active FSS2 challisin could be friable and susceptible to fragmentation, resulting in infective emboli characteristic of endocarditis (Deprèle *et al.*, 2004).

S. gordonii FSS2 challisin is therefore a novel dipeptidyl-carboxypeptidase with potent ACE-like and fibrinogen-degrading activity, and may therefore have a profound effect on both thrombosis and inflammation. Homologues

are commonly found in the streptococcal strains associated with IE and may function as important virulence factors.

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