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Opacity Factor Activity and Epithelial Cell Binding by the Serum Opacity Factor Protein of *Streptococcus pyogenes* Are Functionally Discrete

Abstract

Serum opacity factor (SOF) is a unique multifunctional virulence determinant expressed at the surface of *Streptococcus pyogenes* and has been shown to elicit protective immunity against GAS infection in a murine challenge model. SOF consists of two distinct domains with different binding capacities: an N-terminal domain that binds apolipoprotein AI and a C-terminal repeat domain that binds fibronectin and fibrinogen. The capacity of SOF to opacify serum by disrupting the structure of high density lipoproteins may preclude its use as a vaccine antigen in humans. This study generated mutant forms of recombinant SOF with reduced (100-fold) or abrogated opacity factor (OF) activity, for use as vaccine antigens. However, alterations introduced into the N-terminal SOF peptide (SOFFn) by mutagenesis to abrogate OF activity, abolish the capacity of SOF to protect against lethal systemic *S. pyogenes* challenge in a murine model. Mutant forms of purified SOFFn peptide were also used to assess the contribution of OF activity to the pathogenic processes of cell adhesion and cell invasion. Using latex beads coated with full-length SOF, SOFFn peptide, or a peptide encompassing the C-terminal repeats (FnBD), we demonstrate that adhesion to HEp-2 cells is mediated by both SOFFn and FnBD. The HEp-2 cell binding displayed by the N-terminal SOFFn peptide is independent of OF activity. We demonstrate that while the N terminus of SOF does not directly mediate intracellular uptake by epithelial cells, this domain enhances epithelial cell uptake mediated by full-length SOF, in comparison to the FnBD alone.

Keywords

Streptococcus, *Pyogenes*, Opacity, CMMB

Disciplines

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Opacity factor activity and epithelial cell binding by the serum opacity factor protein of *Streptococcus pyogenes* are functionally discrete

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Running Title: ApoAI and epithelial cell binding by SOF are functionally discrete

SUMMARY

Serum opacity factor (SOF) is a unique multi-functional virulence determinant expressed at the surface of *S. pyogenes* and has been shown to elicit protective immunity against GAS infection in a murine challenge model. SOF consists of two distinct domains with different binding capacities: an N-terminal domain which binds apolipoprotein AI, and a C-terminal repeat domain that binds fibronectin and fibrinogen. The capacity of SOF to opacify serum by disrupting the structure of high density lipoproteins may preclude its use as a vaccine antigen in humans. This study generated mutant forms of recombinant SOF with reduced (100-fold) or abrogated opacity factor (OF) activity, for use as vaccine antigens. However, the structural alterations introduced into the N-terminal SOF peptide (SOF Δ Fn) by mutagenesis to abrogate OF activity, abolish the capacity of SOF to protect against lethal systemic *S. pyogenes* challenge in a murine model. Mutant forms of purified SOF Δ Fn peptide were also used to assess the contribution of OF activity to the pathogenic processes of cell adhesion and cell invasion. Using latex beads coated with full-length SOF, SOF Δ Fn peptide or a peptide encompassing the C-terminal repeats (FnBD), we demonstrate that adhesion to HEp-2 cells is mediated by both SOF Δ Fn and FnBD. The HEp-2 cell binding displayed by the N-terminal SOF Δ Fn peptide is independent of OF activity. We demonstrate that while the N-terminus of SOF does not directly mediate intracellular uptake by epithelial cells, this domain enhances epithelial cell uptake mediated by full-length SOF, in comparison to the FnBD alone.

INTRODUCTION

Streptococcus pyogenes (group A streptococcus; GAS⁴) is an important human pathogen responsible for a wide variety of skin and mucosal infections ranging from pharyngitis and impetigo to more severe invasive infections, such as necrotizing fasciitis and streptococcal toxic shock-like syndrome (1-3). The serum opacity factor (SOF) is a large protein of ~110 kDa which is expressed at the cell surface by approximately half of all clinical isolates (4,5). Similar to a number of other surface proteins expressed by *S. pyogenes*, SOF binds fibronectin via a C-terminal repeated domain (FnBD) (6-8), a function that has been implicated in the adhesion of GAS to epithelial cells (9). In contrast to the conserved C-terminus, the N-terminus of SOF (SOF Δ Fn) is highly variable exhibiting ~55% identity between different serotypes of *S. pyogenes*. The N-terminal domain of SOF was originally thought to cleave apolipoprotein A1 (apoAI) in human serum leading to the precipitation of high-density lipoproteins (HDLs) (10,11). However, it has recently been demonstrated that the OF activity of SOF is not enzymatic; rather, the direct binding of apoAI by SOF triggers the release of the HDL lipid cargo of apoAI, initiating the opacity reaction (12). This OF domain of SOF promotes GAS invasion of epithelial cells (13), but it is not known whether the OF activity itself or a discrete domain within the N-terminus of SOF contributes to this phenotype. SOF is a virulence determinant of GAS, with insertional inactivation or allelic replacement of *sof* reducing mortality in an intraperitoneal and subcutaneous murine infection model (13,14). SOF is also a vaccine candidate - parenteral immunization of mice with SOF

protects against lethal intraperitoneal challenge (15).

It is not known what physiological effect that the precipitation of HDL would have upon the human host or how the interaction of SOF with apoAI contributes to the pathogenesis of GAS. ApoAI exerts a potent anti-inflammatory effect by preventing contact between infected T-cells and monocytes thereby inhibiting cytokine production (namely TNF-alpha and IL-1) in monocytes (16). *In vitro*, both HDL and apoAI exert anti-inflammatory effects against the potent bacterial endotoxins, Gram-negative LPS and Gram-positive lipoteichoic acid, binding strongly to both endotoxins and inhibiting the production of TNF- α (17-19). *In vivo*, transgenic animal studies of the toxicity of LPS have shown that expression of human *apoAI* transgenes protected mice from a lethal dose of LPS (20). ApoAI also possesses specific anti-bacterial and anti-viral properties (21-23).

The administration of active SOF protein as a vaccine when the downstream effects of disrupting HDL and its activity *in vivo* are unknown would not be recommended, as the most effective protection against GAS infection is delivered when the SOF protein is administered parenterally (15) and the localized depletion of HDL may reduce the body's defense against other pathogens or may result in inflammation at the site of immunization. Thus, before SOF could be used as a potential vaccine antigen (alone or as part of a multivalent vaccine formulation) it would be prudent to eliminate the OF activity of the protein. To this end, this study generated mutant forms of recombinant SOF protein with attenuated or eliminated OF activity, for use as vaccine formulations. These mutant SOF proteins are also been used to further delineate OF activity and cell binding activity within the N-terminus of SOF.

MATERIALS AND METHODS

Site directed mutagenesis - A pQE30 based vector encoding a fusion protein of residues 33–872 of SOF from a M75 GAS strain (lacking signal sequence and fibronectin binding repeat region, pSOF75 Δ Fn) (24), was used as the template for all mutagenesis reactions. Site directed mutagenesis was performed as previously described (25). Primers for site directed mutagenesis are given in supplementary material Table 1. Two mutagenesis

strategies were employed, amino acid residues were substituted with alanine (single residues up to 5 residues) and small deletions were made within rSOF75 Δ Fn. Deletions within rSOF75 Δ Fn were generated by using site directed mutagenesis to introduce two *AvrII* restriction sites flanking the region to be deleted, followed by digestion with *AvrII*. The digested fragments were then separated by agarose gel electrophoresis, and the DNA fragment containing the portion of pSOF75 Δ Fn of interest was extracted from the gel and re-ligated.

Expression and purification of wildtype and mutant forms of rSOF75 and rSOF75 Δ Fn protein - Large-scale expression and purification of rSOF75 proteins was conducted essentially according to manufacturer's instructions (Qiagen), and has been previously described (13,26). To ensure correct refolding of proteins was achieved, wildtype purified proteins were subsequently tested for opacity factor activity using an agarose overlay method (7).

Structural characterisation of wildtype and mutant forms of rSOF75 Δ Fn protein - To determine the structural integrity of mutant rSOF75 Δ Fn proteins in comparison to the wildtype, a comparison of the secondary structure of wildtype and mutant rSOF75 Δ Fn proteins was conducted using circular dichroism (CD) spectroscopy. CD spectra were acquired using a Jasco J-810 Spectropolarimeter (Jasco). Experiments were conducted at room temperature with proteins at a concentration of approximately 0.2 mg/ml in 10mM sodium phosphate buffer, pH 7.5 containing 50% trifluoroethanol (27,28). Far UV spectra were recorded from 190-250 nm in a 0.1 cm path length cell (Starna) containing 400 μ l of protein solution. The data shown represents an average of ten scans, corrected for a buffer baseline. Mean residue ellipticity (MRE; $[\theta]$) was calculated using the following formula (29):

$$\text{MRE } [\theta] = \frac{\theta \times 100 \times \text{molecular weight}}{\text{conc (mg/ml)} \times \text{path length} \times 1000 \times \text{no. of residues}}$$

The α -helical content of wildtype and mutant forms of rSOF75 Δ Fn was calculated from the

MRE value at 222 nm using the following equation as described by (30).

$$\% \alpha\text{-Helix} = \frac{\text{MRE}[\theta]_{222} - 2340}{30300} \times 100$$

Opacity Activity Assays – Qualitative opacity factor assays were conducted using the serum agarose overlay method (7). The serum overlay method permits visual confirmation of OF activity, as binding of apoA1 by SOF causes precipitation of apoA1 and HDL, which appears as an opaque white band on the solid serum/agarose medium. Data is presented as an inversion of the actual blot with opacity activity appearing as a dark band on a light background. Quantitative opacity factor assays were conducted using purified HDL or human serum using the method of Courtney *et al.* (12), with opacification measured as absorbance at 405nm.

ApoA1 binding capacity – Wells of a microtiter plate were coated with 20 µg/ml rSOF75 protein or gelatin in 0.01 M sodium bicarbonate for 1 h at 37°C. Plates were washed with PBS and blocked with gelatin (1 mg/ml in PBS) for 1 h at 37°C, 100 µl of biotinylated (Pierce) apoA1 (Calbiochem) was added and incubated at 37°C for 1 h. Following washing 100 µl of avidin-peroxidase (0.5 µg/ml) was added to the wells and incubated at 37°C for 1 h. The plates were washed and 3,3', 5,5'-tetramethylbenzidine substrate added. Colour development was stopped using 1 M phosphoric acid and the absorbance at 650nm was recorded. Binding was assayed in triplicate, binding to gelatin was considered non-specific and was subtracted from the readings.

Interaction of SOF coated latex beads with HEp-2 cells - Assays of the interaction of SOF coated latex beads with HEp-2 cells were conducted as per previously published methods (13,31,32). Preliminary assays were conducted in DMEM HEPES supplemented with either 10% FCS or 1% FCS. However, differential binding was observed between these two assay conditions, the rSOF75ΔFn only mediated binding to HEp-2 cells when incubated in DMEM HEPES 1% FCS, and thus these conditions were used for all further latex bead adherence assays. The efficiency of protein loading on to latex beads was measured by FLUOstar

fluorescent plate reader (BMG Labtech) using anti-SOF75 rabbit serum and fluorescent labeling with goat anti-rabbit Alexa 488 (green) (Molecular Probes) (data not shown), protein loading efficiency was found to be comparable for all protein domains. To determine the effect of exogenous addition of the rFnBD domain on the adhesion and internalization of rSOFΔFn coated latex beads, purified rFnBD at 1, 5 or 10 µg/ml was pre-incubated with the HEp-2 cells for 1 h prior to addition of the coated latex beads, and was maintained throughout the subsequent 4 h incubation with the latex beads.

Confocal microscopy studies - HEp-2 cells (after incubation with the coated latex beads) were fixed for 30 min on ice in 500 µl/well of pre-chilled 4% paraformaldehyde in PBS, and then washed twice in PBS. Cells were then blocked by the addition of 200 µl/well of PBS containing 10% foetal calf serum and incubated for 30 min at room temperature. The blocking solution was then removed and the cells were incubated with either protein-G purified rabbit polyclonal anti-SOF75 antibodies (26) (30 µg) or rabbit polyclonal anti-FnBD antibodies (1:100 dilution) for 45 min at room temperature. Following washing with PBS, cells were incubated with goat anti-rabbit Alexa 488 diluted 1:400 in PBS containing 10% BSA (Molecular Probes) for 1 h at room temperature and subsequently washed with PBS. Cells were permeabilized with 200 µl/well of 0.1% (v/v) triton X-100 in PBS for 30 min on ice, washed in PBS, followed by storage at 4°C overnight. The following day, cells were treated with goat anti-rabbit Alexa 633 diluted 1:400 in PBS containing 10% BSA (Molecular Probes) for 1 h and washed three times in PBS. Cells were then mounted onto a glass slide using Mowiol solution (Calbiochem). Images were recorded using a Leica TCS SP confocal microscope mounted on a Leica DM IRBE inverted microscope with Leica TCS NT software (Version 2.61; Leica Microsystems).

Mouse immunization and challenge - To determine the protective efficacy of rSOF75ΔFn proteins challenge studies were performed. BALB/c mice (n=10) were immunized

subcutaneously with 25 µg of wildtype or mutant forms of rSOF75ΔFn protein in incomplete Freund's adjuvant. Control mice received a subcutaneous injection of PBS. After 2 weeks, the mice were boosted with an intramuscular injection of another 25 µg of each protein in PBS. Control mice received a PBS injection. Two weeks after the booster injections, all mice were challenged by an intraperitoneal injection of $\sim 1 \times 10^9$ CFU of the SOF-positive M49 GAS strain 591 (33). The number of surviving mice was recorded daily. Moribund mice were sacrificed and recorded as dead.

Serum samples were collected on days 0 and 28, and stored at -20°C prior to determination of rSOF75ΔFn-specific antibodies. In brief, 96-well Nunc-Immuno MaxiSorp assay plates (Nunc) were coated with 2 µg/ml of wildtype rSOF75ΔFn in coating buffer (bicarbonate, pH 9.4). After overnight incubation at 4°C , plates were blocked with 1% BSA in PBS (pH 7.4) for 1 h at 37°C . Serial 2-fold dilutions of serum in PBS with 1% BSA were added (100 µl/well), and plates were incubated for 1 h at 37°C . After four washes, secondary biotinylated antibodies were added followed by 1 h incubation at 37°C . After six washes, 50 µl/well of peroxidase-conjugated streptavidin (Pharmingen), diluted 1:1000, was added and plates were further incubated for 45 min at room temperature. After a final six washes, the substrate ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) in 0.1 M citrate-phosphate buffer containing 0.1% H_2O_2 was added, and plates were incubated for 30-60 min at room temperature. The absorbance was measured at a wavelength of 405 nm.

Statistical Analyses - For apoAI binding experiments, an unpaired t-test was used to determine if there was any significant difference in the apoAI binding ability of wildtype and mutant SOF proteins. For latex bead experiments, a one way ANOVA using Bartlett's test for equal variance was used to determine whether there was any significant variation in the median number of beads attached to or taken up by HEp-2 cells, followed by a Tukey's Multiple Comparison Test for individual comparison of adherence and internalisation mediated by two different proteins. For immunization and challenge experiments, a

Kruskal-Wallis test was used to determine whether there was any significant variation in the median titers of the four groups of antisera. Dunn's Multiple Comparison test was used for individual comparison of two groups of antisera. Difference in survival curves was determined by log rank test. All statistics were performed using GraphPad Prism version 4.02 (Graph-Pad Software Inc., San Diego, CA, USA).

RESULTS

SOF is a unique multi-functional protein, capable of binding fibronectin via a C-terminal domain designated FnBD (8,24) and apoAI via an N-terminal domain (12) (Fig. 1A). The N-terminal domain of SOF (SOFΔFn) has been shown to mediate adhesion to HEp-2 cells and promote HEp-2 cell invasion by whole GAS cells. In order to assess the contribution of the OF activity of SOF in the processes of HEp-2 epithelial cell adhesion and invasion, deletion mutagenesis was used to eliminate the OF activity of recombinant rSOF75ΔFn. Deletion was undertaken to remove between 22 and 63 amino acid residues of the rSOF75ΔFn protein (between P¹⁴⁸ and K²¹¹; P²¹⁰ and E²³²; K²³¹ and D²⁸⁶; V²⁸⁵ and E³¹⁵). Each of the rSOF75ΔFn deletion mutants lacked OF activity (Fig. 1B). The rSOF75ΔFn_{DEL}[P210→E232] mutant was also found to lack OF activity when incubated in human serum or human HDL (Fig. 2). In order to delineate specific amino acid residues that contribute to OF activity, site directed mutagenesis to alanine was undertaken on 52 amino acids of SOF75ΔFn that are 100% conserved in 16 different SOF sequences (Supplementary Fig. 1 and Supplementary Table 1). A mutant form of rSOF75ΔFn with attenuated OF activity (100 fold reduction in activity) was constructed by simultaneously substituting 5 amino acids with alanine (D²¹⁸; S²²⁶; K²²⁸; M²²⁹; E²³²) (Fig. 1C). The rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] protein has attenuated OF activity when incubated with either human serum or human HDL (Fig. 2). In order to determine if the loss or attenuation of OF activity was due to a decrease in the apoAI binding capacity of SOF, the ability of rSOF75ΔFn_{WT}, rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] and rSOF75ΔFn_{DEL}[P210→E232] to bind biotinylated

apoAI was assayed (Fig. 2D). There was no significant decrease in apoAI binding by the mutant proteins, suggesting that the loss of OF activity occurs via an alternative mechanism.

The impact of the mutations to rSOF75ΔFn on protein structure was analyzed using far-UV CD spectroscopy. rSOF75ΔFn_{WT} had a CD emission spectrum typical of proteins containing both α helices and β sheets, with a characteristic minimum at ~220 nm, a second larger minimum at ~207 nm and a maximum at 190 nm (34). Of the rSOF75ΔFn_{DEL} mutant proteins generated, rSOF75ΔFn_{DEL}[K231→D286] could not be purified for structural analysis. Far-UV CD spectra obtained for rSOF75ΔFn_{DEL}[P148→K211], rSOF75ΔFn_{DEL}[P210→E232] and rSOF75ΔFn_{DEL}[V285→D315] indicate perturbation to the secondary structure of the proteins. The rSOF75ΔFn_{WT} is predicted to contain 27% alpha helicity, with a predicted 24% alpha helical content in rSOF75ΔFn_{DEL}[P148→K211], 21% in rSOF75ΔFn_{DEL}[P210→E232] and 22% in rSOF75ΔFn_{DEL}[V285→D315]. A concomitant shift towards a more disordered structure was observed for each deletion mutant, as indicated by a shift in the 207 nm minima to a shorter wavelength of 204 nm for rSOF75ΔFn_{DEL}[P148→K211] and rSOF75ΔFn_{DEL}[P210→E232] and 205.5 nm for rSOF75ΔFn_{DEL}[V285→D315] (Fig. 3) (34,35). In contrast to the loss of secondary structural elements in the rSOF75ΔFn_{DEL} mutants, the rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] mutant had increased secondary structure when compared to rSOF75ΔFn_{WT}, with an increase in predicted alpha helicity from 27% to 29% (Fig. 3).

In order to assess the direct contribution of the OF activity of SOF in the processes of HEp-2 epithelial cell adhesion and invasion, the ability of latex beads coated with wildtype OF-positive, mutant OF-negative and OF-attenuated forms of the rSOF75ΔFn protein to bind to the human pharyngeal epithelial cell line HEp-2 was assayed. These studies indicate that the SOFΔFn domain mediates attachment to HEp-2 cells, with the latex beads coated with rSOF75ΔFn_{WT} adhering to HEp-2 cells in numbers equivalent to latex beads coated with the full length rSOF75 (p>0.05), and significantly more latex beads coated with

rSOF75ΔFn_{WT} adhering to HEp-2 cells than latex beads coated with a protein encompassing only the fibronectin binding domain of the SOF protein (rFNBD) (p<0.01). Furthermore, this HEp-2 adherence mediated by the SOFΔFn domain is not dependant on OF activity, with latex beads coated with rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] (attenuated OF activity) and rSOF75ΔFn_{DEL}[P148→K211] (abolished OF activity) mediating adherence at the same level as the rSOF75ΔFn_{WT} protein (p>0.05) (Fig. 4A, C). Latex beads coated with rSOF75ΔFn_{DEL}[P210→E232] and rSOF75ΔFn_{DEL}[V285→D315] have a significantly reduced capacity to attach to HEp-2 cells when compared to rSOF75ΔFn_{WT} (p<0.01), however, there were significantly more latex beads coated with rSOF75ΔFn_{DEL} mutants attached to HEp-2 cells than were observed with the BSA control (p<0.01). It has been previously demonstrated that the SOFΔFn domain of SOF possesses pro-invasive properties when expressed on the surface of non-invasive GAS strains or non-invasive *Lactococcus lactis* (13). However, while the SOFΔFn promotes epithelial cell invasion in these backgrounds, this study has shown that the SOFΔFn protein domain is not sufficient *per se* to mediate intracellular invasion of HEp-2 cells (Fig. 4B, C). However, while it is apparent that the N-terminus of SOF does not directly mediate epithelial cell invasion, it may be concluded that this domain enhances the epithelial cell invasion by full-length SOF, in comparison to the FnBD alone. A significantly greater proportion of latex beads coated with rSOF75 were found to be intracellular (57.1%) than latex beads coated with FnBD alone (31.8% intracellular) (p<0.01) (Fig. 4B).

While SOF is a protective antigen in murine vaccination studies, the capacity of SOF to opacify serum raises questions about its use in humans. The capacity to knock-out OF activity while retaining the structural and functional integrity of the molecule may be a requirement for further evaluation of SOF as a human vaccine candidate. To this end, the protective efficacy of rSOF75ΔFn_{WT}, rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] and the corresponding deletion mutant rSOF75ΔFn_{DEL}[P210→E232] were assessed in a murine model of lethal GAS challenge.

rSOF75ΔFn_{WT}, rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] and rSOF75ΔFn_{DEL[P210→E232]} all elicited significantly elevated titres of anti-rSOF75ΔFn_{WT} serum IgG (p<0.001; p<0.01, p<0.01) (Fig. 5A). In concordance with the results of Courtney *et al.* (15), mice immunized with rSOF75ΔFn_{WT} showed significantly increased survival when challenged with the SOF-positive M49 GAS strain 591 compared with PBS control mice (p = 0.0069). While mice immunized with rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A], and the corresponding deletion mutant rSOF75ΔFn_{DEL[P210→E232]} showed increased survival when compared to the control mice, this increase was not statistically significant (p>0.05) (Fig. 5B). While Courtney *et al.* (15) have previously shown that parenteral immunization with rSOF2ΔFn protects against lethal systemic challenge with an M2 GAS strain, this is the first study to show that immunization with SOF can protect against lethal challenge with a heterologous GAS strain.

DISCUSSION

SOF is a unique multifunctional protein which may contribute to GAS pathogenesis by virtue of its abilities to interact with a variety of components of plasma and the extracellular matrix. A number of GAS surface proteins have a role in promoting host epithelial cell invasion including SfbI (36,37), M protein (31,38,39), PrtF2 (40,41), FbaA (42) and SOF (13). M protein and SfbI mediate intracellular invasion via two distinct pathways, both of which are dependant on fibronectin binding. M protein-mediated ingestion of GAS depends on co-engagement of the CD46 receptor and fibronectin via separate domains of the M protein; fibronectin in turn acts as a bridging molecule binding $\alpha_5\beta_1$ receptors at the host epithelial cell surface. (43-46). The formation of the M protein-fibronectin- $\alpha_5\beta_1$ complex and engagement of the CD46 receptor results in intracellular signaling cascades that lead to cytoskeletal rearrangement for ingestion of the bacteria (31,47). As opposed to M protein, SfbI requires only an interaction with fibronectin to trigger intracellular invasion, since the two fibronectin binding domains on SfbI co-operatively bind fibronectin which in turn binds $\alpha_5\beta_1$ integrins on the host cell surface (44,45). The resultant complex triggers the formation and recruitment of caveosomes which may allow GAS to persist within

host cells without exposure to the acidic environment of phagosomes or lysosomes (48). While it has been clearly demonstrated through specific gene deletion that SOF, PrtF2 and FbaA are mediators of intracellular invasion of epithelial cells, the mechanism via which these surface protein mediate internalisation is not known. The work of Timmer *et al.* (13), using SOFΔFn expressed at the surface of SOF-negative GAS and the heterologous species *L. lactis*, found that the N-terminal OF domain of SOF contributes to epithelial cell invasion independently of the C-terminal fibronectin binding domain. However, using latex beads coated with SOF protein, we demonstrate that the N-terminal OF domain is not intrinsically sufficient to mediate epithelial cell invasion, but will significantly enhance intracellular invasion in the presence of FnBD. Different GAS strains express a wide array of surface anchored proteins that interact with fibronectin, the M49 GAS strain used in the experiments of Timmer *et al.* (13) expresses both FBP54 and PrtF2 (40,49). Thus, in this case the SOFΔFn expressed on the surface of M49 GAS may enhance cellular uptake, as the requirement for fibronectin binding may be achieved by FBP54 and PrtF2 expressed at the surface of the M49 GAS cells. However, the exogenous addition of rFnBD does not induce internalisation of rSOFΔFn (results not shown), suggesting that fibronectin binding must be coupled at the surface of the latex bead or GAS cell for enhanced internalisation to occur.

Incubation of cells with exogenous rSOFΔFn does not enhance uptake of GAS cells but inhibits internalisation in a concentration dependant manner (13), suggesting that the interaction of the N-terminal domain of SOF with the epithelial cell surface occurs via a specific receptor on the surface of HEp-2 cells.

We have previously demonstrated (13) that the N-terminal OF domain of SOF mediates tight adherence to epithelial cells. Thus, SOFΔFn mediated adherence to the surface of epithelial cells most likely occurs via a mechanism independent of that required for the opacity reaction. The finding that these mutations clearly diminished the ability of SOF to opacify HDL but

did not alter its binding to apoA1 indicated that other functions of SOF related to the opacity reaction were altered. Recent data suggest that SOF is a heterodivalent fusogenic protein that opacifies HDL by binding and crosslinking HDL particles resulting in the displacement of apoA1, fusion of the HDL particles, and the extrusion of a delipidated HDL particle (50). This mechanism produces a very large lipid particle that is enriched in cholesterol esters and essentially depleted of apolipoproteins. Thus, we propose that the described mutations of SOF alter the opacity reaction by interfering with one or more of these processes. The expression of multifunctional proteins at the surface of GAS is a common theme. For instance, M protein, SfbI, and the hyaluronic acid capsule each have dual functions in epithelial cell interactions and phagocytosis resistance (31,36-39,51-54), while glyceraldehyde-3-phosphate dehydrogenase functions as a glycolytic enzyme and binds multiple serum proteins including plasmin and fibronectin (55,56). SOF joins an increasing list of multifunctional surface proteins of GAS.

As an immunogenic surface protein of GAS, SOF is a candidate vaccine antigen. While SOF was shown to lack protective efficacy against mucosal challenge when administered intranasally (57), SOF is a promising vaccine against systemic infection as parenteral immunization of mice with SOF2ΔFn protects against lethal intraperitoneal challenge (15). Unlike in Europe and the United States, where the throat is often the primary tissue reservoir, the skin is the major site of infection among a number of populations in which GAS infection is endemic including the Australian aboriginal population (58), populations of India and Trinidad, American Indians and Polynesians living in New Zealand (59-61). Thus, there is a need for protective antigens for use in areas such as the tropical north of Australia, where the skin is the primary route of GAS entry. Two of the prime vaccine candidates from the surface proteome of GAS, SfbI and the conserved C-terminal epitopes of M protein, while effective at eliciting protection against mucosal colonization, have proven ineffectual at reducing the rate of mortality due to systemic GAS infection in murine models (62,63). While SOF has proven to be protective against systemic infection, it is not known what physiological effect the interactions between HDL and SOF would have upon the human

host following vaccination. It would be prudent to eliminate the OF activity of the SOF protein to ensure undesirable side effects do not occur. To this end the OF attenuated (rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A]) and corresponding OF negative (rSOF75ΔFn_{DEL}[P210→E232]) mutants generated in this study were examined to determine the protective efficacy of the mutants against lethal systemic GAS infection. Unfortunately, while the mutant proteins remained immunogenic, the structural alterations introduced upon mutagenesis may have prevented accessibility of protective epitopes or altered the protective epitopes such that mice immunized with rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] or rSOF75ΔFn_{DEL} were not significantly protected. Other protective epitopes in streptococcal antigens including the M protein of GAS and the Group B streptococcus polysaccharide capsule and alpha C protein (64,65), are conformational in nature and as such will only be protective if presented to the host immune system in their native conformation. In addition, maintenance of the conformational structure may enhance the immune response to epitopes of M proteins of GAS (66). The protective efficacy of the main protective epitope of SOF may have been altered by a loss of conformation associated with mutagenesis performed in this study. This study has shown that SOF can protect against intraperitoneal challenge by a heterologous GAS strain. This data in conjunction with previous findings that antiserum against one serotype of GAS was bactericidal for multiple GAS serotypes (15) suggests that SOF contains common protective epitopes.

The SOF protein is a virulence determinant in GAS with affinity for multiple serum proteins including fibrinogen (67), fibronectin (8,24) and apoAI (12). Additionally, SOF plays a role in epithelial cell adhesion and invasion (9,13) and phagocytosis resistance (68). This study has shown that the N-terminus of SOF mediates binding to HEP-2 cells and delineates this binding activity from the ability of the N-terminus of SOF to opacify serum. This study is the first to indicate that vaccination with SOF can protect against infection by a heterologous GAS serotype in an animal model.

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FOOTNOTES

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⁴ The abbreviations used are: GAS, group A streptococcus; SOF, Serum opacity factor; OF, opacity factor; apoAI, apolipoprotein AI; LPS, lipopolysaccharide; SOFΔFn, N-terminal SOF domain; FnBD, C-terminal fibronectin binding domain; WT, wildtype.

FIGURE LEGENDS

FIGURE 1: The domain structure, opacity factor activity and adherence phenotypes of SOF, rSOF75, rSOF75ΔFn_{WT}, and the rSOF75ΔFn mutants generated in this study. *A*, Schematic representation of the SOF75 protein. SS, signal sequence; FnBD, fibronectin binding domain; MSD, membrane spanning domain. The domain mediating opacity factor (OF) activity is indicated by a bracket. Boxes represent the domains encompassed by the rSOF75 proteins used in this study. *B*, Opacity factor phenotype of wildtype and mutant forms of the rSOF75ΔFn peptide as indicated by the serum agarose overlay method.

FIGURE 2: The OF activity and apoAI binding capacity of wildtype and mutant forms of rSOF75ΔFn. *A*, OF activity over 24 h was determined by adding 1 μg/ml of either rSOF75ΔFn_{BD_{WT}}, rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] or rSOF75ΔFn_{DEL[P210→E232]} to human serum and recording the A405nm at timed intervals. *B*, *C*, the opacification of serum (*B*) or HDL (*C*) as a function of protein concentration. Human serum or purified human HDL was treated with the indicated concentration of protein for 24 h and the absorbance determined at 405 nm. *D*, binding of biotinylated apoAI by either rSOF75ΔFn_{BD_{WT}}, rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] or rSOF75ΔFn_{DEL[P210→E232]}.

FIGURE 3: rSOF75ΔFn mutants have perturbed secondary structure. The spectra of wildtype rSOF75ΔFn (solid line) compared to mutant forms of recombinant SOF75ΔFn (dotted line). *A*, rSOF75ΔFn_{DEL[P148→K211]}; *B*, rSOF75ΔFn_{DEL[P210→E232]}; *C*, rSOF75ΔFn_{DEL[V285→D315]}; *D*, rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A]. All proteins exhibit CD emission spectra characteristic of proteins containing both α helices and β sheets, displaying a characteristic minimum at ~220 nm, a second minimum at ~207 nm and a maximum at 190 nm. CD spectra in *A*, *B* and *C* show a shift in the latter minimum to a shorter wavelength indicating an increase in the proportion of the protein having disordered structure.

FIGURE 4: SOFΔFn promotes association of latex beads to HEp-2 cells independent of OF activity *A*, The total number of SOF coated latex beads per 10 HEp-2 cells. SOF coated latex beads were incubated with HEp-2 cells for 4 hours in DMEM HEPES supplemented with 1% FCS. Asterisks indicate adherence significantly lower than that mediated by the recombinant full length SOF, rSOF75 ($p < 0.001$) *B*, *C*, Binding and uptake of SOF-coated latex beads by HEp-2 cells. Following incubation with latex beads, internal and external beads were differentiated using double immunofluorescence confocal microscopy. External beads were labeled by incubation with SOF specific rabbit anti-serum, followed by goat anti-rabbit Alexa-488 conjugated antibody (green). Internal beads were labelled by subsequently permeabilizing the cells using 0.1% Triton X-100, and subsequently incubating the cells with SOF-specific rabbit antiserum, followed by goat anti-rabbit Alexa-633 conjugated antibody (red). *C*, A sample of images obtained using double immunofluorescence microscopy. Binding and uptake mediated by rSOF75, rFnBD and rSOF75ΔFn peptides is shown. Binding mediated by SOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] with attenuated OF activity and rSOF75ΔFn_{DEL[P148→K211]} with abrogated OF activity is also shown.

FIGURE 5: The murine response to parenteral immunization with wildtype and mutant forms of rSOF75ΔFn and subsequent lethal intraperitoneal challenge *A*, Antibody titres directed against rSOF75ΔFn_{WT} 28 days post intravenous immunisation with either rSOF75ΔFn_{WT}, rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] or rSOF75ΔFn_{DEL[P210→E232]}. Bars represent specific IgG present in the serum of control and vaccinated mice, with results expressed as geometric means, error bars represent standard error of the mean (n=10). Asterisks indicate

titres significantly greater than the control. *B*, Survival of intravenously immunized mice after lethal challenge with a heterologous *S. pyogenes* strain. Mice (n=10) vaccinated with either rSOF75ΔFn_{WT}, rSOF75ΔFn_[D218A-S226A-K228A-M229A-E232A] or rSOF75ΔFn_{DEL}[P210→E232] were challenged with ~1x10⁹ CFU of virulent M49 *S. pyogenes* strain 591. Mortality was recorded daily. Significance was determined using the log rank test.

SUPPLEMENTARY FIGURE 1: ClustalW alignment of the amino acid sequence of the N-terminal domain of SOF from 16 different GAS strains. 100% conserved amino acid residue are highlighted in black.

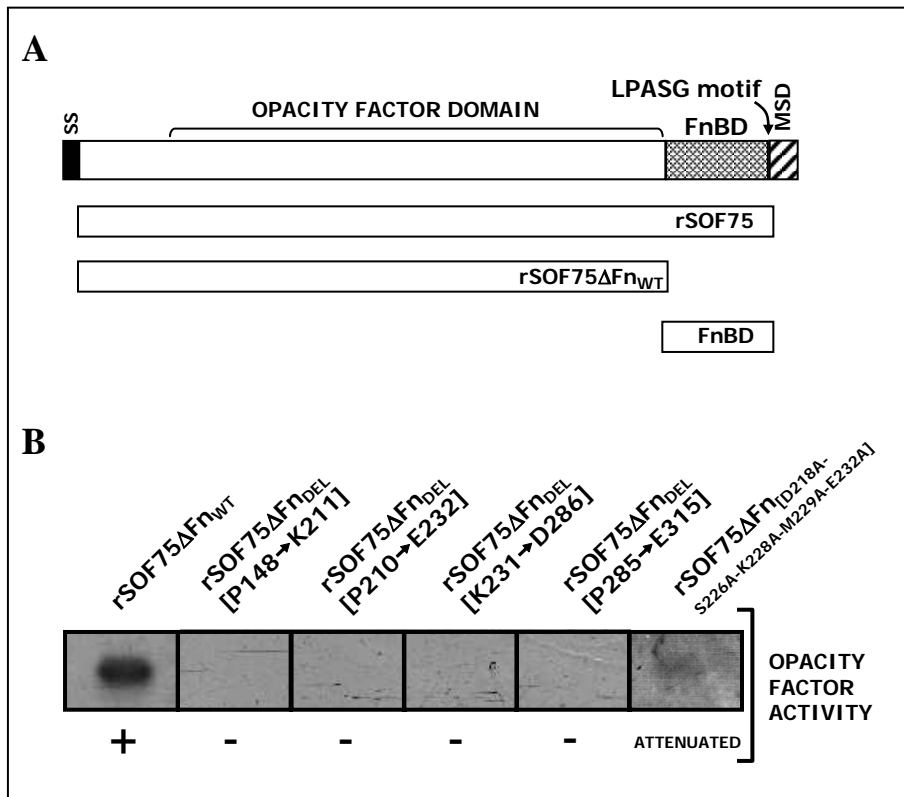


Figure 1

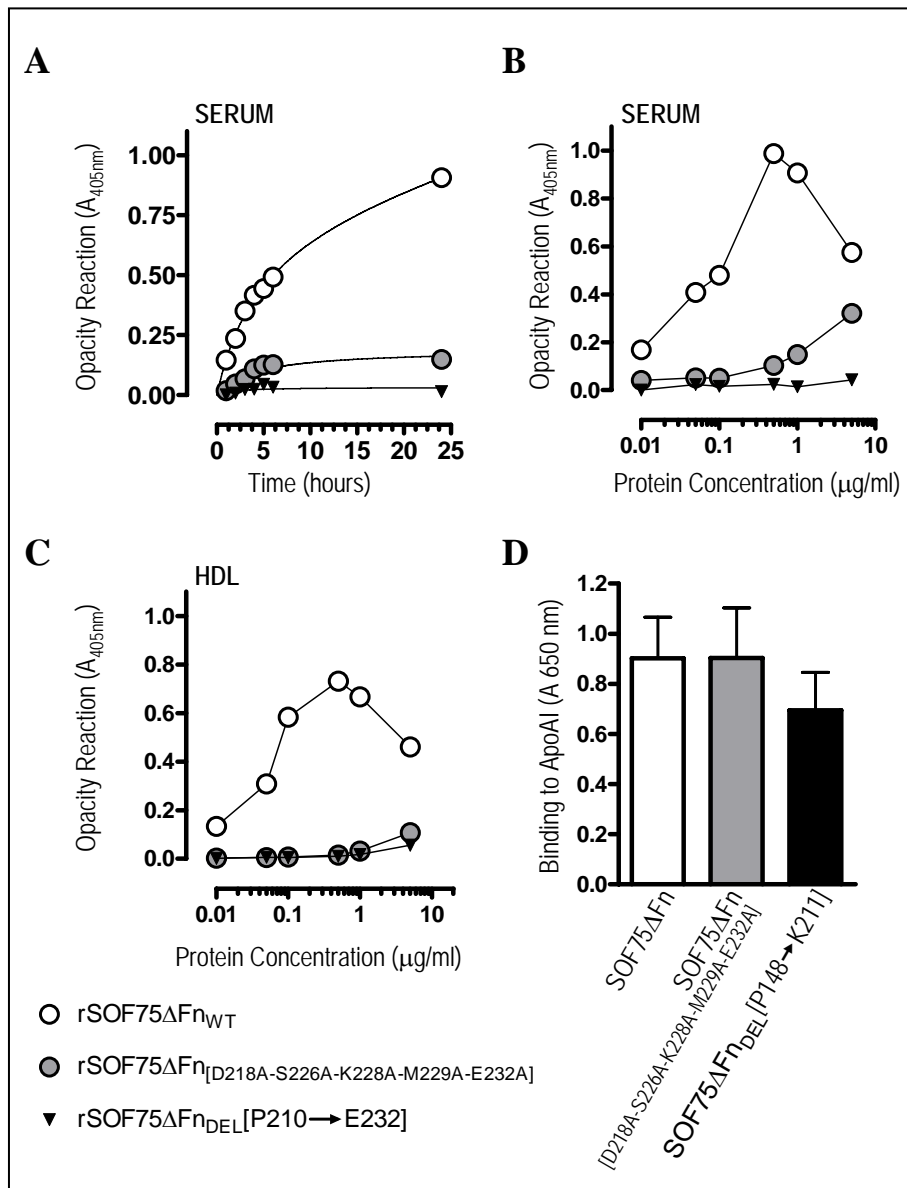
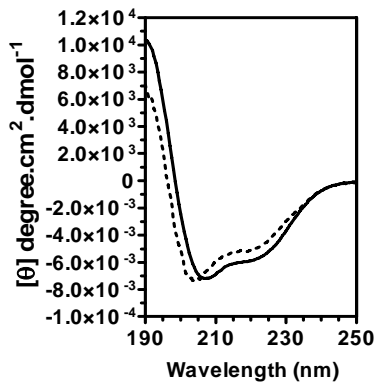
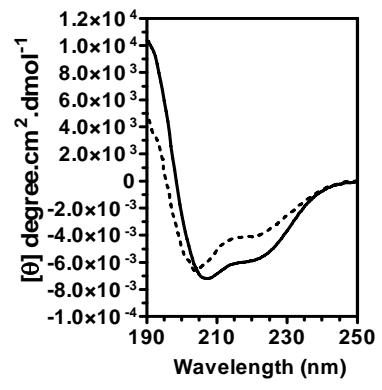


Figure 2

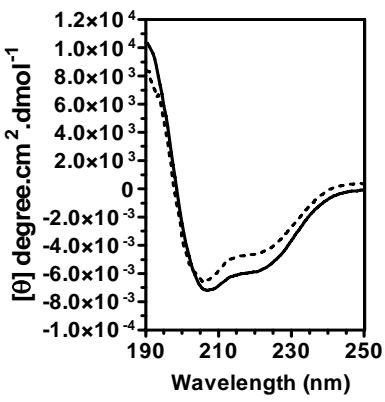
A rSOF75ΔFn_{DEL}[P148→K211]



B rSOF75ΔFn_{DEL}[P210→E232]



C rSOF75ΔFn_{DEL}[V285→E315]



D rSOF75ΔFn
[D218A-S226A-K228A-M229A-E232A]

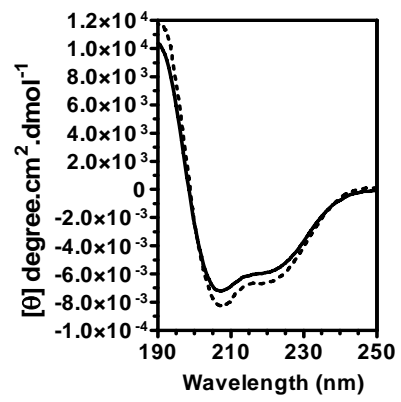


Figure 3

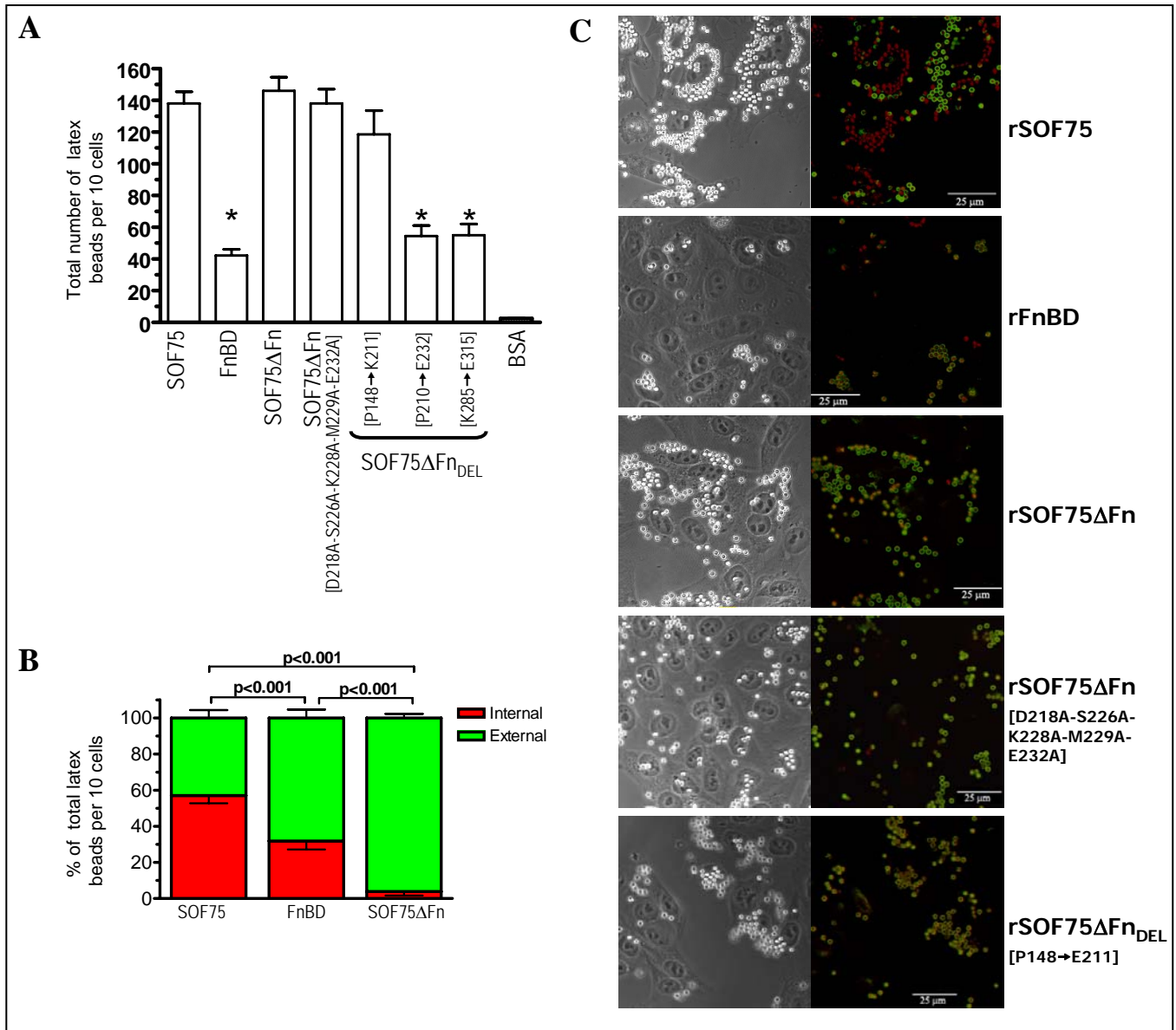


Figure 4

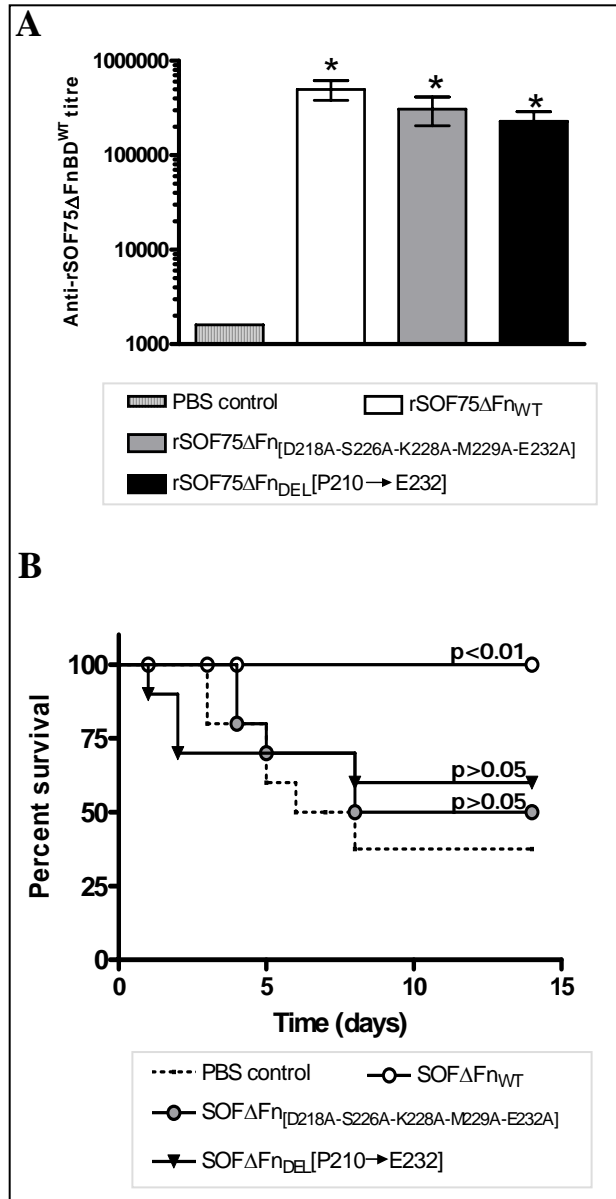


Figure 5

SOF90		33 ST---STETSTAS-----ASA	45
SOF90		33 ST---STETSTAS-----ASA	45
SOF13		33 ST-GASSTETS-----AST	45
sof75		33 NA---STETSASS-----TTS	45
sof22		33 NA---STETSASS-----TTS	45
SOF4		33 S--TTGSTETS-----AAS	44
SOF4		33 S--TTGSTETS-----AAS	44
sof2		33 SA-STSTETS-----	42
SOF4.2		33 TA---NTETSTTP-----ATT	45
sof28		33 SA-S-SSTESST-----TTA	45
SOF11		33 S--TTGSTETS-----AAS	44
SOF22		33 SA-NTSTETSTPSTTE-TSVSASAGTS	58
SOFSTNS554		33 SA-STSTETS-----AASAGTT	49
SOF76		33 STGASSSTETS-----ASSNNTN	50
sof63		33 ST--TGSTETS-----AASTTTS	48
sof49		33 ST-GVSNTEASASSTNTNTASADATASG	59
SOF100		33 ST-GATSTESSTTSTAS-----ASTD	52
SOF90	46	GATTNETTATIPASASTGTGEEAAGSGTGSEAN-----GASSVVSSEESQSSESAPASP	97
SOF90	46	GATTNETTATIPASASTGTGEEAAGSGTGSEAN-----GASSVVSSEESQSSESAPASP	97
SOF13	46	NTST-----ASAGTSTSETAASGTGSEA-----AVVSSEGSQSSESAPASP	86
sof75	46	TASTAET---STP-TGTSGTAASGASGEAT-----VATANGGPQSAPATSEATPQP	92
sof22	46	TASTAET---STP-TGTSGTAASGASGEAT-----VATANGGPQSAPATSEATPQP	92
SOF4	45	TASTAETS-----TTTGTNGTAASGASGESS-----DVSVTSSGGQSSESAPASP	90
SOF4	45	TASTAETS-----TTTGTNGTAASGASGESS-----DVSVTSSGGQSSESAPASP	90
sof2	43	TASAGVGT----SGTAASET-GSGAAVTTAT----TTTATNGGPQSTPAVAEAT--P	89
SOF4.2	46	TPSAGTGT----ATTSGTATTTPSATTDAGGEAGSGTNGASSVTSVSSGGQSSTGTPASP	100
sof28	46	NTGTGTAS----GMTATTPSATTDGTGEEAAGSGARSEANGASSVVSSEESQSSTGTPASP	100
SOF11	45	TASVDATT-----SGTTANGASGESS-----DASVASSEGSQSSEKAPASP	85
SOF22	59	TSGTVATTPS---ASTGTDGAAGSGTNGASS-----VTSVSSGGQSSESAQTLPL	103
SOFSTNS554	50	ASGTTATA---PSATTGTGEEAAGSGTNGASS-----VTSVSSGGQSSESAQAST	94
SOF76	51	TASTVETS-----TTTSTSGTAASGTGSEAA-----VVSSEGSQSSESAQASP	93
sof63	49	TASTVETS-----TTTGTSVTAASEASSESSD-----V--SVVSSEGSQSSESAPASP	94
sof49	60	TAATT-----PSAGTSTSTGEAAGSGLSSEANW----SDAAVASSGGQSSTGTPASP	108
SOF100	53	TATTSGAAT---TTTGTSGTAASGTASGTNG-----ASSVTSVSSGGQSSESAQASP	100
SOF90	98	-----QAHTASAATSTST-----SASSSNE-K---TLKTATTSTSTSTPVA	134
SOF90	98	-----QAQTASAATSTST-----SASSSNE-K---TLKTATTSTSTSTPVA	134
SOF13	87	-----QAQTASAATSTST-----SASSSNE-K---TLKTATTSTSTSTPVA	123
sof75	93	-----QAQAAPAASAPT-----TVTSSSS-SDSDAKTPKAASITSSATVA	132
sof22	93	-----QAQAAPAASAPT-----TVTSSSS-SDSDAKTPKAASITSSATVA	132
SOF4	91	QP-----QAQTATVAATSA-----SSTSSSS-EEQAPKAV--TSSTPPSTPVA	130
SOF4	91	QP-----QAQTATVAATSA-----SSTSSSS-EEQAPKAV--TSSTPPSTPVA	130
sof2	90	QP-----QAQIAPVAATS-----TSSASSSSDGKAPQAV---TSSTSPSTPAA	130
SOF4.2	101	QP-----QAQTTPAATSTST-----SNSSS---DGGIPK---TATSPSTPVA	136
sof28	101	-----QAQTAPAATSTST-----SVSSS---NEKTPKT---ATTTSTPVA	135
SOF11	86	QPQPQPQAQTAPAATSTAS-----SKAKT---EEQTPKAASITSPSTPAA	129
SOF22	104	-----QAQTATVASAST-----IASPSSSSASASDVKAPRAATSTPSTPAA	145
SOFSTNS554	95	-----QPQTQTATATSS-----SSSNTNGPSSSSEKTPKTATSTSTSTST	136
SOF76	94	-----QAQTAPVAATSA-----SSTSSSSSEAN-SDVKAPQAASITSSATVA	134
sof63	95	-----QPQAQTTPAATST-----TSSASSSSSE--D-KASKAATSTSTSTPAA	133
sof49	109	-----QAQTAPAATTTT-----SSASSSNEKPLKTATTTTSTPAA	144
SOF100	101	-----QAQTAPVAEATPKPQAQTGVASPSNLSNTNGSSSSETQTTPKSAGTTSSAPAA	154
SOF90	135	STSNN---SNKVTSTEAETPMMDVEQYTVDKKDSSVTQTDDK-----KLLKIRR	180
SOF90	135	STSNN---SNKVTSTEAETPMMDVEQYTVDKKDSSVTQTDDK-----KLLKIRR	180
SOF13	124	STSNN---SNKVTSTEAETPMMDVEQYTVDKKDSSVTQTDDK-----KLLKIRR	169
sof75	133	SPSNG---SNKEANAETAPQMDVEQYKIKDENSSITVADKA-----KQLKIRR	178
sof22	133	SPSNG---SNKEANAETAPQMDVEQYKIKDENSSITVADKA-----KQLKIRR	178
SOF4	131	ASTSS--NSNQVTGTEASPQMDVERYTVDKESSELKVKDKGNPKNGSKVN--KDTKLIR	186
SOF4	131	ASTSS--NSNQVTGTEASPQMDVERYTVDKESSELKVKDKGNPKNGSKVN--KDTKLIR	186
sof2	131	ASSNG---SNQEASAEETEPQTMVEKQYTVDKENSKLNIKDKGTPKTGSSVNNKDKTLIR	187
SOF4.2	137	ASN---SNQEASAEETEPQTMVEKQYTVDKEN-QAKYKDKGDKPKNGSSVD--DKDTKLIR	190
sof28	136	STSNN---SNKVTSTEAETQTVDVERYTVDKENSKLNIKDKGTPKTRSSVN--KDTKLIR	190
SOF11	130	SSSSN---SNQEASAEETEPQMDVEKQYTVDKESSELKVKDKGKPKNENKVD--KDTKLIR	184
SOF22	146	STSS--N-SNQVTGTEASPQMDVEQYTVDKKETELKVKDKGKE-TNGSGVN--KLLIRNR	199
SOFSTNS554	137	PAAASN--SNQEASAEETEPQMDVEQYTVDKKETELTVKDKGN-LNSSGSD--K--QLIN	190
SOF76	135	AASN--N-SNKVTGTEVEPQMDVEQYKVNKEKTELTVKDKGTQPKNGRTVNQKNIKLIR	191
sof63	134	VASSSSN-SNQATGTEVEPQMDVEQYTVNKESELKVKDKGKE-MNGSGVS--K--KLIR	187

sof49	145	SSSS--N-GNQVTGTEVEPQMMDVEQYKVKDKENSELTVKVDRR-----QLKIRK	190
SOF100	155	ASN---N-SNKVTSTEAETPMMDVEQYTVDDKKSSSVTQTDDK-----KLLKIRR	199
SOF90	181	DGNE-KTRDLYDVKREVKDNGDGTLDVTLK-VTPKQIDEGADVMALLDVSQKMTKENFDK	238
SOF90	181	DGNE-KTRDLYDVKREVKDNGDGTLDVTLK-VTPKQIDEGADVMALLDVSQKMTKENFDK	238
SOF13	170	DGNE-KTRDLYDVKREVKDNGDGTLDVTLK-VTPKQIDEGADVMALLDVSQKMTKENFDK	227
sof75	179	DDNP-KDKDLFDVKREVKDNGDGTLDVTLK-VMPKQIDEGADVMALLDVSQKMTKENFDK	236
sof22	179	DDNP-KDKDLFDVKREVKDNGDGTLDVTLK-VMPKQIDEGADVMALLDVSQKMTKENFDK	236
SOF4	187	NRDD-EQRDIVDIKREVKTNADGTIDVTVT-VTPKEIDKADVMALLDVSQKMTEDDFKN	244
SOF4	187	NRDD-EQRDIVDIKREVKTNADGTIDVTVT-VTPKEIDKADVMALLDVSQKMTEDDFKN	244
sof2	188	NRDG-KLRDIVDVTRIVKTNEDGTIDVTVT-VKPKQIDEGADVMALLDVSQKMSSEDDFNN	245
SOF4.2	191	NRDG-KQRDIVDVTRIVKTNEDGTIDVTVT-VKPKQIDEGADVMALLDVSQKMTTEEDFNN	248
sof28	191	NRDD-KQRDIVDVTRIVETNEDGLLMFTGNGLKPKQIDEGADVMALLDVSQKMTKENFDK	249
SOF11	185	NRDG-EQRDIVDIKREVKTNADGTIDVTVT-VTPKEIDKADVMALLDVSQKMTKENFDK	242
SOF22	200	--DG-EQRDIVDIKREVKTNADGTIDVTVT-VTPKEIDKADVMALLDVSQKMTTEEDFKN	255
SOFSTNS554	191	-RDG-EQRDIVDIKREVKTNADGTIDVTVT-VTPKEIDKADVMALLDVSQKMTTEEDFNN	247
SOF76	192	NRDG-EQRDIVDIKREVKDNGDGTLDVTLK-VTPKEIDKADVMALLDVSQKMSSEDAFNN	249
sof63	188	NRDG-EQRDIVDIKREVKTNADGTIDVTVT-VTPKEIDKADVMALLDVSQKMSKEDFNN	245
sof49	191	DVDNPKDKDLFDVKREVKDNGDGTLDVTLK-VTPKQIDEGADVMALLDVSQKMTQENFDK	249
SOF100	200	DGDE-KTRDLYDVKREVKDNGDGTIDVTLK-VTPKQIDEGADVMALLDVSQKMTRENFDK	257
SOF90	239	AKEQIKKMVTTLTGEPDGDGKE---NHNRRNSVRLMTFYRKVNDPIELT---TKNVDAKL	291
SOF90	239	AKEQIKKMVTTLTGEPDGDGKE---NHNRRNSVRLMTFYRKVNDPIELT---TKNVDAKL	291
SOF13	228	AKEQIKKMVTTLTGEPDGDGKE---NHNRRNSVRLMTFYRKVNDPIELT---TKNVDAKL	280
sof75	237	AKEQIKKMVTTLTGEPDGDGKE---NHNRRNSVRLMTFYRKVSDPIELT---TKNVDAKL	289
sof22	237	AKEQIKKMVTTLTGEPDGDGKE---NHNRRNSVRLMTFYRKVSDPIELT---TKNVDAKL	289
SOF4	245	AKDKIKKLVKTLTSSKASNSDNDDEHKHNSRNSVRLMTFYREISDPIDISGKTDAAELDELL	304
SOF4	245	AKDKIKKLVKTLTSSKASNSDNDDEHKHNSRNSVRLMTFYREISDPIDISGKTDAAELDELL	304
sof2	246	AKNKIKKLVKTLTSSKASNSDNDDEHKYNSRNSVRLMTFYREISNPIDISGKTEEQLDKLL	305
SOF4.2	249	AKEKIKKLV--TLTG--T-GKNGGE--HKARNSVRLMTFYREISKPIDISGKTEEQLDTLL	302
sof28	250	AKEQIKKMVTTLTGEPDGDGKE---NHNRRNSVRLMTFYRKVNEPIELT--AENVDKTLL	302
SOF11	243	AKEQIKKMVTTLTGEPDGDGKE---NHNRRNSVRLMTFYRKISEPIDLSGKTSSEVDKEL	298
SOF22	256	AKDKIKKLVKTLTSSKSVSKSTDSQPNHNARNNSVRLMTFYREISDPIDISGKTDDDELDKLL	315
SOFSTNS554	248	AKDKIKKLVTTLT---SKSADGQQNLNRRNSVRLMTFYREISDPIDI-GKTETQLDQLL	303
SOF76	250	AKEKIKKLVTTLTSSKASNSDNDDEHKHNSRNSVRLMTFYRKVNEPIEL---TAENVDKTLL	306
sof63	246	AKTKIKKLVKTLTEK-----NGE-NHNSRNSVRLMTFYREISDPIDISGKTEEQLDKIL	298
sof49	250	AKGQIKKVVTTLTGESTDGGK---NHNRRNSVRLMTFYRKVSDPIELTTKT---IGAKL	302
SOF100	258	AKEQIKKMVTTLTGEPDGDGKE---NHNRRNSVRLMTFYREISDPIDISGKTDEELNSIL	313
SOF90	292	NEVWEQAKK-DWDWGVDLQGAHKKAREIFKN---EKNSK--KRQHIVLFSQGESTFYSYDI	345
SOF90	292	NEVWEQAKK-DWDWGVDLQGAHKKAREIFKN---EKNSK--KRQHIVLFSQGESTFYSYDI	345
SOF13	281	NEVWEQAKK-DWDWGVDLQGAHKKAREIFKN---EKNSK--KRQHIVLFSQGESTFYSYDI	334
sof75	290	KEVWDQAKK-DWDWGVDLQGAHKKAREIFKN---EKNSK--KRQHIVLFSQGESTFYSYDI	343
sof22	290	KEVWDQAKK-DWDWGVDLQGAHKKAREIFKN---EKNSK--KRQHIVLFSQGESTFYSYDI	343
SOF4	305	DKLRQEAQ-DEYDWGVDLQGAHKKAREIFKN---DQEK--KSGKRQHIVLFSQGESTFYSYDI	360
SOF4	305	DKLRQEAQ-DEYDWGVDLQGAHKKAREIFKN---DQEK--KSGKRQHIVLFSQGESTFYSYDI	360
sof2	306	DDLRRKAK-ANYDWGVDLQGAHKKAREIFKN---EKEKFGKRRIHIVLFSQGESTFYSYEL	361
SOF4.2	303	NKLRQEAQ-DEYDWGVDLQGAHKKARDVFNK---EKEKSKKRQHIVLFSQGESTFYSYDI	358
sof28	303	DEVWKKAK-EDWDWGVDLQGAHKKAREIFKN---EKEKSKKRQHIVLFSQGESTFYSYDI	358
SOF11	299	DNIWDKVKKEDWDWGVDLQGAHKKARDIFKN---EKESK--KRQHIVLFSQGESTFYSYDI	353
SOF22	316	DELRRKAK-ANYDWGVDLQGAHKKARTVFNKNNNDNKKKSGKRQHIVLFSQGESTFAYEL	374
SOFSTNS554	304	DELREKAK-ANYDWGVDLQGAHKKAREIFKN---DQEK--KSGKRQHIVLFSQGESTFYSYDI	359
SOF76	307	DEVWKKAK-EDWDWGVDLQGAHKKAREIFKN---EKEKNSGKRQHIVLFSQGESTFYSYDI	362
sof63	299	NDLRRKAK-ANYDWGVDLQGAHKKAREIFKN---DQEK--KSGKRQHIVLFSQGESTFYSYDI	354
sof49	303	EEVWEQAKK-DWDWGVDLQGAHRRARDIFRG---EKGSK--KRQHIVLFSQGESTFYSYDI	356
SOF100	314	DDVWKKAKK-DWDWGVDLQGAHKKARDIFKN---EKNSK--KRQHIVLFSQGESTFYSYEL	367
SOF90	346	NANS---KANLKAITEDKITTNSPLLPWLPPIFNHTNRKAEIINDVEKVLNMAEKLGIKLP	402
SOF90	346	NANS---KANLKAITEDKITTNSPLLPWLPPIFNHTNRKAEIINDVEKVLNMAEKLGIKLP	402
SOF13	335	NANS---KANLKAITEDKITTNSPLLPWLPPIFNHTNRKAEIINDVEKVLNMAEKLGIKLP	391
sof75	344	HNKSD--SKILKTRVNIITNSPLFPWLPPIFNHTNRKADMIDDVKYLIKWGEKLGIEGL	401
sof22	344	HNKSD--SKILKTRVNIITNSPLFPWLPPIFNHTNRKADMIDDVKYLIKWGEKLGIEGL	401
SOF4	361	KNK--NDRVTVKTRITEKVTTNSPLLPWLPPIFNHTNQEDMMLDDFAKLVNKLKSLGLDSL	418
SOF4	361	KNK--NDRVTVKTRITEKVTTNSPLLPWLPPIFNHTNQEDMMLDDFAKLVNKLKSLGLDSL	418
sof2	362	QNSV-REDKTKLSRSGAVTSSNPLFPWLPPIFNHTKNDIMLDDVKLVKLGQTLGIEGL	420
SOF4.2	359	KN---KDDKKLQKTLTTVTTNSPLFSWLPPIFDHTNRKADMIDDIKYLIKWGEALGIEGL	414
sof28	359	KN---KM-ILQKLPITEKVTTSSPLFPWLPPIFNHTNRKAEIIGDLEKVLDMAEKVGISLP	414
SOF11	354	NDKD-KNNTVRKNRITGKVTTNSPLFPWLPPIFNHTNQAEVIDDVKLLDFAEKMGISLP	412
SOF22	375	QYGVKDKTSLKSRLE-TVTTNSPLFPWLPPIFDHTKNGDILNDVRLHLSFADRLGKLSL	433
SOFSTNS554	360	KN--KNDSKLLKARLT-TVTTNSPLFSWLPPIFDHTNRKADMMLDDFDKLLSIAQKFGVEIP	416
SOF76	363	ND--KDKSLTKSRITGKVTTNSPLLRWLPPILEHTKNGDMINDVKTLIAFASKLGIKLL	420

sof63	355	KN--KNDSTVTKTRITEKVTISNPLLPWPIFDHHTQNADMLED	SAKLIKLLKSLGLESL	412
sof49	357	SD-KDNGASVRVPSITGNVTASNPLFPWLPFPPTPHPAEVIDD	VDKLLGFAENLGLSLP	415
SOF100	368	D--K--SKLSK-SSLTEVTTISNPLLHWPIFNFTNRKADMLDD	ITKVIKKVKDLGVKSV	421
SOF90	403	KGLKTAVQTVGATNAAFGSLLGG-LTQYLALKEYSS-DNLDGGGF	YSKR/VGEGYYYS	460
SOF90	403	KGLKTAVQTVGATNAAFGSLLGG-LTQYLALKEYSS-DNLDGGGF	YSKR/VGEGYYYS	460
SOF13	392	KGLKTAVQTVGATNAAFGSLLGG-LTQYLALKEYSS-DNLDGGGF	YSKR/VGEGYYYS	449
sof75	402	NLDNLTLAGAASGIVGGFLGGSLTEYLSLKEYQS-DRLNASQ	FNERRVGEYYS	460
sof22	402	NLDNLTLAGAASGIVGGFLGGSLTEYLSLKEYQS-DRLNASQ	FNERRVGEYYS	460
SOF4	419	KTVDSVLQVLR-ATSSVGKLLG-QSLTEYLTSEYNS-DKLGSEW	FDTKRVGEGYYYS	475
SOF4	419	KTVDSVLQVLR-ATSSVGKLLG-QSLTEYLTSEYNS-DKLGSEW	FDTKRVGEGYYYS	475
sof2	421	DNLQSTLSLISTGSSLAGAFLGGSLTEYLTKEYKS-GDLKENQ	FDTKRVGEGYHHS	479
SOF4.2	415	SGLKDKLSLAGLASSALGGFLGGSLTEYLSLKEYQS-DSLKADQ	FNMEKRVGEGYYYS	473
sof28	415	SSLKSAVKVGLTNSAIGSILG-KGLTEYLTSEYNS-DNLDGGGF	YSKR/VGEGYYYS	472
SOF11	413	KGLRAGVQAIGLSNSFLSTFTG-SGLTEYLTSEYNS-DILKEKQ	FDTKRVGEGYYYS	470
SOF22	434	NLDKAPLDAISIGNGLFSGPLGEYLTKEYNS-RKLNEDQ	FDTKRVGEGYYYS	492
SOFSTNS554	417	NGLKTGLKAAATNSLLSFTGGDGLTDYLTREYMA-DKLOETD	FNMSNRVGEYHHS	475
SOF76	421	SWIEAPLSMLSLTNDLFGSVLGGGPGYLTSEYDS-HKLNEDQ	FDTKRVGEGYHHS	479
sof63	413	QTADNILQALQAAAN-RIGSLFG-KSPTEYLTSEYNS-NKLGEE	SFDTSKHVGEYYS	469
sof49	416	KGLREGVTAIGLRRGLSFTSG-LTEYLTSEYNS-AILYYAQ	FDTKRVGEGYYYS	473
SOF100	422	GTAESVLTALKAMNSLGSFFTGS--LTEYLTKEYYSDSKL	LEEKYFDTKRVGEGYYYS	479
SOF90	461	FSERTHEDKMPLESAIREALKTSVPKLS---DTWFFGILNSFV	DK-DTVEKAKLDVIMQ	515
SOF90	461	FSERTHEDKMPLESAIREALKTSVPKLS---DTWFFGILNSFV	DK-DTVEKAKLDVIMQ	515
SOF13	450	FSERTHEDKMPLESAIREALKTSVPKLS---DTWFFGILNSFV	DK-DTVEKAKLDVIMQ	504
sof75	461	FSER-KTAEMPNRALIKKQLEGLFKGKE---GKWFKSILEKLS	SLT-DDYQKAKEEAILK	514
sof22	461	FSER-KTAEMPNRALIKKQLEGLFKGKE---GKWFKSILEKLS	SLT-DDYQKAKEEAILK	514
SOF4	476	FSDRESENTMPLENEIKTALKTYLPKFE---EKSWFTNVLK	YFELE-EKAEQAKLDVIMK	531
SOF4	476	FSDRESENTMPLENEIKTALKTYLPKFE---EKSWFTNVLK	YFELE-EKAEQAKLDVIMK	531
sof2	480	FSBRKKTGEIPFKSEIEPKIKELFENKNNQDKSWTEWIFD	KLSTL-ERIQKAKQETLMK	538
SOF4.2	474	FSKRE-TADMPNRDLIKQNLGLFKE---KDGWFKSILDKLS	SLT-DKYQKAKEAALLK	527
sof28	473	LSDRKYENTMPLEEAIRTALASNFPKLT---DNWFFDILNSF	VNK-DTVEKAKLDVIMK	527
SOF11	471	YSKRTHGDKMPFEKQIREVLEQFLPKIE---SREWAKKFID	IFGLQGQKVDQIGVDVIMK	527
SOF22	493	FSNRNSDNPLESELKVKLETYLPKFE---ENWFTSVLQYF	GLKKEAEQ-TKLDVIMK	548
SOFSTNS554	476	FSERNTHD-MPKETLEKLLDSQIPRLGK---ESWFGWALD	KLSTLTYRN-GQKAALMK	530
SOF76	480	FSDRESEDRIPELEIKALKALPRFDE---NNWFTKVLTY	FKLKDCAEQ-AKLDVIMK	535
sof63	470	FSDRKSENTMPLESAIKALTNSFPKIP---DSWFFGILKS	SDIKAKVEK-AKLDVIMQ	524
sof49	474	YSVRTHGDMLPFESEIRKALEQVLPKIED---REWAMPFI	DIFGLPIQKVNQSGIDVIMK	530
SOF100	480	FSNREYNAEMPFERELKLALETALSSVG---EDWFTSIL	NRLGLKKEKTEK-AKVDVIMK	534
SOF90	516	VLNSIFYKREYRYNHNLSAIAEAKMAQKEGITYFYSVDVT	SLAK--SKR-----S	563
SOF90	516	VLNSIFYKREYRYNHNLSAIAEAKMAQKEGITYFYSVDVT	SLAK--SKR-----S	563
SOF13	505	VLNSIFYKREYRYNHNLSAIAEAKMAQKEGITYFYSVDVT	SLAK--SKR-----S	552
sof75	515	VLDYFFYKRDYIYNNHLSAIAEAKMAQEGVTFYYSVDV	DFNS-ASKRAKRQVKSEEDK	573
sof22	515	VLDYFFYKRDYIYNNHLSAIAEAKMAQEGVTFYYSVDV	DFNS-ASKRAKRQVKSEEDK	573
SOF4	532	VLKSVFYKROYYNHNLSAIAEAKMAQKEGITYFYSVDVT	SLAK--SNRSKRSEKTEAD	589
SOF4	532	VLKSVFYKROYYNHNLSAIAEAKMAQKEGITYFYSVDVT	SLAK--SNRSKRSEKTEAD	589
sof2	539	LLEYLFYKREYHYNHNLSAIAEAKMAQKEGITYFYSVDVT	DLKTT-SKRVRQVESTEDK	597
SOF4.2	528	VLDYLFYKREYHYNHNLSAIAEAKMAHR--ASPLLVDVT	DLT---ARVKQTATYTD	581
sof28	528	VLNSIFYKREYRYNHNLSAIAEAKMAQKEGITYFYSVDVT	DLNS-ASKRVRQAARVKG	586
SOF11	528	VINSIFYKROYYNHNLSAIAEAKMAQKEGITYFYSVDVT	DLKPP---KRAKRAQAVLQKT	584
SOF22	549	VIKSVFYKROYYNHNLSAIAEAKMAQKEGITYFYSVDVT	DFNS-ALKRAKRQVESEKSR	607
SOFSTNS554	531	ILDYLFYKREYHYNHNLSAIAEAKMAQKEGITYFYSVDVT	DFETT-SKRVRQVGVLQET	589
SOF76	536	VIKSVFYKROYYNHNLSAIAEAKMAQKEDITYFYSVDVT	SPKQADNKTRRSADTPGE	594
sof63	525	VLKSVFYKREYRYNHNLSAIAEAKMAQKDGITYFYSVDVT	SPNQATT--KRSRRSTE--	580
sof49	531	VINSIFYKROYYNHNLSAIAEAKMAQKEGITYFYSVDVT	DLSSA-SKRAKRQAVPQKT	589
SOF100	535	ILNTLFYRROYYNHNLSAIAEAKMAQKEGITYFYSVDVT	DLK---PKRAKRQAVPQKT	591
SOF90	564	KRFSEKTEAEKRND-KFDGYLKEMSEG-KKFLLEDKDVVNK	KDFKDLTGLTIKDEF	621
SOF90	564	KRFSEKTEAEKRND-KFDGYLKEMSEG-KKFLLEDKDVVNK	KDFKDLTGLTIKDEF	621
SOF13	553	KRFSEKTEAEKRND-KFDGYLKEMSEG-KKFLLEDKDVVNK	KDFKDLTGLTIKDEF	610
sof75	574	KKAKEKENIEKKRDEKFDNYLQKQSEGGKEFFNDVDKAEN	--FKDITLASVTVTETFGNNV	631
sof22	574	KKAKEKENIEKKRDEKFDNYLQKQSEGGKEFFNDVDKAEN	--FKDITLASVTVTETFGNNV	631
SOF4	590	RR-----NDKFDYLLKQSEEDG-TFLEAKGVENKDK	FKDAVTKLTIKDEF	637
SOF4	590	RR-----NDKFDYLLKQSEEDG-TFLEAKGVENKDK	FKDAVTKLTIKDEF	637
sof2	598	KKEKDREDIEKERNEKFDNYLQKQSEGGKDFEDVDKA	--EKFKDILTNTVTET	655
SOF4.2	582	KK-KEIEKIEEERNKFDAYLRKQSEEDN-DFLSDVEDK	--DKFKDILTNTVTET	637
sof28	587	KE--ENKNEEERNTKFDYLLKQSEEGN-NFLSNVEER	--DFFKDILTNTVTET	641
SOF11	585	TK----KESEEDRNKFDYLLKQSEGGKEFFNDVDEA	--DKFKDILTNTVTET	638
SOF22	608	KKKKIRKTLKENGTEKFDYLLKQSEEGN-FNLDIDVKNQ	KDFKDLTLEVKVTET	666
SOFSTNS554	590	AKK--EPEKER-NDKFDYLLKQSEEG-KEFLKIDID	--NQDKFKDILTNTVTET	642

SOF76	595	-KR-----NEQFAKYLREBMSG-KDFLKNTEIKDKDKFKDITLTELTIKDEF	642
sof63	581	KKEAEE-----NEKFDKYLKEMSEGGKFFNDVD--KTRDFKDTLTELKIKDEF	632
sof49	590	TKK----ESEEDRNNKFDGYLKKMSEGGKEFFTGVD--KADKFKDITLTELTIKDEF	643
SOF100	592	TKK----ESEEDRNNKFDGYLKKMSEGGNAFFNDVD--KADKFKDITLTELTIKDEF	645
SOF90	622	TVQESSEGGKYYHKTSLISETPTVNYT----AAATAK-----WFSSPTKESL	672
SOF90	622	TVQESSEGGKYYHKTSLISETPTVNYT----AAATAK-----WFSSPTKESL	672
SOF13	611	TVQESSEGGKYYHKTSLISETPTVNYT----AAATAK-----WFSSPTKESL	661
sof75	632	SVESGS-----WKTSLGNSGSSSRE----VSYKGRDSGSLSLFGSTKESL	682
sof22	632	SVESGS-----WKTSLGNSGSSSRE----VSYKGRDSGSLSLFGSTKESL	682
SOF4	638	NVDKSS-----YELSVGNVRKLDSTT---SINHQPYP--AGWLASATKESL	687
SOF4	638	NVDKSS-----YELSVGNVRKLDSTT---SINHQPYP--AGWLASATKESL	687
sof2	656	NVKDNS-----WQVSSENN-NSLHSN-YKSVTHKAASDA-SWWSLYSNKESL	707
SOF4.2	638	NVKENL-----YQTSLSNSNSGNSNSGGDVRHTKPSGS-SWTFSTKESL	691
sof28	642	TVEKDS-----WKSITDGLKNSNNN---NVKHQQANTS-TWSFFSSSKESL	692
SOF11	639	SVENQS-----WKVSSNSN---HKEN---VKYTNAQKN-RWWSFSTKESL	685
SOF22	667	SVENQ-----SWKVSSNSN---HKE---NVKYTNAKEK--GWFSSSEKESL	712
SOFSTNS554	643	AAGSD-----SWSNSHG-----VVKYQKNENG--GWFTTSKESL	683
SOF76	643	TVETNSDCKKNYKTNLNN-----TL---KVNHTPSRAGGWGLSSSTTKESL	693
sof63	633	TVEENSWNTLSTAGLKNNSN---KNK---DVQHQKASQPSVWFSSTKESL	685
sof49	644	TVETNSEGK-KNYKTNLKG---NTL---KVNHTPSKAGSLSWFSSATKESL	695
SOF100	646	TVENNS-----WNTLSTAG---LKN---SVNHQASSS-SSWFSRSTKESL	692
SOF90	673	-LKTAFESGQPLTLTYLKLKVEKEKFKALQER--SR--KKRAASSESENTVTD	727
SOF90	673	-LKTAFESGQPLTLTYLKLKVEKEKFKALQER--SR--KKRAASSESENTVTD	727
SOF13	662	-LKTAFESGQPLTLTYLKLKVEKEKFKALQER--SR--KKRAASSESENTVTD	716
sof75	683	-LKQAFEGKPLTLTYLKLKVDKDKFKRETLLKQESRRIKKRAASSESENTV	741
sof22	683	-LKQAFEGKPLTLTYLKLKVDKDKFKRETLLKQESRRIKKRAASSESENTV	741
SOF4	688	-LKEAFKNGFLTFFKYKLVQNKDK---LLKDK-KR--IKRDTSTENKTSVTAD	739
SOF4	688	-LKEAFKNGFLTFFKYKLVQNKDK---LLKDK-KR--IKRDTSTENKTSVTAD	739
sof2	708	-LKEAFKNSLTFKYKLVQNKQK---LLDKNKR--TKRDTSTENKTSVTKDI	760
SOF4.2	692	-LKEAFKNGFLTFFKYKLVQNKDK---LLKDI-KR--TKRGTSTENKTSVTKD	743
sof28	693	-LKETFEKNGSLTFKYKLVQNKDK---LLDLKKE--TKRDTSTENKTSVTANI	745
SOF11	686	-LKQAFEDGKPLTLTYLKLKVDNNKLLKALDDKRKNR--PKRSIPTENENS	742
SOF22	713	-LKEAFKNSLTFKYKLVQNKD---KLLDKDK-R--TKRDTPTEDKTSVTAKI	765
SOFSTNS554	684	-LKKAFEDGKPLTFKYKLVQNKDKFKTALAEESKQR--TKRSAPAEENSVTK	740
SOF76	694	-LKKAFEDGKPLTLTYLKLKVDNAKFKKALEENNKKK--TKGDTSTGNKTS	750
sof63	686	-LKEAFKNGSLTFKYKLVQNKD---KLLDKNKN-I--TKRDTSTEDKTSVT	738
sof49	696	GRKAFEDGKPLTLTYLKLKVDNGKFKKSLEENNKKR--TKRSAPTENENS	753
SOF100	693	-LKKAFEDGKPLTLTYLKLKVDNNKFKTALAEK-KKR--IKR-LPYRNENS	747
SOF90	728	SYKINNGNDTNDNKNLLEDVKMSYSKFKMPIPELDIEVVVPKVPKEPLV	787
SOF90	728	SYKINNGNDTNDNKNLLEDVKMSYSKFKMPIPELDIEVVVPKVPKEPLV	787
SOF13	717	SYKINNGNDTNDNKNLLEDVKMSYSKFKMPIPELDIEVVVPKVPKEPLV	776
sof75	742	SYKINNGKDTNNNK--LEEVKMSYSKFKMPIPELDIEVVVPKVPKEPLV	799
sof22	742	SYKINNGKDTNNNK--LEEVKMSYSKFKMPIPELDIEVVVPKVPKEPLV	799
SOF4	740	TYKINN-QEVKGNK--LDDVKLTYTKETVVPDVEGEVVP--IPEKPLV	794
SOF4	740	TYKINN-QEVKGNK--LDDVKLTYTKETVVPDVEGEVVP--IPEKPLV	794
sof2	761	NYKINN-QEVKGNK--LDDVNLTYTKETVVPDVEGEVVP--IPEKPLV	815
SOF4.2	744	TYKINE-QVKGNK--LENVKLTYTKETVVPDVEGEVVP--IPEKPLV	798
sof28	746	NYKINN-QEVKGNK--LDVNLTYTKETVVPDVEGEVVP--IPEKPLV	800
SOF11	743	NYKINK-QEVKGNK--LDDVKLTYTKETVVPDVEGEVVP--IPEKPLV	797
SOF22	766	TYNINGQE-VKDNK--LDDVNLTYSKFKVPVPOIDGHVIE---PQRHYS-	818
SOFSTNS554	741	TYKINNQE-VKGN--LGDVKLTYSKFKVPVPOIDGVIE---PQAPKLP	794
SOF76	751	TYKINDQE-VKGNK--LDEVNLTYSKLKVVPVPOIDGVIE---PQAPKLP	804
sof63	739	TYKINDQE-VKGN--LDDVNLTYSKFKVPVPOIDGHVIE---PQAPLP	792
sof49	754	TYKINNQGQTKK--LDDVNLTYSKLKVVPVPOIDKVEIE---QEP	808
SOF100	748	TYKINNQGQTKK--LDDVNLTYSKLKVVPVPOIDGHVIE---PQAPLP	802
SOF90	788	NYPT-----POLPKDEDELEISGGHGP	842
SOF90	788	NYPT-----POLPKDEDELEISGGHGP	842
SOF13	777	NYPT-----POLPKDEDELEISGGHGP	831
sof75	800	NYPT-----POLPKDEDELEISGGHGP	854
sof22	800	NYPT-----POLPKDEDELEISGGHGP	854
SOF4	795	NYPT-----POLPKDEDELEISGGHGP	849
SOF4	795	NYPT-----POLPKDEDELEISGGHGP	849
sof2	816	NYPTPDIPPOLPKDEDELEISGGHGPSVDIVEDTGTGAEVGGAGQNGV	875
SOF4.2	799	NYPT-----POLPKDEDELEISGGHGP	853
sof28	801	NYPT-----POLPKDEDELEISGGHGP	855
SOF11	798	NYPT-----POLPKDEDELEISGGHGP	852
SOF22	819	VLDYTEESIYRLPLEH-----GSNAPDTQVTIEEDTVQP--RPDILVGG	868

SOFSTNS554	795	VLDYTEESIYR	LELKH----	CSNAPDTQVTIEEDTVPP--	TSDILVGGQSGP---	VDIT	844
SOF76	805	VLDYKEESIYR	LELEH----	CSNAPDTQVTIEEDTVPQ--	RPDILVGGQSGP---	VDIT	854
sof63	793	NFEYEEETGYQ	LELKH----	CSNAPDTQVTIEEDTVPQ--	RPDILVGGQSGP---	VDIT	842
sof49	809	NFEYEEETGYQ	LELKH----	GRNAPDTQVTIEEDTVPQ--	RPDILVGGQSDP---	IDIT	858
SOF100	803	NFEYEEETGYQ	LELEH----	CSNAPDIQVTIEEDTVPQ--	RPDILVGGQSGP---	VDIT	852

Supplementary Table 1: Forward primers used for PCR construction of SOF site-directed mutants. Underlined sequences indicate introduced mutations. For site-directed mutants containing four or more mutations not encoded in a single primer, mutations were sequentially introduced using previously mutated DNA as a template.

Introduced mutation	Forward Primer
Alanine substitutions	
K ²¹¹ -Q ²¹² -D ²¹⁴ -D ²¹⁸ /A	5' CCTTAAAGTAATGCCTGCAGCAATTGCCGAAGGTGCCGCTGTTATGGCC 3'
D ²¹⁸ /A	5' CAAATTGACGAAGGTGCCGCTGTTATGGCCCTTTTAGAT 3'
M ²²⁰ /A	5' GACGAAGGTGCCGATGTTGCGGCCCTTTTAGATGTCTCT 3'
D ²²⁴ /A	5' GATGTTATGGCCCTTTTAGCTGTCTCTCAAAAGATGACA 3'
S ²²⁶ /A	5' ATGGCCCTTTTAGATGT <u>CGCT</u> CAAAAGATGACAAAAGAG 3'
K ²²⁸ /A	5' GCCCTTTTAGATGTCTCTCAAGCGATGACAAAAGAGAATTTTGAT 3'
M ²²⁹ /A	5' GCCCTTTTAGATGTCTCTCAAAAGCGGACAAAAGAGAATTTTGATAAGGCT 3'
S ²²⁶ -K ²²⁸ -M ²²⁹ -E ²³² /A	5' GCCCTTTTAGATGT <u>CGCT</u> CAAGCGCGGACAAAAGCGAATTTTGATAAGGC 3'
E ²³² /A	5' GCCCTTTTAGATGTCTCTCAAAAGTGACAAAAGCGAATTTTGATAAGGCT 3'
S ²²⁶ -K ²²⁸ -M ²²⁹ -E ²³² /A	5' GCCCTTTTAGATGT <u>CGCT</u> CAAGCGCGGACAAAAGCGAATTTTGATAAGGC 3'
K ²³⁸ /A	5' TGATAAGGCTGCAGAACAAATAAAAAAATGGTTACAACCTTACAGGC 3'
K ²⁴² /A	5' GCTAAAGAACAATAGCAAAAATGGTTACAACCTTAAACAGGCGAGCC 3'
K ²⁴³ /A	5' GAACAAATAAAAGCAATGGTTACAACCTTAAACAGGCGAGCCA 3'
T ²⁴⁷ -T ²⁴⁹ /A	5' AAAATGGTTACAGCTTTAGCAGGCGAGCCAACCTGATGGTAAGGAA 3'
R ²⁶² -N ²⁶³ /A	5' GGTAAGGAAAATCATAATAGGGCTGCTTCTGTACTGTCTAATGACT 3'
R ²⁶⁶ /A	5' CATAATAGGCGTAATTCTGTAGCTCTAATGACTTTTTACCCTAAG 3'
M ²⁶⁸ /A	5' CGTAATCTGTACGTCTAGCGACTTTTTACCCTAAGGTTAGC 3'
R ²⁷² /A	5' GTACGTCTAATGACTTTTTACGCTAAGGTTAGCGATCCGATTGAG 3'
K ²⁹⁷ /A	5' GAAGTTTGGGATCAGGCGCAAAAAGATTGGGACTGGGGT 3'
D ³⁰¹ /A	5' GATCAGGCCAAAAAAGATTGGGCTGGGGTGTGATTTACAAGGC
D ³⁰⁵ /A	5' TGGGACTGGGGTGTGCTTTACAAGGCGCTATC
Q ³⁰⁷ /A	5' GACTGGGGTGTGATTTAGCAGGCGCTATCCATAAAGCT 3'
H ³¹¹ /A	5' GTTGATTTACAAGGCGCTATCGCTAAGGCTCGAGAAAATTTTAAG 3'
R ³²⁶ -H ³²⁸ /A	5' AAGTCAAAAAAGCCCAAGCTATCGTCTGTCTCTCAAGGCGAG 3'
S ³³³ /A	5' CATATCGTCCTGTTGCTCAAGGCGAGTCAACC 3'
E ³³⁶ /A	5' GTCCTGTTCTCTCAAGGCGCTCAACCTTTAGTTATGAC 3'
S ³³⁷ /A	5' GTCCTGTTCTCTCAAGGCGAGGCAACCTTTAGTTATGACATTCAT 3'
T ³⁶¹ -S ³⁶³ /A	5' AGGGTAAATGAAAATATCGCAACTGCTAACCCTGTTCCCTGG 3'
S ⁴⁶² -R ⁴⁶⁴ /A	5' GGGTATTATTACCATAGTTTGTGCTGAAGCGAAAACCTGCTGAAATGCCG 3'
N ⁵³¹ -S ⁵³³ /A	5' GACTATATTTACTACAATCACGCTCTCGCAGCAATAGCTGAAGCC 3'
E ⁵³⁷ /A	5' CTCTCAGCAATAGCTGCAGCCAAAATGGCTCAA 3'
K ⁵³⁹ /A	5' TCAGCAATAGCTGAAGCGCAATGGCTCAACAAGAGGGG 3'
D ⁵⁵² /A	5' GGGGTCACCTTCTATTCCGTTGCTGTTACTGATTTCAACTCAGCT 3'
M ⁵⁹⁷ -S ⁵⁹⁸ -E ⁵⁹⁹ /A	5' GATAATTACTCAAAACAAGCGGCTGCAGGCGGTAAGAA 3'
K ⁶¹⁵ -D ⁶¹⁶ /A	5' GTGGATAAGGCAGAGAATTTCCGAGCTACCCTAGCCAGTGTG 3'
S ⁶⁷⁹ -K ⁶⁸⁰ /A	5' AGTCTCACTTGGACTATTGCCGACAGCAGTTGAAAACAAGCCTTTGAAGAG 3'
Y ⁶⁹⁷ /A	5' GGTAAGCCGCTAACCCCTCACCGCTAAGCTGAAAAGTTGATAAAGAT 3'
K ⁶⁹⁸ /A	5' CCGCTAACCCCTCACCTATGCGCTGAAAGTTGATAAAGAT 3'
L ⁶⁹⁹ /A	5' CCGCTAACCCCTCACCTATAAGGCGAAAAGTTGATAAAGATAAAGAT 3'
V ⁷⁰¹ /A	5' ACCCTCACCTATAAGCTGAAAGCTGATAAAGATAAATTTAGAGAA 3'
L ⁷¹⁰ /A	5' GATAAATTTAGAGAACTGCTAAAAAGCAACAAGAATCTCG 3'
K ⁷²¹ /A	5' GAATCTCGTCGTATAAAGGCACGACGAGCATCTTCGGAAAGT 3'
R ⁷²² /A	5' GAATCTCGTCGTAAAAAGAAAGCAGCAGCATCTTCGGAAAGTGAG 3'
AvrII restriction sites (CCTAGG)	
P ¹⁴⁸ -E ¹⁴⁹ /P-R	5' GCTAATGCTGAGACTGCACCTAGGATGATGATGGACGTGG 3'
K ²¹¹ /R	5' CCTTAGATGTAACCTTAAAGTAATGCCTAGGCAAATGACGAAGGTGCC 3'
T ²³⁰ -K ²³¹ /P-R	5' GATGTCTCTCAAAAGATGCCTAGGGAGAATTTTGATAAGGC 3'
T ²³⁰ -K ²³¹ -E ²³² /T-L-G	5' GATGTCTCTCAAAAGATGACCCTAGGGAATTTTGATAAGGCT 3'
V ²⁸⁵ -A ²⁸⁶ /L-G	5' CCGATTGACCTACTACAAAAACCTAGGTGCTAAATTAAGG 3'
A ³¹³ -R ³¹⁴ -E ³¹⁵ /A-L	5' GGTGTTGATTTACAAGGCGCTATCCATAAGGCCCTAGGAATTTTAAGAAAG 3'