The fibrinogen-binding protein (FgBP) of *Streptococcus equi* subsp. *equi* additionally binds IgG and contributes to virulence in a mouse model

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The major cell-wall-associated protein of the equine pathogen *Streptococcus equi* subsp. *equi* is an M-like fibrinogen-binding protein (FgBP) which binds equine fibrinogen (Fg) avidly, through residues located at the extreme N-terminus of the molecule. In this study, it is shown that FgBP additionally binds equine IgG-Fc. When tested against polyclonal IgG from ten other animal species, it was found that FgBP binds human, rabbit, pig and cat IgG, but does not bind mouse, rat, goat, sheep, cow or chicken IgG. Through the use of a panel of recombinant FgBP truncates containing defined deletions of sequence, it was shown that residues in the central regions of FgBP are important in IgG binding. An *fbp* knockout mutant which does not express FgBP on the cell surface was also constructed. Mutant cells failed to autoaggregate, bound no detectable equine Fg or IgG-Fc, were rapidly killed in horse blood, and showed greatly decreased virulence in a mouse model. Results suggest that FgBP is the major surface structure responsible for binding either Fg or IgG, that the molecule has pronounced antiphagocytic properties, and that it is a likely factor contributing to the virulence of wild-type *S. equi* subsp. *equi*.

Keywords: IgG-binding protein, phagocytosis, autoaggregation, ligand-binding domains, strangles

INTRODUCTION

Lancefield group A, C and G streptococci express on their cell surface a group of fibrous proteins, termed M or M-like proteins, which form α -helical coiled-coil dimers and which bind host serum or matrix proteins such as fibrinogen (Fg), immunoglobulins, albumin, kininogen and plasminogen (reviewed by Navarre & Schneewind, 1999). One or more of these proteins can be expressed by individual bacterial strains. In the case of the group A streptococci there are > 80 serotypes of M proteins, and these have been shown to be major virulence determinants and protective antigens. Although the precise mechanism by which M proteins contribute to virulence is unclear, there is evidence to suggest that M proteins can be involved in adhesion, invasion and evasion of the host defences. Indeed, the

major functional characteristic of these proteins is their ability to confer on streptococci the capacity to resist phagocytosis in whole blood in the absence of specific antibody. This has variously been attributed to: (i) a reduction in deposition of complement C3b on the streptococcal cell surface due to binding by M proteins of fibrinogen and/or complement regulators, e.g. factor H, factor H-like protein and C4b-binding protein; (ii) the promotion of large bacterial aggregates which are more difficult to phagocytose; and (iii) the ability of Fg to compete with streptococcal-associated C3bi binding to CD11b–CD18-expressing cells and prevent phagocytosis (Frick *et al.*, 2000; Kehoe, 1994; Navarre & Schneewind, 1999; Ringdahl *et al.*, 2000).

The Lancefield group C streptococcus *Streptococcus equi* subsp. *equi* is the causative agent of strangles, which is a highly contagious disease of the upper respiratory tract of the family *Equidae* and one of the most frequently reported equine diseases world-wide. The disease is initially characterized by nasal discharge and fever, followed by abscess formation in local lymph

Abbreviations: Em, erythromycin; Fg, fibrinogen; FgBP, fibrinogen-binding protein; HRP, horseradish peroxidase; i.p., intraperitoneal(ly); Km, kanamycin.

nodes. Morbidity rates of up to 100% have been reported, and in up to 10% of cases mortality can occur as a result of disseminated abscessation (bastard strangles; Timoney, 1993).

S. equi has the potential to express at least five wall-associated proteins, viz. two fibronectin-binding proteins (FNE and SFS; Lindmark & Guss, 1999; Lindmark *et al.*, 2001), an α_2 -macroglobulin/albumin/IgG-binding protein (ZAG; Lindmark *et al.*, 1999) and two Fg-binding proteins termed FgBP or SeM and SzPse (Meehan *et al.*, 1998; Timoney *et al.*, 1997). Of these, FgBP is by far the most dominant wall-associated protein expressed by virulent *S. equi* (Meehan *et al.*, 1998). FgBP is a highly immunogenic protein that behaves as a multimer (220 000 Da) during SDS-PAGE, reacts with convalescent horse serum, and is protective in a small animal model against lethal *S. equi* challenge. The sequence of the corresponding gene (*fbp*) has been determined and shown to encode a protein of 534 amino acids (58 344 Da), which possesses some structural and sequence similarities to other streptococcal cell wall proteins. Based on experimentation and computer predictions, these have been shown to include a 36-residue signal sequence, a cell wall/membrane anchoring domain and two blocks (A and B) of degenerate repeated sequences (Meehan *et al.*, 1998, 2000a). In addition, computer-assisted secondary structure analysis predicts that FgBP, like M proteins, possesses a high proportion (over 60%) of α -helical coiled-coil (dimer) structure (Meehan *et al.*, 1998). However, the protein shows little significant sequence similarity to other M(-like) proteins, except for the Fg/IgG-binding DemA protein from *Streptococcus dysgalactiae*, where some similarities in the A-repeat and C-terminal regions have been noted (Vasi *et al.*, 2000).

The region within FgBP required for maximum binding of Fg has been located, using a panel of 20 recombinant hexahistidyl (His₆)-tagged FgBP truncates possessing overlapping N- and C-terminal deletions of sequence (FgBP1–17) and specific internal deletions of the A repeat and/or B repeats (FgBP[A⁻], FgBP[B⁻] and FgBP[A⁻B⁻]). This has been shown to extend over a large aspect (the N-terminal half) of the mature protein. Maximum ligand binding is not dependent on the presence of the A and/or B repeats, but is critically dependent on residues within the first 19 N-terminal amino acids and also on an extended region of (stabilizing) coiled-coil structure (Meehan *et al.*, 1998, 2000a, b). This contrasts with the situation for M1 and M5 proteins of group A streptococci where the centrally located B repeats appear to be critical for Fg binding (Ringdahl *et al.*, 2000).

Previous studies have shown that *S. equi* cells are resistant to non-immune phagocytosis, and there is some indirect evidence that FgBP may be involved (Boschwitz & Timoney, 1994a, b; Chanter *et al.*, 1994). Thus, Timoney and co-workers showed that an isolate of *S. equi* expressing high levels of M protein (very likely FgBP) bound four times more complement C3b and

survived 100-fold better than an unrelated isolate which apparently expressed lower levels of the antigen. In addition, specific antiserum inhibited survival of *S. equi* in whole blood, whereas the presence of fibrinogen enhanced the ability of *S. equi* to resist killing by equine neutrophils (Boschwitz & Timoney, 1994a, b; Chanter *et al.*, 1994).

In this paper we show for the first time that FgBP binds IgG as well as Fg. In addition, we describe the construction of an *fbp* insertion mutant of *S. equi* subsp. *equi* and demonstrate convincingly that FgBP plays a role in resistance to phagocytosis, and in virulence.

METHODS

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are detailed in Table 1. Strains of *S. equi* were grown at 30, 37 or 39 °C as appropriate on Columbia base agar supplemented with 5% (v/v) horse blood, on Todd-Hewitt agar supplemented with 0.2% (w/v) yeast extract (THYE agar), or statically overnight (15 h) in THYE broth supplemented where appropriate with kanamycin (Km; 200 µg ml⁻¹) and erythromycin (Em; 1.5 µg ml⁻¹). For autoaggregation experiments, cells were grown to stationary phase (20–24 h). *Escherichia coli* strains were grown at 37 °C in L-broth (Sambrook *et al.*, 1989) medium or in 2 × YT medium (Lech & Brent, 1997) supplemented, where appropriate, with ampicillin (100 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), tetracycline (10 µg ml⁻¹), Km (25 µg ml⁻¹), Em (400 µg ml⁻¹) and IPTG (1 mM).

Recombinant DNA techniques. Genomic DNA was isolated from *S. equi* TW by a modification (Meehan *et al.*, 1998) of the method of Yu & Ferretti (1989). Plasmid DNA was purified from *E. coli* by the modified alkaline lysis method of Feliciello & Chinali (1993), or with a plasmid purification kit (Qiagen). DNA digestions, ligations, transformations and electroporations were carried out by standard methods (Caparon & Scott, 1991; Sambrook *et al.*, 1989). Southern hybridizations of genomic DNA, digested with *Sac*I and *Nde*I, were carried out at 65 °C in standard hybridization buffer (Boehringer Mannheim). Probes (fragments of *fbp*, pG + host9 and Ω Km2) were labelled with digoxigenin by random priming, as described by the manufacturer (Boehringer Mannheim). Forward (F1, 5'-CCGGAATTCGGTCCTTATCAAATACTG-3'; F2, 5'-GACCAAGCTTAGACCGCTTGGTAGAGTC-TGC-3') and reverse (R1, 5'-GACCAAGCTTCTGCAAG-TTAGCCTCAAGAAG-3'; R2, 5'-GAGCACGGGCCC GTGTTAGAACGATAGTCGCTC-3'; R3, 5'-CGC-GGATCCGCGCAGAAAACCATTAGCC-3') oligonucleotide primers (Sigma-Genosys) complementary to selected *fbp* sequence and to sequence regions upstream and downstream of *fbp* and containing, where appropriate, engineered restriction endonuclease cleavage sites (underlined), were used to amplify *fbp* fragments. Amplified fragments were gel-purified using the Wizard PCR Preps DNA purification kit (Promega).

Construction of *S. equi fbp* mutants. An insertion mutation in the *fbp* gene was constructed by replacement of the central 407 bp of the *fbp* sequence with the Ω Km2 interposon (Fig. 1b; Perez-Casal *et al.*, 1991; Prentki & Krisch, 1984), and involved using the broad-host-range thermosensitive plasmid pG + host9 to mutagenize the wild-type chromosomal copy of *fbp* via a double-crossover integration event. pG + host9

Table 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Relevant genotype or property	Source
Strains		
<i>S. equi</i> TW	Virulent isolate from a case of strangles	Meehan <i>et al.</i> (1998)
<i>S. equi</i> <i>fbp</i> :: Ω Km2	<i>S. equi</i> TW <i>fbp</i> :: Ω Km2; Km ^r Em ^s	This study
<i>S. equi</i> <i>fbp</i> :: Ω Km2(pFBP4)	Complemented derivative of <i>S. equi</i> <i>fbp</i> :: Ω Km2; Km ^r Em ^r Cm ^s	This study
<i>E. coli</i> XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> (F' <i>proAB lacI</i> ^q Z Δ M15 Tn10); Tc ^r ; used to propagate pBluescript and pQE30	Stratagene
<i>E. coli</i> T61	<i>rep⁺ recA</i> (pGK12); Km ^r , Tc ^r ; used to propagate pG + host9	E. Maguin*
Plasmids		
pFBP1	1584 bp ligated PCR fragment carrying Δ <i>fbp</i> 461–866 with an internal <i>Hind</i> III site in pBluescript II SK(+); Ap ^r	This study
pFBP2	2200 bp Ω Km2 interposon inserted into <i>Hind</i> III-digested pFBP1; Ap ^r Km ^r	This study
pFBP3	3784 bp <i>fbp</i> :: Ω Km2 fragment in <i>Eco</i> RI– <i>Apa</i> I-digested pG + host9; Em ^r Km ^r	This study
pFBP4	2063 bp PCR fragment encompassing the complete <i>fbp</i> gene in pVA838; Em ^r Cm ^s	This study
pBluescript II SK(+)	ColE1 <i>oriV lacZ</i> α phagemid cloning vector; Ap ^r	Stratagene
pG + host9	Thermosensitive broad-range cloning vector; Em ^r	Maguin <i>et al.</i> (1996)
pBR322 Ω Km2	pBR322 containing the Ω Km2 interposon; Km ^r Tc ^r	Perez-Casal <i>et al.</i> (1991)
pVA838	<i>E. coli</i> –streptococcal shuttle plasmid; Em ^r Cm ^r	Macrina <i>et al.</i> (1982)
pQE30	Cloning and expression vector to produce N-terminal His ₆ -tagged proteins; Ap ^r	Qiagen

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cannot replicate at temperatures above 35 °C (Maguin *et al.*, 1996). Primers F1 and R1 were used to amplify a DNA fragment corresponding to the first 460 bp of *fbp* (encoding amino acids 1–153 of unprocessed FgBP) together with 323 bp of upstream sequence; primers F2 and R2 were used to amplify a DNA fragment corresponding to the final 739 bp of *fbp* (encoding amino acids 290–534 of unprocessed FgBP) plus 62 bp of downstream sequence. One hundred microlitre PCR reactions were performed in *pfu* polymerase buffer (Promega) containing 3 U *pfu* polymerase, 250 ng of forward and reverse primers, 250 μ M dNTPs and 500 ng *S. equi* genomic DNA. Amplification conditions consisted of 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2.5 min, followed by a final extension at 72 °C for 10 min. Gel-purified PCR fragments were cleaved as appropriate with *Eco*RI, *Hind*III and *Apa*I. The cleaved products were then ligated and cloned into pBluescript cleaved with *Eco*RI and *Apa*I, to generate the plasmid pFBP1. pFBP1 was then cleaved with *Hind*III, filled in with DNA polymerase I large (Klenow) fragment, and ligated with the purified Ω Km2 obtained from *Sma*I-digested pBR322 Ω Km2. The resultant plasmid (pFBP2) was cut with *Eco*RI and *Apa*I, and the 3784 bp *fbp*:: Ω Km2 DNA fragment (Fig. 1b) was gel-purified and then ligated to plasmid pG + host9 (Em^r) digested with *Eco*RI and *Apa*I. The resultant plasmid (pFBP3) was purified, electroporated into *S. equi* TW, and Km^r Em^r transformants were grown at 30 °C in order to select derivatives carrying the replicating plasmid. A double-cross-over event between homologous sequences on pFBP3 and the *S. equi* chromosome was facilitated by a temperature shift to 39 °C, resulting in the loss of pG + host9, integration of Ω Km2 into chromosomal *fbp* and a Km^r Em^s phenotype. For this procedure, an overnight culture of *S. equi* TW(pFBP3) grown at 30 °C was diluted 1:100 into fresh THYE broth, grown for

24 h at 39 °C (subculturing once after 7 h), and finally plated onto THYE agar containing Km. Out of 150 Km^r transformants of *S. equi*, six were found to be Km^r Em^s. PCR experiments using several primer sets covering different regions of *fbp* and Southern hybridization of restriction enzyme digested genomic DNA using probes specific for *fbp*, pG + host9 and the Ω Km2 element confirmed that these six transformants did not possess pG + host9 and were the result of integration of the Ω Km2 element into the *fbp* gene (data not shown). One of these *S. equi* *fbp*:: Ω Km2 mutants was selected for further study.

The stability of the Ω Km2 fragment in *S. equi* *fbp*:: Ω Km2 was determined after growth of *S. equi* *fbp*:: Ω Km2 for about 40 generations in the absence of antibiotic selection, followed by plating onto THYE agar. The resultant colonies were then replica-plated onto agar containing Km. All 600 colonies screened retained the Km^r phenotype. Southern hybridization experiments of restricted genomic DNA from eight randomly selected Km^r colonies showed that all retained the Ω Km2 fragment.

To construct a complemented derivative of *S. equi* *fbp*:: Ω Km2, a DNA fragment encompassing the entire *fbp* gene together with 323 bp of upstream and 136 bp of downstream sequence was amplified using primers F1 and R3, and *S. equi* genomic DNA as a template. Amplification conditions consisted of 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 30 s and extension at 72 °C for 4 min, followed by a final extension at 72 °C for 5 min. The gel-purified product was cleaved with *Eco*RI and *Bam*HI and cloned into pVA838 cleaved with *Eco*RI and *Bam*HI, to generate pFBP4. pFBP4 was transformed into *S. equi* *fbp*:: Ω Km2 by electroporation and complemented derivatives possessing a Km^r Em^r phenotype were selected.

Phagocytosis assay. Actively growing cultures of *S. equi* derivatives ($OD_{600} \sim 0.14$) were diluted in THYE broth to approximately $1000\text{--}3000$ c.f.u. ml^{-1} . Aliquots ($100\text{ }\mu\text{l}$) were added to $800\text{ }\mu\text{l}$ of either heparinized horse blood or plasma (from horses with no history of strangles) and the suspensions were incubated at $37\text{ }^{\circ}\text{C}$ for 3 h, with end-over-end rotation. One hundred microlitre aliquots of the resultant suspensions were then plated onto blood agar and incubated at $37\text{ }^{\circ}\text{C}$ to determine viable counts, as described by Lancefield (1962).

Mouse challenge experiments. All animal experiments were performed in compliance with EC directive 86/609/EC as implemented in Ireland under Statutory Instrument 17/94. Groups of Laca mice (12 weeks old) were challenged by intraperitoneal (i.p.) injection of $200\text{ }\mu\text{l}$ vols PBS containing either *S. equi* TW or *S. equi fbp::\Omega\text{Km}2*. Mice were monitored for 30 d post challenge and, for humane reasons, were killed if considered terminally ill with body temperatures below $32\text{ }^{\circ}\text{C}$. Survival times among the challenge groups were analysed statistically using the Mann–Whitney U-test.

Isolation of bacterial cell envelopes and purification of FgBP and recombinant FgBP truncates. Bacterial cell envelopes were isolated from stationary-phase *S. equi* cells, using a method based on French pressure cell lysis, as described previously (Meehan *et al.*, 1998). Native (wild-type) FgBP was purified from mutanolysin extracts of *S. equi* envelopes using horse Fg affinity chromatography, as previously described by Meehan *et al.* (1998). The construction of recombinant plasmids expressing different affinity-tagged FgBP truncates (FgBP1–17, FgBP[A[−]], FgBP[B[−]] and FgBP[A[−]B[−]]) has been described in detail elsewhere. Briefly, this involved ligation of appropriate PCR-amplified *fbp* fragments into the pQE30 plasmid vector. His₆-tagged FgBP derivatives were then purified from the soluble fractions of transformed *E. coli* XL-1 Blue lysates by metal-chelate affinity chromatography in the presence of protease inhibitors (Meehan *et al.*, 1998, 2000a, b; see Fig. 1a).

Biochemical procedures. SDS-PAGE was performed using both 7.5% and 12.5% (w/v) polyacrylamide separating gels and a 4.5% (w/v) polyacrylamide stacking gel, as described by Laemmli (1970). Samples were routinely heated for 3 min at $100\text{ }^{\circ}\text{C}$ in sample buffer (Laemmli, 1970) prior to electrophoresis. Proteins were detected by staining with Coomassie brilliant blue. Molecular masses were determined from the relative mobilities of 15 standard molecular mass marker proteins (BenchMark protein ladder; Gibco-BRL). Protein concentrations of purified FgBP truncates and of cell envelope preparations of *S. equi* strains were estimated by a modification (Dulley & Grieve, 1975) of the Lowry method, using BSA as a standard. The protein concentrations of IgG solutions were based on details provided by the suppliers.

Immunochemical and affinity procedures. Test tube precipitation experiments were performed using a modification of the immunoprecipitation technique described by Doherty *et al.* (1986). Purified IgGs ($100\text{ }\mu\text{g}$), in 50 mM sodium phosphate (pH 7.4) containing 0.1 M NaCl, were preincubated for 1 h at $25\text{ }^{\circ}\text{C}$ with Complete protease inhibitor cocktail (Roche) and then centrifuged (13000 g , 15 min, $20\text{ }^{\circ}\text{C}$). Purified FgBP truncate ($20\text{ }\mu\text{g}$) and Triton X-100 (final concn 2%, v/v) were added to the cleared supernatant, and the solutions were mixed and incubated for 4 h at $25\text{ }^{\circ}\text{C}$. The resultant affinity precipitates were harvested by centrifugation (13000 g , 15 min, $20\text{ }^{\circ}\text{C}$), washed twice in $200\text{ }\mu\text{l}$ 0.1 M NaCl containing 0.5% (v/v) Triton X-100, and finally resuspended in Laemmli sample buffer with and without 2-mercaptoethanol (Laemmli, 1970). Affinity diffusion assays were conducted as described previously (Meehan *et al.*, 1998).

Western immunoblotting was done as described by Caffrey *et al.* (1988) using the following reagents: 5% (w/v) dried skimmed milk as blocking reagent; mouse anti-recombinant FgBP1 (1:30000 dilution) as primary antibody; and horse radish peroxidase (HRP)-labelled affinity-purified goat anti-mouse IgG as localizing antibody. Fg-affinity blotting using HRP-labelled horse Fg in 2% (w/v) dried skimmed milk was performed as described previously (Meehan *et al.*, 1998, 2000a). For IgG-affinity blotting, nitrocellulose blots were blocked with a solution of 2.5% (w/v) dried skimmed milk followed by incubation, as appropriate, with unlabelled horse IgG-Fab, unlabelled or HRP-labelled horse IgG-Fc, HRP-labelled Fc of human or rabbit origin, or HRP labelled Fab fragments of either human or rabbit origin ($2\text{--}10\text{ }\mu\text{g ml}^{-1}$). Where appropriate HRP-labelled affinity-purified goat anti-horse IgG (H+L) was used as localizing antibody (Meehan *et al.*, 1998). For FgBP1-affinity dot blot experiments, IgGs were probed with HRP-labelled FgBP1 ($10\text{ }\mu\text{g ml}^{-1}$). In whole-cell dot blots, *S. equi* suspensions ($OD_{600} 0.4$) were subjected to doubling dilutions and $150\text{ }\mu\text{l}$ aliquots were transferred onto nitrocellulose using the Bio-Dot apparatus (Bio-Rad). Dried blots were then blocked and developed as described above for Western immunoblotting or equine Fg/IgG affinity electroblots. All peroxidase labelled probes were detected using 4-chloro-1-naphthol as developing reagent (Meehan *et al.*, 1998). For immunofluorescence microscopy (Henderson *et al.*, 1997), glutaraldehyde-fixed bacterial cells were probed with mouse anti-FgBP1 antiserum (1:400 dilution) followed by FITC-conjugated goat anti-mouse immunoglobulins. All experimentation involving Western and dot immunoblotting, immunoprecipitation and immunofluorescence microscopy was repeated two to four times to ensure reproducibility, and representative data are shown in Figs 2–5.

It is appropriate to stress that the antibody-based detection systems for FgBP described above (viz. Western immunoblotting and immunofluorescence microscopy) utilized, as primary and secondary probes, antibodies from animal species (mouse and goat, respectively) which failed to show non-immune reactions with FgBP.

Immunochemical and affinity reagents. Bovine IgG was from Chemicon. All other purified IgGs, together with horse Fg and HRP- and FITC-conjugated affinity-purified goat anti-mouse immunoglobulins (Fab-specific), were from Sigma. Goat anti-horse IgG (H+L) was obtained from ICN. Unlabelled horse IgG-Fc/Fab and HRP-labelled human and rabbit IgG-Fc/Fab fragments were obtained from Jackson ImmunoResearch Laboratories. Horse Fg, horse IgG-Fc and FgBP1 were labelled with HRP, as previously described (Meehan *et al.*, 1998). Mouse anti-FgBP1 was generated by immunizing mice subcutaneously on days 0 and 28 with $50\text{ }\mu\text{g}$ purified FgBP1 emulsified in $200\text{ }\mu\text{l}$ MPL+S-TDCM Ribi adjuvant (active ingredients monophosphoryl lipid A and trehalose dimycolate; RIBI Immunochem Research). On day 35, mice were exsanguinated and serum was obtained.

RESULTS

FgBP binds IgG

Agar gel-diffusion and test tube precipitation experiments performed by incubating recombinant FgBP1 or FgBP12 (which lacks the 19 N-terminal residues critical for Fg binding; Fig. 1a) with purified IgGs from different animal species clearly showed that FgBP is capable of forming insoluble complexes with horse, human, rabbit,

Fig. 1. Schematic representation of (a) FgBP derivatives and (b) the *fbp::ΩKm2* DNA fragment used to construct *S. equi fbp* mutants. (a) The respective amino acid positions of the signal sequence (S), the A and B repeats and the cell wall/membrane-spanning domain (W/M) are shown in the structure of pre-FgBP. Below the primary structure of FgBP are scaled representations of the mature (wall-associated) FgBP (N/C-) and of the principal recombinant FgBP truncates used in this study, together with their Fg-binding abilities. Fg-binding ability equivalent to, or half that of wild-type is indicated by + + + + +. Four- to eightfold, 16-fold, 32-fold, 64-fold and ≥ 128 -fold reductions in Fg-binding potential are indicated by + + + +, + + +, + +, + and -, respectively (Meehan *et al.*, 2000a, b). (b) The position (in bp) of the Ω Km2 element in relation to the *fbp* coding sequence (hatched boxes) and flanking sequence (open boxes) is indicated.

pig and cat IgG, but not with IgG from mouse, rat, goat, sheep, cow or chicken (see Fig. 2a, b). These results were confirmed in affinity dot blotting experiments in which the various IgGs were probed with HRP-conjugated FgBP1 (data not shown). Since *S. equi* does not cause natural infection in animal species other than the *Equidae*, the results strongly suggest that FgBP is capable of binding IgG via a non-immune mechanism. However, a cautionary note has to be struck in relation to (commercial) horse IgG because of the uncertainties regarding the medical history of donor animals (i.e. whether they had a history of strangles).

Most other IgG-binding proteins, such as protein A from *Staphylococcus aureus*, protein H from *Streptococcus pyogenes*, and protein G from group C and G streptococci, are known to bind to the Fc region of IgG (Frick *et al.*, 1992). To ascertain whether FgBP binds equine IgG by a similar non-immune mechanism, Western blots were performed using FgBP1 probed with either purified Fab or Fc fragments of horse/human/rabbit IgG. The results showed that FgBP1 bound strongly to Fc fragments of equine, human and rabbit origin. In contrast, FgBP1 showed only faint reactions with equine Fab fragments and showed no detectable binding to either human or rabbit Fab fragments (Fig. 2c). These results were confirmed using affinity dot blots and ELISA tests (data not shown). Whether the faint reaction observed between equine IgG-Fab and FgBP1 is

a consequence of immune or non-immune binding remains to be determined.

Localization of the IgG-binding domain

To localize the equine IgG-Fc binding domain within FgBP, use was made of a panel of 20 recombinant FgBP proteins containing defined N-terminal, C-terminal and internal deletions of sequence (Meehan *et al.*, 2000a, b; Fig. 1a). Initial Western IgG-affinity blotting experiments showed that FgBP3 and FgBP4 bound equine IgG-Fc weakly or not at all and that the native protein and remaining truncates (Fig. 1a) all bound detectable levels of equine IgG-Fc (data not shown). In order to place these observations on a more quantitative basis and to directly relate IgG-Fc binding with comparable Fg-affinity experiments (Meehan *et al.*, 2000a, b), IgG-affinity dot blots were performed using undenatured FgBPs (Fig. 3). These semi-quantitative experiments confirmed the general trend observed during Western affinity blotting and revealed the following in the absence of SDS: (a) wild-type FgBP, FgBP1 (which lacks the wall/membrane anchor domain), FgBP2 (missing 113 amino acids from its C-terminus), FgBP12–13 (missing 19 and 34 residues, respectively, from the N-terminus) and FgBP[A⁻], FgBP[B⁻] and FgBP[A⁻B⁻] all bound similar levels of equine IgG-Fc to a first approximation; (b) FgBP3 (missing 182 amino acids from

Fig. 2. IgG-binding by purified recombinant FgBP. (a) SDS-PAGE analysis of affinity precipitates obtained following incubation of FgBP12 with purified IgG from different animal species. FgBP12 was used as it lacks the 19 N-terminal amino acid residues critical for Fg binding and is clearly resolvable from unreduced IgG on SDS-PAGE gels. An analogous profile of IgG precipitation was obtained with FgBP1 (data not shown). Samples were analysed on a 7.5% (w/v) polyacrylamide separating gel in the absence of 2-mercaptoethanol. Lanes: 1, FgBP12 (4 µg); 2, equine IgG (8 µg); 3, washed precipitate obtained following incubation of FgBP12 with equine IgG; 4, washed precipitate obtained following incubation of either FgBP12 only or equine IgG only in precipitation buffer; 5, washed precipitate obtained following incubation of FgBP12 with mouse IgG [similar (negative) reactions were observed for IgGs from rat, goat, sheep, cow and chicken (not shown)]; 6–9, washed precipitates obtained following incubation of FgBP12 with IgGs from rabbit, human, pig and cat, respectively. (b) IgG-affinity agarose diffusion experiment conducted with FgBP1 (15 µg, central well) and IgGs (38 µg) from horse, rabbit, human, pig, cat and sheep (clockwise from top well; for clarity the middle of each well is indicated with a dot). Equine IgG-Fc gave a similar (positive) reaction to that observed for equine IgG. IgGs from mouse, rat, goat, cow and chicken gave similar (negative) reactions to that observed for sheep IgG (not shown). (c) Western affinity blot of FgBP1 (2 µg) probed with equine IgG-Fab (lane 1), equine IgG-Fc (lane 2), human IgG-Fab (lane 3) and human IgG-Fc (lane 4). Rabbit IgG-Fab and rabbit IgG-Fc fragments gave identical reactions to those observed for the corresponding fragments of human origin (not shown). The positions of the 200 kDa and 90 kDa forms of FgBP1 are indicated.

its C-terminus) bound 32-fold less IgG-Fc than FgBP1; (c) FgBP4 (missing 255 residues from the C-terminus) bound no detectable IgG-Fc; (d) N-terminal truncates FgBP14, 15 and 16 (which have 70, 102 and 148 residues, respectively, deleted from their N-termini) bound approximately fourfold less IgG-Fc than their FgBP2 control; and (e) FgBP17 (missing 182 N-terminal residues) bound approximately 32-fold less IgG-Fc than its FgBP2 control. Very similar results were obtained when blots were probed with human IgG-Fc; the major difference being that FgBP3 exhibited wild-type binding to human IgG-Fc (data not shown). These data suggest that a large central region of FgBP encompassing residues 185–421 and including the A and B repeats is important for IgG-Fc binding, but that neither the A nor B repeats are essential for that binding.

Fig. 3. Equine IgG-Fc-affinity dot blot analysis of undenatured FgBP truncates. The first well in each row of doubling dilutions contains 60 pmol truncate. Sample identity is indicated at the side of the blot with FgBPwt representing wild-type FgBP. Samples were probed with HRP-labelled equine IgG-Fc. FgBPs 14–16 gave identical profiles.

Construction and characterization of *S. equi* *fbp::ΩKm2*

Insertional inactivation of the *fbp* gene was accomplished by replacement of the central 406 bp with the ΩKm2 element (Perez-Casal *et al.*, 1991; Prentki & Krisch, 1984; Methods section and Fig. 1b). The Ω-interposon is stably maintained in the chromosome of *S. equi* in the absence of selective pressure and contains strong terminators of transcription and translation. Theoretically, this construct could secrete a 12.9 kDa FgBP truncate lacking the C-terminal three-quarters of the native protein. However, several lines of evidence confirm that *S. equi fbp::ΩKm2* no longer expresses any FgBP and that, under laboratory conditions, FgBP is likely to be the major Fg-binding protein expressed by wild-type *S. equi*. Firstly, SDS-PAGE and Western immuno/affinity blot analyses of mutanolysin-extracted bacterial cell envelopes showed that wild-type *S. equi*, but not *S. equi fbp::ΩKm2*, possessed the mature wall-associated 220 kDa FgBP which reacted with specific antiserum to FgBP and bound Fg. Secondly, no cross-reacting FgBP polypeptides or any Fg-binding protein could be detected in the envelopes of the *fbp* mutant strain (Fig. 4) or in the soluble cytoplasmic extracts and culture supernatants of either the wild-type or an isogenic mutant (data not shown). Finally and im-

Fig. 4. SDS-PAGE, Western immunoblotting and Fg-affinity blot analysis of mutanolysin-extracted *S. equi* cell envelopes. Mutanolysin-extracted bacterial cell envelopes (25 µg protein) of *S. equi* (lanes 1, 4 and 7), *S. equi fbp::ΩKm2* (lanes 2, 5 and 8) and the complemented derivative *S. equi fbp::ΩKm2(pFBP4)* (lanes 3, 6 and 9) were analysed by SDS-PAGE using 12.5% (w/v) polyacrylamide separating gels. Lanes 1–3 were stained with Coomassie brilliant blue. Lanes 4–9 were electrotransferred onto nitrocellulose and probed with mouse anti-recombinant FgBP1 (lanes 4–6) or HRP-conjugated horse Fg (lanes 7–9). The position and apparent molecular mass (in kDa) of FgBP are indicated.

(a)

(b)

(c)

Fig. 5. Analysis of *S. equi* and *S. equi fbp::ΩKm2* by immunofluorescence microscopy, affinity dot blotting and test tube autoaggregation. (a) Immunofluorescent photomicrographs of exponentially growing cultures of *S. equi* (left) and *S. equi fbp::ΩKm2* (right). Cells were probed with mouse anti-recombinant FgBP1 followed by fluorescein-labelled anti-mouse immunoglobulin. Note that all of the *S. equi* cells, but none of the *S. equi fbp::ΩKm2* cells, react with the specific anti-FgBP1 antiserum and that the wild-type, but not the mutant, shows a tendency to clump. (b) Fg-affinity and equine IgG-Fc-affinity dot blots of doubling dilutions of whole cells of *S. equi* (rows 1 and 3) and *S. equi fbp::ΩKm2* (rows 2 and 4). Bacterial cells were probed directly with HRP-labelled horse Fg (rows 1 and 2) or HRP-labelled equine IgG-Fc (rows 3 and 4). (c) *S. equi* (left) and *S. equi fbp::ΩKm2* (right) were grown overnight to stationary phase at 37 °C, resuspended and allowed to settle for 2 h at room temperature.

portantly, immunofluorescence microscopy and whole-cell dot blot analysis performed with specific anti-FgBP1 serum, horse Fg or horse IgG-Fc revealed that *S. equi fbp::ΩKm2* cells, in contrast to those of the wild-type, no longer express FgBP on their cell surface and do not bind detectable levels of either Fg or IgG-Fc (Fig. 5a, b). It should also be noted that both *S. equi* and its *fbp::ΩKm2* derivative were encapsulated and grew at the same rate in broth culture (data not shown). However, a characteristic feature of the *fbp* mutant was its failure to autoaggregate in liquid media during stationary phase in a manner observed for wild-type

cells (Fig. 5c). This phenomenon was also evident but to a less dramatic extent in exponential phase (compare Fig. 5a left and right panels).

Analogous experimentation conducted with the complemented derivative *S. equi fbp::ΩKm2(pFBP4)* revealed that it expressed mature functional FgBP as anticipated, but at considerably lower levels than the wild-type strain (Fig. 4; lanes 3, 6 and 9). This was confirmed by immunofluorescence microscopy, whole-cell dot blots and settling experiments (data not shown). The reason for the low expression is unclear, but was a

Table 2. Survival of *S. equi* and *S. equi fbp::ΩKm2* in horse blood

Assays were performed in triplicate and are representative of experiments performed on four other occasions.

Bacterial strain*	Initial inoculum (c.f.u. ml ⁻¹)	Final count in:	
		Whole blood (c.f.u. ml ⁻¹)*	Plasma (c.f.u. ml ⁻¹)*
<i>S. equi</i>			
Donor 1	1.43 ± 0.28 × 10 ²	3.20 ± 0.67 × 10 ³	2.58 ± 0.72 × 10 ³
Donor 2	1.42 ± 0.08 × 10 ²	3.33 ± 0.26 × 10 ³	1.82 ± 0.48 × 10 ³
<i>S. equi fbp::ΩKm2</i>			
Donor 1	2.35 ± 0.33 × 10 ²	0	3.16 ± 0.56 × 10 ³
Donor 2	1.97 ± 0.17 × 10 ²	0	1.35 ± 0.22 × 10 ³

* Donor 1 and 2 indicates the identity of the horse from which blood was obtained in the experiment.

consistent feature in attempts to create such constructs. pVA838 does not appear to affect parental levels of FgBP, since *S. equi* TW(pVA838) expressed wild-type levels of the protein as determined by immunofluorescence microscopy and whole-cell dot blot analysis (data not shown). Furthermore, the complemented mutant harboured replicating recombinant pVA838 with full length *fbp* as evidenced by: (a) Southern blot analysis of restriction enzyme digested-genomic DNA of *S. equi fbp::ΩKm2*(pFBP4) using pVA838- and *fbp*-specific probes; (b) PCR analysis of genomic DNA of *S. equi fbp::ΩKm2*(pFBP4); (c) restriction enzyme digestion analysis of pFBP4 purified from *S. equi fbp::ΩKm2*(pFBP4); and (d) retention of appropriate antibiotic resistance markers. Moreover, there was no evidence of any recombination between recombinant pVA838 and the chromosomal copy of *fbp* (data not shown). Others in the field have also experienced the problem of poor expression of surface proteins from complemented streptococcal derivatives (Kihlberg *et al.*, 1999).

FgBP is antiphagocytic

To assess whether FgBP plays a role in resistance of *S. equi* to phagocytosis, the ability of wild-type *S. equi* TW to survive and grow in non-immune horse blood was compared with that of its isogenic *fbp::ΩKm2* derivative (Table 2). The experiment was conducted using blood from two different donor horses that had no history of strangles. As expected both strains grew well in horse plasma. In contrast, only wild-type *S. equi* survived and grew (equally well) in whole blood. The *fbp::ΩKm2* derivative lacking FgBP was killed. As anticipated (Boschwitz & Timoney, 1994b; Timoney *et al.*, 1997), the survival of wild-type *S. equi* was reduced substantially (9- to 45-fold) following the addition of (opsonizing) rabbit anti-FgBP antibodies (data not shown). From this it can be concluded that FgBP plays an important role in resistance of *S. equi* to killing by phagocytes (and/or other blood factors) in the absence of specific antibody.

Fig. 6. Virulence of *S. equi* TW and *S. equi* TW *fbp::ΩKm2* in mice. Groups of five mice were challenged (i.p.) with 3.3×10^5 c.f.u. *S. equi* TW (○), or with 2.5×10^5 c.f.u. (△), 2.5×10^6 c.f.u. (△) or 2.5×10^7 c.f.u. (□) *S. equi* TW *fbp::ΩKm2*. The response of mice to challenge with 2.5×10^5 and 2.5×10^6 c.f.u. *S. equi* TW *fbp::ΩKm2* was identical. The level of significance between the test (*S. equi* TW *fbp::ΩKm2*) and control (*S. equi* TW) challenge groups was $P \leq 0.01$.

FgBP contributes to virulence in mice

It has been previously shown that *S. equi* is virulent in mice and that vaccination with purified FgBP can protect against i.p. infection (Meehan *et al.*, 1998). In order to determine whether FgBP plays a role in virulence, the ability of *S. equi* to cause lethal infection of mice was compared with that of the *fbp* knockout mutant (*S. equi fbp::ΩKm2*). The results of these experiments (Fig. 6) show clearly that the *fbp* mutant is considerably more attenuated than wild-type *S. equi*. Thus, all mice challenged with up to 2.5×10^6 c.f.u. of *S. equi fbp::ΩKm2* remained healthy and survived for over 30 d, whereas mice challenged with a 7.6-fold lower dose of virulent *S. equi* all died within 5 d. Raising the i.p. challenge dose to 2.5×10^7 c.f.u. of *S. equi fbp::ΩKm2* resulted in 60% mortality with an extended time to death (Fig. 6).

DISCUSSION

One of the principal observations of this study is that FgBP of *S. equi* binds IgG as well as Fg, and does so by a non-immune mechanism involving the Fc aspect of IgG. FgBP somewhat resembles the Mrp proteins of group A streptococci in its ligand-binding properties (Navarre & Schneewind, 1999), although FgBP appears to lack the pronounced glutamate/glutamine-rich EQ domain observed for Mrp (and class II Emm) proteins (Pack *et al.*, 1996). FgBP also shows a rather unique profile of IgG binding compared to other IgG binding proteins, showing strong affinity for horse, human, rabbit, pig and cat IgG, but no observable binding to rat, mouse, goat, sheep, bovine and chicken IgG (Åkesson *et al.*, 1994; Björck & Kronvall, 1984; Gomi *et al.*, 1990; Jonsson *et al.*, 1995; Lindmark *et al.*, 1983). Preliminary competition experiments confirm that FgBP probably binds to the same region (C γ 2–C γ 3 interface) of IgG-Fc as other IgG-binding proteins, such as protein A, protein G and protein H (data not shown).

The binding of Fg and IgG to FgBP clearly requires distinct structural features. Binding of Fg is critically dependent on the N-terminal 19 aa and additionally requires a significant stretch of (stabilizing) downstream coiled-coil structure (Meehan *et al.*, 2000a, b). In contrast, IgG-Fc binding does not appear to require residues at the immediate N-terminus, but instead is dependent on a sizeable central domain of FgBP which encompasses both the A and B repeat regions. However, in common with the situation for Fg, it is evident that neither the A nor B repeats regions are essential for IgG-Fc binding. In other IgG-binding proteins, the actual location of and residues involved in IgG binding varies. Thus, IgG binding is located closer to the N-terminus in the case of protein H and to the C-terminus for protein G. Furthermore, the IgG-binding domains in protein A and protein G are located in repeat regions, whereas protein H and M1 protein appear to have two separate binding sites for IgG which are not present in repeat domains and which appear to bind to different human IgG subsets (Åkerström *et al.*, 1987; Frick *et al.*, 1994; Navarre & Schneewind, 1999; Raeder *et al.*, 1998; Uhlén *et al.*, 1984). Searches of databases for homologies to the amino acid sequence of the putative IgG-binding domain of FgBP reveals significant similarities only with (a) a new protective antigen (Spa) of group A streptococci which shows a curious and remarkable (98%) homology with *S. equi* FgBP over the C-terminal half of the molecule (Dale *et al.*, 1999; GenBank accession no. AF0876813), and (b) the central regions of the Fg/IgG-binding protein DemA from *S. dysgalactiae*. Perhaps significantly, efficient binding of bovine IgG to DemA appears to require this region of the protein (Vasi *et al.*, 2000).

Ligand-binding studies performed with *S. equi* and its *fbp* insertion mutant provide convincing evidence that FgBP is the dominant surface antigen responsible for the binding of both Fg and IgG by wild-type cells. This confirms and extends previous studies showing that

FgBP in whole cells is accessible to both specific antiserum and Fg and that it is the major Fg-binding protein in mutanolysin-extracted bacterial cell envelopes (Meehan *et al.*, 1998; Timoney *et al.*, 1997). *S. equi* has the potential to express other surface proteins capable of binding Fg (viz. SzPse; Timoney *et al.*, 1997) or IgG (ZAG; Jonsson *et al.*, 1995; Lindmark *et al.*, 1999). However, these are clearly relatively minor ligand-binding species under the conditions of laboratory growth, since *S. equi fbp:: Ω Km2* binds no detectable Fg and IgG-Fc.

Previous studies have provided evidence that the M protein of *S. equi* is antiphagocytic (see Introduction). Here, through use of an isogenic *fbp* derivative, we show conclusively and for the first time that FgBP contributes in a major way to the survival of *S. equi* in whole horse blood. The precise mechanism by which this occurs remains to be elucidated. There is evidence that reduction in C3b deposition and Fg binding contribute to the ability of *S. equi* to resist killing by equine neutrophils (Boschwitz & Timoney, 1994a, b; Chanter *et al.*, 1994), although in our own hands the levels of enhanced survival in the presence of Fg are marginal (data not shown). The ability of FgBP to bind IgG-Fc suggests another possible mechanism for resistance to phagocytosis. Certainly, for the clinically important (M1) serotype of group A streptococcus it has been convincingly established that the IgG-binding protein H can block C3 deposition due to inhibition of the classical complement pathway, and that the presence of either protein H or the Fg-binding M1 protein on the cell surface is sufficient for survival in human blood (Berge *et al.*, 1997; Kihlberg *et al.*, 1999). In other cases, reduction in C3b deposition has been attributed to the ability of M proteins to bind complement regulators (see Introduction).

Some streptococcal M proteins can also mediate bacterial autoaggregation, and this property has been shown to be crucial for adherence and for resistance to phagocytosis (Frick *et al.*, 2000). Such a mechanism cannot be ruled out for virulent *S. equi*. Comparative microscopic and sedimentation analysis shows that wild-type *S. equi* routinely grows as large sedimentable aggregates, whereas the *fbp:: Ω Km2* knock-out mutant does not (see Fig. 5; data not shown). FgBP does not possess the conserved 19 amino acid residue (AHP) sequence which has been implicated in the homophilic protein-protein interactions responsible for formation of *S. pyogenes* aggregates (Frick *et al.*, 2000); however, it can form apparent multimers under certain conditions and the B-repeats of FgBP have been heavily implicated in this process (Meehan *et al.*, 2000b).

It is relevant to note that other streptococcal surface components, such as the hyaluronic acid capsule, have also been implicated in resistance to phagocytosis. In the case of *S. equi*, strains expressing lower amounts of capsule seem to be more susceptible to phagocytosis and to cause a less invasive disease than heavily capsulated strains (Anzai *et al.*, 1999b). The virulent strain of *S.*

equi TW used in the current studies does express capsule under conditions of laboratory growth; however, it is clear from the experimental data that the level of capsule produced is insufficient to prevent efficient phagocytic killing in the absence of both FgBP and specific antibody.

The M-like protein (FgBP) of *S. equi* has been recognized for a number of years as a likely virulence determinant. It is structurally and functionally similar to the well-studied M protein virulence factors of group A streptococci, possesses anti-phagocytic properties (see above), and elicits strong serum and mucosal antibody responses in infected horses. However, direct evidence that FgBP contributes to virulence has been lacking until now. The results of the small animal experiments conducted with *S. equi* and *S. equi fbp::ΩKm2* provide compelling evidence that this is indeed the case. Supporting evidence that FgBP is important in *S. equi* infection of the target species comes from a recent study of horses that were outwardly healthy but were shown to be persistent carriers of *S. equi*. Streptococcal isolates from about 25% of these carriers expressed truncated forms of FgBP (lacking the N-terminal Fg-binding region) and were more sensitive to phagocytosis (Chanter *et al.*, 2000). Clearly, the balance of evidence presented here and elsewhere strongly suggests that the antiphagocytic properties of FgBP contribute to the virulence of *S. equi*. This is not to exclude other possible roles. Certainly, there is a multitude of studies suggesting that Fg- and/or IgG-binding M proteins of group A streptococci are involved in adhesion, invasion and colonization (Cleary & Retnoningrum, 1994; Dombek *et al.*, 1999; Molinari & Chhatwal, 1999; Navarre & Schneewind, 1999). Furthermore, wall-anchored M protein has recently been implicated in the maturation of cysteine proteinase (Collin & Olsén, 2000). In turn, cysteine proteinase has been shown to release, from the cell surface, biologically active fragments of M protein and protein H-IgG complexes (Berge & Björck, 1995; Berge *et al.*, 1997). It has been suggested that the deposition of the protein H-IgG complexes onto body organs may cause localized inflammation and tissue damage, and thus contribute to severe complications of suppurative *S. pyogenes* infection. Interestingly, purpura haemorrhagica, a fatal sequel to strangles, has been proposed to be an immune-complex-mediated disease (Galán & Timoney, 1985). Clearly the role of FgBP and its IgG-binding properties in complications associated with this disease appear to warrant further investigation. Challenge trials in horses using *S. equi fbp::ΩKm2* and similar derivatives should also provide a further insight into the role of FgBP and other potential *S. equi* virulence factors in the disease process (Anzai *et al.*, 1999a, b; Chanter *et al.*, 1999; Flanagan *et al.*, 1998; Harrington *et al.*, 2000; Muhktar & Timoney, 1988).

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REFERENCES

- Åkerström, B., Nielsen, B. & Björck, L. (1987). Definition of IgG- and albumin-binding regions of streptococcal protein G. *J Biol Chem* **262**, 13388–13391.
- Åkesson, P., Schmidt, K.-H., Cooney, J. & Björck, L. (1994). M1 protein and protein H: IgG- and albumin-binding streptococcal surface proteins encoded by adjacent genes. *Biochem J* **300**, 877–886.
- Anzai, T., Sheoran, A. S., Kuwamoto, Y., Kondo, T., Wada, R., Inoue, T. & Timoney, J. F. (1999a). *Streptococcus equi* but not *Streptococcus zooepidemicus* produces potent mitogenic responses from equine peripheral blood mononuclear cells. *Vet Immunol Immunopathol* **67**, 235–246.
- Anzai, T., Timoney, J. F., Kuwamoto, Y., Fujita, Y., Wada, R. & Inoue, T. (1999b). *In vivo* pathogenicity and resistance to phagocytosis of *Streptococcus equi* strains with different levels of capsule expression. *Vet Microbiol* **67**, 277–286.
- Berge, A. & Björck, L. (1995). Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. *J Biol Chem* **270**, 9862–9867.
- Berge, A., Kihlberg, B.-M., Sjöholm, A. G. & Björck, L. (1997). Streptococcal protein H forms soluble complement-activating complexes with IgG, but inhibits complement activation by IgG-coated targets. *J Biol Chem* **272**, 20774–20781.
- Björck, L. & Kronvall, G. (1984). Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. *J Immunol* **133**, 969–974.
- Boschwitz, J. S. & Timoney, J. F. (1994a). Characterization of the antiphagocytic activity of equine fibrinogen for *Streptococcus equi* subsp. *equi*. *Microb Pathog* **17**, 121–129.
- Boschwitz, J. S. & Timoney, J. F. (1994b). Inhibition of C3 deposition on *Streptococcus equi* subsp. *equi* by M protein: a mechanism for survival in equine blood. *Infect Immun* **62**, 3515–3520.
- Caffrey, P., McVeigh, T. & Owen, P. (1988). Western immunoblotting. In *Immunochemical and Molecular Genetic Analysis of Bacterial Pathogens*, pp. 255–266. Edited by P. Owen & T. J. Foster. Amsterdam: Elsevier.
- Caparon, M. G. & Scott, J. R. (1991). Genetic manipulation of pathogenic streptococci. *Methods Enzymol* **204**, 556–586.
- Chanter, N., Collin, N. C. & Mumford, J. A. (1994). Resistance of *Streptococcus equi* *in vitro* to equine polymorphonuclear leucocytes. In *Equine Infectious Diseases VII: Proceedings of the Seventh International Conference, Tokyo, Japan, 8th–11th June 1994*, pp. 201–210. Edited by H. Nakajima & W. Plowright. Newmarket: R & W Publications.
- Chanter, N., Ward, C. L., Talbot, N. C., Flanagan, J. A., Binns, M., Houghton, S. B., Smith, K. C. & Mumford, J. A. (1999). Recombinant hyaluronate associated protein as a protective immunogen against *Streptococcus equi* and *Streptococcus zooepidemicus* challenge in mice. *Microb Pathog* **27**, 133–143.
- Chanter, N., Talbot, N. C., Newton, J. R., Hewson, D. & Verheyen, K. (2000). *Streptococcus equi* with truncated M-proteins isolated from outwardly healthy horses. *Microbiology* **146**, 1361–1369.
- Cleary, P. & Retnoningrum, D. (1994). Group A streptococcal immunoglobulin-binding proteins: adhesins, molecular mimicry or sensory proteins? *Trends Microbiol* **2**, 131–136.
- Collin, M. & Olsén, A. (2000). Generation of a mature streptococcal

- cysteine proteinase is dependent on cell wall-anchored M1 protein. *Mol Microbiol* **36**, 1306–1318.
- Dale, J. B., Chiang, E. Y., Liu, S., Courtney, H. S. & Hasty, D. L. (1999). New protective antigen of group A streptococci. *J Clin Invest* **103**, 1261–1268.
- Doherty, H., Yamada, H., Caffrey, P. & Owen, P. (1986). Identification, immunochemical characterization, and purification of a major lipoprotein antigen associated with the inner (cytoplasmic) membrane of *Escherichia coli*. *J Bacteriol* **166**, 1072–1082.
- Dombek, P. E., Cue, D., Sedgewick, J., Lam, H., Ruschkowski, S., Finlay, B. B. & Cleary, P. P. (1999). High-frequency intracellular invasion of epithelial cells by serotype M1 group A streptococci: M1 protein-mediated invasion and cytoskeletal rearrangements. *Mol Microbiol* **31**, 859–870.
- Dulley, J. R. & Grieve, P. A. (1975). A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Anal Biochem* **64**, 136–141.
- Feliciello, I. & Chinali, G. (1993). A modified alkaline lysis method for the preparation of highly purified plasmid DNA from *Escherichia coli*. *Anal Biochem* **212**, 394–401.
- Flanagan, J., Collin, N., Timoney, J., Mitchell, T., Mumford, J. A. & Chanter, N. (1998). Characterization of the haemolytic activity of *Streptococcus equi*. *Microb Pathog* **24**, 211–221.
- Frick, I.-M., Wikström, M., Forsén, S., Drakenberg, T., Gomi, H., Sjöbring, U. & Björck, L. (1992). Convergent evolution among immunoglobulin G-binding bacterial proteins. *Proc Natl Acad Sci USA* **89**, 8532–8536.
- Frick, I.-M., Åkesson, P., Cooney, J. & 7 other authors (1994). Protein H – a surface protein of *Streptococcus pyogenes* with separate binding sites for IgG and albumin. *Mol Microbiol* **12**, 143–151.
- Frick, I.-M., Mörgelin, M. & Björck, L. (2000). Virulent aggregates of *Streptococcus pyogenes* are generated by homophilic protein–protein interactions. *Mol Microbiol* **37**, 1232–1247.
- Galán, J. E. & Timoney, J. F. (1985). Immune complexes in purpura hemorrhagica of the horse contain IgA and M antigen of *Streptococcus equi*. *J Immunol* **135**, 3134–3137.
- Gomi, H., Hozumi, T., Hattori, S., Tagawa, C., Kishimoto, F. & Björck, L. (1990). The gene sequence and some properties of protein H. A novel IgG-binding protein. *J Immunol* **144**, 4046–4052.
- Harrington, D. J., Greated, J. S., Chanter, N. & Sutcliffe, I. C. (2000). Identification of lipoprotein homologues of pneumococcal PsaA in the equine pathogens *Streptococcus equi* and *Streptococcus zooepidemicus*. *Infect Immun* **68**, 6048–6051.
- Henderson, I. R., Meehan, M. & Owen, P. (1997). Antigen 43, a phase-variable bipartite outer membrane protein, determines colony morphology and autoaggregation in *Escherichia coli* K-12. *FEMS Microbiol Lett* **149**, 115–120.
- Jonsson, H., Lindmark, H. & Guss, B. (1995). A protein G-related cell surface protein in *Streptococcus zooepidemicus*. *Infect Immun* **63**, 2968–2975.
- Kehoe, M. A. (1994). Cell-wall-associated proteins in Gram-positive bacteria. In *New Comprehensive Biochemistry*, vol. 27, *Bacterial Cell Wall*, pp. 217–261. Edited by J.-M. Ghuysen & R. Hakenbeck. Amsterdam: Elsevier.
- Kihlberg, B.-M., Collin, M., Olsén, A. & Björck, L. (1999). Protein H, an antiphagocytic surface protein in *Streptococcus pyogenes*. *Infect Immun* **67**, 1708–1714.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lancefield, R. C. (1962). Current knowledge of type-specific M antigens of group A streptococci. *J Immunol* **89**, 307–313.
- Lech, K. & Brent, R. (1997). Media preparation and bacteriological tools. In *Current Protocols in Molecular Biology*, pp. 1.1.1–1.1.6. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley.
- Lindmark, H. & Guss, B. (1999). SFS, a novel fibronectin-binding protein from *Streptococcus equi*, inhibits the binding between fibronectin and collagen. *Infect Immun* **67**, 2383–2388.
- Lindmark, H., Jonsson, P., Engvall, E. & Guss, B. (1999). Pulsed-field gel electrophoresis and distribution of the genes *zag* and *fzn* in isolates of *Streptococcus equi*. *Res Vet Sci* **66**, 93–99.
- Lindmark, H., Nilsson, M. & Guss, B. (2001). Comparison of the fibronectin-binding protein FNE from *Streptococcus equi* subspecies *equi* with FNZ from *S. equi* subspecies *zooepidemicus* reveals a major and conserved difference. *Infect Immun* **69**, 3159–3163.
- Lindmark, R., Thoren-Tolling, K. & Sjöquist, J. (1983). Binding of immunoglobulins to protein A and immunoglobulin levels in mammalian sera. *J Immunol Methods* **62**, 1–13.
- Macrina, F. L., Tobian, J. A., Jones, K. R., Evans, R. P. & Clewell, D. B. (1982). A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. *Gene* **19**, 345–353.
- Maguin, E., Prévost, H., Ehrlich, S. D. & Gruss, A. (1996). Efficient insertional mutagenesis in lactococci and other gram-positive bacteria. *J Bacteriol* **178**, 931–935.
- Meehan, M., Nowlan, P. & Owen, P. (1998). Affinity purification and characterization of a fibrinogen-binding protein complex which protects mice against lethal challenge with *Streptococcus equi* subsp. *equi*. *Microbiology* **144**, 993–1003.
- Meehan, M., Muldowney, D. A., Watkins, N. J. & Owen, P. (2000a). Localization and characterization of the ligand-binding domain of the fibrinogen-binding protein (FgBP) of *Streptococcus equi* subsp. *equi*. *Microbiology* **146**, 1187–1194.
- Meehan, M., Muldowney, D. A., O'Meara, F. & Owen, P. (2000b). Neither the A- nor B-repeat regions of the fibrinogen-binding protein of *Streptococcus equi* subsp. *equi* are essential for fibrinogen binding. *FEMS Microbiol Lett* **190**, 317–321.
- Molinari, G. & Chhatwal, G. S. (1999). Streptococcal invasion. *Curr Opin Microbiol* **2**, 56–61.
- Muhktar, M. M. & Timoney, J. F. (1988). Chemotactic response of equine polymorphonuclear leucocytes to *Streptococcus equi*. *Res Vet Sci* **45**, 225–229.
- Navarre, W. W. & Schneewind, O. (1999). Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* **63**, 174–229.
- Pack, T. D., Podbielski, A. & Boyle, M. P. D. (1996). Identification of an amino acid signature sequence predictive of protein G-inhibitable IgG3-binding activity in group-A streptococcal IgG-binding proteins. *Gene* **171**, 65–70.
- Perez-Casal, J., Caparon, M. G. & Scott, J. R. (1991). Mry, a trans-acting positive regulator of the M protein gene of *Streptococcus pyogenes* with similarity to the receptor proteins of two-component regulatory systems. *J Bacteriol* **173**, 2617–2624.
- Prentki, P. & Krisch, H. M. (1984). *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**, 303–313.
- Raeder, R., Woischnik, M., Podbielski, A. & Boyle, M. D. (1998). A secreted streptococcal cysteine protease can cleave a surface-expressed M1 protein and alter the immunoglobulin binding properties. *Res Microbiol* **149**, 539–548.
- Ringdahl, U., Svensson, H. G., Kotarsky, H., Gustafsson, M.,

- Weineisen, M. & Sjöbring, U. (2000).** A role for the fibrinogen-binding regions of streptococcal M proteins in phagocytosis resistance. *Mol Microbiol* **37**, 1318–1326.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Timoney, J. F. (1993).** Strangles. *Vet Clin North Am Equine Pract* **9**, 365–374.
- Timoney, J. F., Artiushin, S. C. & Boschwitz, J. S. (1997).** Comparison of the sequences and functions of *Streptococcus equi* M-like proteins SeM and SzPSe. *Infect Immun* **65**, 3600–3605.
- Uhlén, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L. & Lindberg, M. (1984).** Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. *J Biol Chem* **259**, 1695–1702.
- Vasi, J., Frykberg, L., Carlsson, L. E., Lindberg, M. & Guss, B. (2000).** M-like proteins of *Streptococcus dysgalactiae*. *Infect Immun* **68**, 294–302.
- Yu, C.-E. & Ferretti, J. J. (1989).** Molecular epidemiologic analysis of the type A streptococcal exotoxin (erythrogenic toxin) gene (*speA*) in clinical *Streptococcus pyogenes* strains. *Infect Immun* **57**, 3715–3719.

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