Mutation of the maturase lipoprotein attenuates the virulence of *Streptococcus equi* to a greater extent than does loss of general lipoprotein lipidation.

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Abstract

Streptococcus equi is the causative agent of strangles, a prevalent and highly contagious disease of horses. Despite the animal suffering and economic burden associated with strangles little is known about the molecular basis of *S. equi* virulence. Here we have investigated the contribution of a specific lipoprotein and the general lipoprotein processing pathway to the ability of *S. equi* to colonise equine epithelial tissues *in vitro* and to cause disease in both a mouse model and the natural host *in vivo*. Colonisation of air-interface organ cultures was significantly reduced after inoculation with a mutant strain deficient in the maturase lipoprotein ($\Delta prtM_{138\text{-}213}$) compared to infection with wild-type *S. equi* strain 4047 or a mutant that was unable to lipidate preprolipoproteins ($\Delta lgt_{190\text{-}685}$). Moreover mucus production was significantly greater in both wild-type-infected and $\Delta lgt_{190\text{-}685}$-infected organ cultures. Both mutants were significantly attenuated in a mouse model of strangles compared with the wild-type strain, although 2/30 mice infected with the $\Delta lgt_{190\text{-}685}$ mutant did still exhibit signs of disease. In contrast only the $\Delta prtM_{138\text{-}213}$ mutant was significantly attenuated in a pony infection study with 0/5 infected ponies exhibiting pathological signs of strangles compared with 4/4 infected with the wild-type and 3/5 infected with the $\Delta lgt_{190\text{-}685}$ mutant. We believe that this is the first study to evaluate the contribution of lipoproteins to the virulence of a Gram positive pathogen in its natural host. These data suggest that the PrtM lipoprotein is a potential vaccine candidate and further investigation of its activity and its substrate(s) are warranted.

Introduction

The Group C streptococcus, *Streptococcus equi* (*S. equi*) is the aetiological agent of strangles, one of the most prevalent and important diseases of the horse (52). Nearly 30% of all reported equine infections worldwide may be attributable to this organism (8). Strangles is so-called because of the pharyngeal constriction which occurs as a consequence of lymph node swelling (often accompanied by abscessation) in the upper respiratory tract of the horse following the spread of infection from the nasopharynx. In approximately 5% of cases systemic spread of the organism leads to abscess formation in other organs resulting in the usually fatal condition known as 'bastard strangles' (44). There is comparatively little information regarding the molecular basis of virulence in *S. equi* (22). As in other pathogenic streptococci (4, 14, 32, 38) much work has focussed on the identification of secreted and surface-located components that may interact with the host (2, 16, 22, 27, 35). In this respect, a major development in the study of this important veterinary pathogen has been the availability of data from the *S. equi* genome project (http://www.sanger.ac.uk/Projects/S_equi/).

One major mechanism by which Gram-positive bacteria can retain exported proteins within their cell envelopes is through lipid modification, which anchors these lipoproteins to the outer face of the plasma membrane (6, 48). Bioinformatic analysis of Gram-positive bacterial genomes, including those of *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Streptococcus agalactiae*, has revealed that lipoproteins are a numerically significant feature (ca. 2%) of their predicted proteomes (3, 46, 47, 50, 51). Moreover, the predicted functions of these putative lipoproteins include roles in nutrient acquisition, adherence, protein maturation and cell signalling. Thus lipoproteins are predicted to play important roles in the

interactions between pathogenic bacteria and their hosts. Three *S. equi* lipoproteins have been characterised to date: LppC, a lipoprotein acid phosphatase enzyme (21); MBL, a putative metal-binding lipoprotein homologous to pneumococcal PsaA that is predicted to participate in ABC transporter-mediated uptake of manganese (23); and HAP, initially identified as hyaluronate (capsule) associated protein (10) but which is likely to act as a substrate-binding lipoprotein for ABC transporter-mediated uptake of oligopeptides (22). Bioinformatic analysis of the draft *S. equi* genome sequence has allowed us to identify at least 32 other putative lipoproteins (Sutcliffe and Harrington, unpublished observations) including a homologue (PrtM) of the pneumococcal vaccine candidate PpmA (33). Recently, the signal sequences of five of these putative lipoproteins were recovered in a screen for signal peptides using a phage display technique (27).

Bacterial lipoproteins are synthesised with distinctive Type II signal peptides that direct them into the Sec pathway for protein export (6, 17) and thence into a unique modification pathway which requires a minimum of two specific enzymes. Firstly, prolipoprotein diacylglyceryl transferase (Lgt) transfers a diacylglycerol moiety from membrane phospholipid substrates onto a critical cysteine residue in the cleavage region ('lipobox') of the type II signal peptide (36, 41). The lipid-modified prolipoprotein is then acted upon by a dedicated lipoprotein signal peptidase (Lsp) which cleaves the signal sequence preceding the thioether-modified cysteine, thereby leaving the lipid anchor unit at the N-terminus of the mature lipoprotein (41, 54). These two steps are sufficient for lipoprotein anchoring and appear to be the extent of the pathway in many Gram-positive bacteria (46, 47).

This pathway for lipid modification of bacterial proteins is an attractive target for antimicrobial drug development as both the Lgt and Lsp enzymes are unique to

prokaryotes. Likewise, as lipoproteins are likely to play important roles in host colonisation and bacterial virulence, individual lipoproteins have potential as targets for novel therapeutic or prophylactic (vaccine) strategies (28). Consistent with this are the findings that although Lgt is not apparently an essential enzyme during *in vitro* growth of Gram-positive bacteria (12, 29, 34, 39), an *lgt* mutant of *S. pneumoniae* was attenuated for virulence in a mouse model of infection (34) and the normal functions of *Bacillus subtilis* lipoproteins in protein secretion, sporulation and germination processes are impaired in *lgt* mutants (12, 25, 29, 39). Moreover, an Lgt mutant of *Staphylococcus aureus* that grew normally in rich media exhibited growth defects in minimal media, consistent with defects in lipoprotein-mediated nutrient uptake systems, notably ABC transporters (45). Similarly, although *Lsp* is dispensable for the growth of Gram-positive bacteria *in vitro* (18, 37, 40, 53, 55) studies using cell culture or animal models of infection show that Lsp is required for full virulence of *Listeria monocytogenes* (37), *Mycobacterium tuberculosis* (40) and *S. aureus* (11, 30). In contrast, inactivation of *Lsp* in *Streptococcus suis* did not appear to lead to an attenuation in virulence in a co-colonisation model of piglet infection (18).

We hypothesised that the lipid modification pathway is essential for full virulence of *S. equi*. As the Lgt enzyme catalyses the first and committed step on this path, we initially studied the contribution of this enzyme to the physiology and virulence of *S. equi*. In parallel we have investigated the deletion of a single, specific lipoprotein, namely the putative maturase lipoprotein (PrtM). We believe that this is the first study to evaluate the contribution of lipoproteins to the virulence of a Gram positive bacterial pathogen in its natural host.

Materials and Methods

Bacterial strains and culture conditions

S. equi strain 4047 was originally isolated in 1990 from a submandibular abscess of a New Forest pony and has been maintained in the culture collection of the Animal Health Trust, Newmarket, UK. This strain is the subject of the *S. equi* genome sequencing project. *Escherichia coli* TG1 *repA*+, which allows the stable replication of the plasmid pG+host9 at 37°C, was kindly supplied by Dr Emmanuelle Maguin (Institut Nationale de la Recherche Agronomique, Jouy en Josas, France). *S. equi* was cultured at 37°C (unless otherwise stated). Liquid cultures were grown in Todd Hewitt broth (THB) plus 0.2% (w/v) yeast extract in an atmosphere containing 5% CO_2. Semi-solid cultures were grown on Todd Hewitt agar (THA) or Columbia base agar containing 5% defibrinated horse blood in an atmosphere containing 5% CO_2. Mutant *S. equi* strains containing recombinant plasmids were grown on THA or in THB containing erythromycin at 0.5 or 1.0 μg ml^{-1} (THAE and THBE, respectively). *E. coli* strains were cultured in Luria-Bertani (LB) broth or agar at 37°C.

Plasmids and primers

The plasmids and primers used in this study are shown in Table 1.

Construction of in-frame deleted *lgt* and *prtM* alleles

In order to generate Lgt-deficient and PrtM-deficient mutants of *S. equi* 4047 by allelic replacement, copies of the *S. equi lgt* and *prtM* genes containing in-frame deletions were constructed. For the Lgt mutant, PCR primers (SELGTAR 1U, 2L, 3U and 4L; Table 1) were designed based upon sequences found within the *lgt* gene and adjacent sequences. The 22 nucleotides at the 5' end of primer SELGTAR 3U were

designed to complement the SELGTAR 2L primer sequence. PCR using *Pfu* polymerase (Promega), *S. equi* 4047 chromosomal DNA and primers SELGTAR 1U and SELGTAR 2L generated the expected 526 bp DNA fragment. A second PCR reaction with the SELGTAR 3U and SELGTAR 4L primers generated the expected 496 bp DNA fragment. The PCR products from each reaction were diluted, mixed and allowed to anneal via their overlapping, complementary ends. A third PCR reaction was then carried out with these annealed DNA fragments as template and with primers SELGTAR 1U and SELGTAR 4L, again using the *Pfu* polymerase. The product of this reaction was a DNA fragment of 1022 bp containing the 5' 189 base pairs and 3' 96 base pairs (plus upstream and downstream sequences) but lacking the central 495 bp of the *lgt* gene. The fragment was digested with the restriction endonucleases *Apa*I and *Sac*II and cloned into the corresponding restriction sites of the pG+Host9 vector to give the recombinant plasmid pAH08. To generate a PrtM mutant, a copy of the *prtM* gene was constructed that lacked bases 411 to 639, which includes the sequence encoding most of the parvulin-like domain of the protein. Sequences flanking the deletion were generated by PCR using Vent DNA polymerase (New England Biolabs) with the primer pairs 5'PRTM/PRTM-NDEL and 3'PRTM/PRTM-CDEL (Table 1). The corresponding 342bp and 376bp PCR products were then digested with the restriction endonucleases *Eco*RI and *Eco*RV (5' product) and *Sal*I and *Eco*RV (3' product) and the digested products ligated into *Eco*RI and *Sal*I digested pG+Host9:IS*S1* plasmid in a three-way ligation to form the deletion construct, pGprtMΔ. The engineering of an *Eco*RV site into primers as part of the cloning strategy results in the introduction of non-*prtM* DNA sequence encoding the amino acids aspartic acid and isoleucine at the site of the deletion. Plasmids pAH08

and pGprtMΔ were transformed into *E. coli* TG1repA+ and transformants selected at 37°C on LB plates containing erythromycin (150 μg ml^{-1}).

Allelic replacement mutagenesis.

Transformation of the encapsulated *S. equi* strain 4047 with plasmids pAH08 and pGprtMΔ was achieved using a modification of the method described by Simon and Ferretti (43). Briefly, an overnight culture of *S. equi* 4047 grown in THB containing hyaluronidase (30 μg ml^{-1}) was diluted 20-fold in 200 ml of the same medium and grown to an OD_{595} of 0.125. Bacterial cells were harvested by centrifugation and washed three times in 10 ml volumes of ice-cold 0.5 M sucrose. After the final wash the pellet was resuspended in 1 ml of ice-cold 0.5 M sucrose and 100 μl aliquots of the competent cells were used in transformation reactions. Transformations were performed with 1-5 μg plasmid DNA using a Gene Pulser electroporater (BioRad, UK) with pulse settings of 2.5 kV cm^{-1}, 200Ω and 25 μF, typically giving a pulse time of 4-6 ms. Ice-cold THB was added to the transformed cells which were then incubated at 37°C for 3 h to allow cell recovery. Transformants were selected by plating serial dilutions of the cells on THAE followed by overnight incubation at 28°C (the permissive temperature) to allow plasmid replication.

To replace the wild-type *lgt* and *prtM* genes with their respective in-frame deleted alleles, transformants containing either pAH08 or pGprtMΔ were subjected to two rounds of homologous recombination as previously described by Biswas *et al*. (5). The first recombination event, leading to the integration of pAH08 or pGprtMΔ into the strain 4047 chromosome, was achieved by growing transformants at 28°C overnight and then increasing the temperature to 37°C for 3 h. Integrants were selected following growth on THAE overnight at 37°C. Integrants were then

inoculated into THBE and grown at 37°C overnight followed by dilution into THB and incubation at 28°C for a further 48 h. Incubation at the permissive temperature allows plasmid replication and facilitates the second recombination event. Bacteria were plated on THA and grown at 37°C to ensure excision of free plasmid. Putative mutant colonies were sub-cultured onto fresh THA and THAE plates to confirm their erythromycin sensitivity. The presence of the mutant allele in the chromosome of putative mutants was determined by PCR using the primers SELGTAR 1U and SELGTAR 4L for the *lgt* mutants and 5'PRTM and 3'PRTM primers for the *prtM* mutants. PCR products, representing the deletion derivatives of each allele, were generated using proof-reading DNA polymerases and the predicted deletions confirmed by DNA sequencing. DNA sequencing was performed by the University of Newcastle Central Facility for Molecular Biology using an ABI Prism 377 DNA sequencer or at the AHT using an ABI3100 DNA sequencer with BigDye fluorescent terminators. A representative mutant for each deleted allele was chosen for subsequent studies and designated $\Delta lgt_{190\text{-}685}$ and $\Delta prtM_{138\text{-}213}$ respectively.

Analysis of the presence and localisation of lipoproteins

Lack of Lgt activity in the $\Delta lgt_{190\text{-}685}$ mutant was confirmed by radiolabelling lipoproteins. Radiolabelling of *S. equi* lipoproteins was performed as previously described by Sutcliffe et al. (49).

In order to demonstrate the presence of surface located lipoproteins in *S. equi* 4047 and *S. equi* $\Delta lgt_{190\text{-}685}$ strains, TEM was performed as described by Dixon et al. (13). Western blotting was used to indicate the presence of lipoproteins in either cell extract or secreted protein profiles. The preparation of bacterial cell extracts, SDS-PAGE and Western blotting were all performed as previously described (21). SDS-solubilised cell extracts contain a mixture of both soluble and membrane-asscoiated proteins. The primary anti-LppC antibody used in this study was kindly provided by Dr Horst Malke and used at the recommended dilution. Cell-associated and supernatant acid phosphatase activities of wild type and mutant strains were determined spectrophotometrically as previously described (21).

Investigation of virulence of *S. equi* mutants in an *in vitro* colonisation model

Air interface respiratory organ cultures were constructed using equine upper respiratory tract tissues (nasal turbinate, guttural pouch and trachea) using methods described for human (26) and canine (1) tissue with some modifications. Tissues were obtained from an abattoir and washed in Dulbecco's modified Eagle's medium (DMEM) containing antibiotics (penicillin, 100 U ml^{-1}; streptomycin, 50 μg ml^{-1}; gentamicin, 100 μg ml^{-1}; amphotericin, 2.5 μg ml^{-1}) for 4 h to remove commensal flora. Following further washing in DMEM to remove residual antibiotics and amphotericin tissues were dissected into pieces approx 5 mm^2 and mounted at an air interface on agarose platforms surrounded by 2.5 ml DMEM, in 6 well cell culture

plates. Organ cultures were maintained in a humidified 5% CO_2 incubator. Viability of air interface organ cultures was assessed using polystyrene bead clearance (1). Contamination was monitored by running a bacteriology loop around all 4 edges of the culture pieces and streaking onto horse blood agar plates. Any tissue pieces in which contamination was detected were discarded. Organ culture pieces were infected with a 10 μl suspension containing 1 x 10^5 colony forming units (cfu) of wild type *S. equi* 4047, Δ*lgt*$_{190\text{-}685}$ or Δ*prtM*$_{138\text{-}213}$ or were mock-infected with THB. Colonisation of organ culture pieces was assessed by measuring viable counts (6 organ culture pieces per time point) of adherent bacteria at 4 h and 24 h post infection (p.i.). Organ culture pieces were vortexed for 15 s in PBS to remove non-adherent bacteria and then homogenised before plating serial 10-fold dilutions onto THA and enumerating colonies. Changes in the surface features of organ culture pieces (2 per time point) in response to infection with wild type *S. equi* or the two mutants at 24 h p.i. were assessed by morphometric analysis of scanning electron microscopy (SEM) images of the epithelial surface. Tissues were processed and surface morphometry were carried out using standard methods (26). The percentage of the epithelial surface covered with mucus was recorded. Data represent the means and standard deviations of 6 independent experiments using tissues from different horses. Differences in colonisation and surface morphometry data were tested for statistical significance using Mann-Whitney U tests and are reported at the 5% level.

Investigation of the virulence of *S. equi* mutants in a mouse model of strangles

Mice were challenged intranasally as described by Chanter *et al.* (9). Briefly, thirty 3 to 4 week old female Balb/C mice were challenged with $4x10^6$ cfu of fresh cultures of wild-type 4047, $\Delta prtM_{138\text{-}213}$ or $\Delta lgt_{190\text{-}685}$ *S. equi* strains and clinical signs of disease including weight loss and sneezing were compared with a group of 10 unchallenged controls over a period of five days. At the end of this period, mice were euthanased and examined for signs of *S. equi* infection (measured as viable *S. equi* counts) and pathology by histological examination of lymph nodes and tissues of the head and neck. The extent of pathology in each mouse was then graded, on the basis of pathological features most pertinent to *S. equi* infection, using the following scoring system: lymphadenitis [1], lymph node abscess [5], rhinitis [1], marked rhinitis [5], pharyngitis [3], meningitis [5], otitis media [3], lung lesions [5] and splenic lesions [5].

Investigation of the virulence of *S. equi* mutants in a pony challenge study

Groups of 5 naïve, male yearling Welsh mountain ponies were challenged with either $\Delta prtM_{138\text{-}213}$ or $\Delta lgt_{190\text{-}685}$ and a similar control group of 4 male ponies with *S. equi* 4047. Each group was housed separately throughout the challenge period with strict infection control measures in place to ensure no cross-contamination between the groups. Fresh cultures of each strain were grown in THB supplemented with 10% foetal calf serum (THB10) at 37°C with 5% CO_2 to an OD_{600nm} of 0.3. Previous studies have shown that this density of bacteria corresponds to approx. 2×10^8 cfu ml^{-1} of *S. equi* 4047 (unpublished observations). At this point cultures were diluted 1:8 in fresh pre-warmed and pre-gassed THB10 and 2 ml of challenge inocula were administered via both nostrils using a flexible tube and spray nozzle, in order to

administer approximately 1 x 10^8 cfu/pony. Clinical signs of disease including fever, swelling of the lymph nodes and nasal discharge were monitored daily for up to three weeks. Ponies were considered to be pyrexic when their temperature exceeded 39.0^{o}C. Clinical scores were calculated based on the scoring system presented in Table 2. Blood samples were collected to enable monitoring of the neutrophil levels present in challenged ponies. Normally these range from 3 to 6.5 x 10^6 ml^{-1} in healthy ponies, but frequently exceed 1 x 10^7 ml^{-1} during *S. equi* infection. At the end of the study period, all of the ponies were euthanased and the extent of their disease quantified on post mortem examination using the following scoring system: abscess in a lymph node [15], micro-abscess in a lymph node [10], enlarged lymph node [1], empyaema of the guttural pouch [5], follicular hyperplasia of the guttural pouch [1]. Samples of lesions at post mortem were used to re-isolate the challenge organisms in order to confirm their identity by PCR of the *lgt* and *prtM* genes.

Animal ethics

These studies were performed under a Home Office project license after ethical review and following strict welfare guidelines.

Results

Identification of the *lgt* gene and construction of an Lgt-deficient allelic replacement mutant

Our initial studies allowed the amplification of a 261 bp internal fragment of the *S. equi* 4047 *lgt* gene (Genbank accession number AJ403973), using degenerate primers based upon conserved amino acid sequences in the Lgt proteins of *S. mutans*, *S. pneumoniae* and *S. pyogenes*. The sequence was completed by subsequent PCR experiments and verified by reference to an early release of the *S. equi* 4047 genome project. Putative promoter and ribosome binding site sequences were identified upstream of the *lgt* gene, which is located downstream of the *hprK* gene as in several other Gram positive bacteria (7, 24). The *lgt* gene of *S. equi* 4047 encodes a 259 amino acid protein with a molecular weight of approximately 29.8 kDa. The derived protein sequence contains the Lgt Prosite motif G-R-X-[GA]-N-F-[LIVMF]-N-X-E-X(2)-G (PS01311/PDOC01015) and matches the Pfam profile (PF01790) for Lgt. An overlap-deletion PCR strategy was used to create a mutant *lgt* allele with a 495 bp in-frame deletion which removed this Prosite motif and was thus predicted to produce a non-functional Lgt enzyme. Replacement of the wild-type allele with the in-frame deletion derivative in *S. equi* Δ$lgt_{190\text{-}685}$ was confirmed by PCR and sequencing.

Radiolabelling of lipoproteins in *S. equi* 4047 and *S. equi* Δ$lgt_{190\text{-}685}$

To confirm the absence of Lgt activity in the allelic replacement mutant, *S. equi* 4047 and *S. equi* Δ$lgt_{190\text{-}685}$ were grown in the presence of [^{14}C] palmitate. Palmitate is incorporated into endogenous membrane lipids which are used as the

substrate for lipid modification of preprolipoproteins by Lgt, thereby resulting in the radiolabelling of mature lipoproteins. Electrophoresis of cell extract proteins of the parent strain 4047 revealed the presence of at least 10 distinct, radiolabelled lipoproteins following autoradiography (Fig. 1, lane 1). In contrast there was an absence of labelled protein bands in equivalent cell extracts of the mutant strain (Fig. 1, lane 2). Intensive labelling at the bottom of each lane indicated comparable incorporation of the labelled palmitate into bacterial lipids (Fig. 1). This result confirmed the absence of functional Lgt activity in the mutant.

Investigation of the effect of Lgt mutation on the processing of a known *S. equi* lipoprotein

In order to determine the effect of Lgt mutation on the processing of an individual lipoprotein, the presence of the *S. equi* LppC acid phosphatase (21) was investigated in the wild-type and mutant strains by Western blot analysis. As expected a single cross-reacting band representing the mature form of LppC was seen in cell extracts of the parent 4047 strain probed with an antibody to the *Streptococcus equisimilis* acid phosphatase LppC (Fig 2A, lane 2). When a cell extract of *S. equi* 4047 which had been treated with globomycin was analysed, a second cross-reacting band representing the pro-LppC form of the protein was seen (Fig. 2A, lane 1). The appearance of this additional, higher molecular weight band is consistent with the inhibition of lipoprotein signal peptidase II by globomycin (21). A cross-reacting doublet was seen in cell extracts of *S. equi* Δ$lgt_{190\text{-}685}$ (Fig 2A, lane 3) although the cross-reacting bands were considerably less intense for this strain, despite an equivalent total protein load compared to the wild-type. Moreover, neither of the bands corresponded in molecular weight with the pro-LppC form seen in the

globomycin-treated culture suggesting that the prepro-LppC form of the protein, which is unlipidated but retains its signal peptide, migrates faster than the pro-LppC form. The lower amount of cell-associated LppC observed for *S. equi* Δ*lgt*$_{190\text{-}685}$ could be explained by a reduced retention of prepro-LppC in the cell membrane as a consequence of the inability of the mutant strain to modify this protein with lipid. Consequently we investigated the release of unlipidated LppC by performing Western blots on concentrated culture supernatants obtained from the cultures from which the cell extracts had been derived. There was a minor but detectable cross-reacting protein in the supernatant of *S. equi* 4047 but not in the supernatant of *S. equi* Δ*lgt*$_{190\text{-}685}$ (data not shown). It was also noticeable that the band detected in the supernatant of *S. equi* 4047 was smaller than the mature form of the protein seen in cell extracts of the same strain suggesting that a proportion of the membrane-anchored LppC is released by proteolytic processing in the parent strain. Whole-cell acid phosphatase assays were also performed on each strain. As previously observed for *S. equi* strain 9682 (21), a peak of acid phosphatase activity at a pH optimum of 5 was readily detectable for *S. equi* 4047 but this activity was significantly reduced in the mutant strain *S. equi* Δ*lgt*$_{190\text{-}685}$ (Fig. 2B). However, acid phosphatase activity was undetectable in the culture supernatants of both strains (data not shown) suggesting that the protein detected in Western blots of *S. equi* 4047 culture supernatants is probably not active. Further confirmation of a reduced level of LppC in the cell envelope of the mutant compared to the wild-type came from LppC-specific immunogold labelling experiments. Single cocci of *S. equi* 4047 and Δ*lgt*$_{190\text{-}685}$ (n=10 for each) were labelled with 234 ± 20 and 54 ± 20 gold particles, respectively. Cumulatively, these data suggested that there was a significant defect in LppC

localisation within the cell envelope of *S. equi* Δ*lgt*$_{190\text{-}685}$ compared to the parent strain.

Construction of a PrtM-deficient allelic replacement mutant

The *S. equi* Δ*lgt*$_{190\text{-}685}$ mutant had been shown to be defective in the processing of lipoproteins generally (Fig. 1). To gain further insight into the significance of specific lipoproteins in *S. equi*, we created a *S. equi* mutant strain defective in the function of the putative maturase lipoprotein, PrtM. The PrtM sequence was identified from the *S. equi* genome project and, in addition to its homology to pneumococcal PpmA (33), it also exhibits significant homologies to the maturase proteins of other Gram positive bacteria (15, 19, 56, 57). This family of sequences belong to the parvulin family of PpiC-type peptidyl-prolyl cis-trans isomerases (PPIase). A *S. equi* mutant (Δ*prtM*$_{138\text{-}213}$) was constructed with an in-frame internal deletion in the *prtM* coding sequence corresponding to the central (parvulin-like) PPIase domain (57). This mutant is predicted to synthesise a non-functional PrtM protein, although the absence of an *in vitro* assay for PrtM function precludes experimental confirmation of this. Growth of both the *S. equi* Δ*lgt*$_{190\text{-}685}$ and the Δ*prtM*$_{138\text{-}213}$ mutants in nutrient rich broth was comparable to that of the wild type *S. equi* (data not shown).

Colonisation of air interface organ cultures by *S. equi* strains

Following inoculation of nasal turbinate, guttural pouch and tracheal organ culture pieces with 1 x 10^5 cfu wild type *S. equi* 4047 or the two mutants, all three strains were recovered from all three tissues at 4 h p.i (Fig. 3A). At 24 h p.i. wild type *S. equi* and Δ*lgt*$_{190\text{-}685}$ were again recovered whereas Δ*prtM*$_{138\text{-}213}$ was not detected.

Wild type bacteria were recovered in statistically significantly higher numbers at both 4 h and 24 h p.i. from nasal turbinate (3.8 ± 0.35; 3.4 ± 0.55) and guttural pouch (4.0 ± 0.60; 4.2 ± 0.70) cultures than from tracheal cultures (2.8 ± 0.25; 1.3 ± 0.80). The numbers of $\Delta lgt_{190\text{-}685}$ recovered at 4 h and 24 h p.i. from turbinate (3.9 ± 0.50; 2.8 ± 0.70), guttural pouch (3.2 ± 0.45; 3.62 ± 0.80) and tracheal (2.4 ± 0.45; 1.1 ± 0.60) cultures were not significantly different from those of wild type *S. equi*. However, there were significantly fewer $\Delta prtM_{138\text{-}213}$ recovered at 4 h and 24 h p.i. from nasal turbinate (1.8 ± 0.30; <0.7 ± 0), guttural pouch (1.4 ± 0.45; <0.7 ± 0) and tracheal (0.7 ± 0.50) cultures than both wild type *S. equi* and $\Delta lgt_{190\text{-}685}$.

Changes in surface epithelial morphology of air interface organ cultures exposed to *S. equi* strains

The surface morphology of uninfected organ culture pieces from nasal turbinate, guttural pouch and trachea was predominantly ciliated epithelium. The guttural pouch and tracheal cultures were densely and uniformly ciliated whereas nasal turbinate tissue exhibited a mixture of ciliated and non-ciliated epithelial cells. In all three tissues after 24 h in culture a small percentage of the total epithelial surface area was covered with mucus (Fig. 3B) and the amount of surface coverage in the uninfected control pieces was not significantly different to that at the start of the experiment. Wild-type *S. equi* induced a marked mucus response which resulted in a significantly greater proportion of the epithelial surface being covered by mucus in all three tissues (nasal turbinate 86 ± 18%; guttural pouch 95 ± 22%; trachea 90 ± 14%). The mucus formed a dense layer that obscured the underlying ciliated epithelium (Fig. 4B). Inoculation of $\Delta lgt_{190\text{-}685}$ also induced a mucus response at 24 h pi in all three tissues. The amount of mucus coverage of the epithelial surface was significantly greater (nasal turbinate 75 ± 12%; guttural pouch 86 ± 16%; trachea 82 ±

20%) than the uninfected control pieces but was not significantly different from pieces infected with wild-type *S. equi* (Fig. 3B). Qualitatively the mucus layer produced appeared less dense than that produced in response to wild-type *S. equi* (Fig. 4C). In contrast to infection with both wild-type *S. equi* and $\Delta lgt_{190\text{-}685}$, inoculation of $\Delta prtM_{138\text{-}213}$ did not result in a significant increase in mucus production compared to uninfected control pieces (Fig. 3B), with the result that the ciliated epithelial surface was not obscured by mucus (Fig. 4D).

Virulence of *S. equi* mutants in a mouse model of strangles

The virulence of the mutants was determined in a mouse intranasal infection model of strangles (9). As expected, approximately 60% of mice challenged with the parent 4047 strain lost or failed to gain weight over the 5-day study period, indicative of *S. equi* infection (Fig. 5A). *S. equi* 4047 also induced sneezing from 3 days post challenge (Fig. 5B) and had induced significant levels of disease in mice as determined by post mortem examination 5 days post challenge (Fig. 5C & 5D). Deletion of either the *lgt* or *prtM* genes significantly attenuated *S. equi* on intranasal challenge of mice as measured by weight gain, sneezing rate, pathological score and the overall incidence of disease (Fig. 5A-D). Mice challenged with either $\Delta lgt_{190\text{-}685}$ or $\Delta prtM_{138\text{-}213}$ generally continued to gain weight in line with mock-challenged controls. However, 2 of 30 mice challenged with the $\Delta lgt_{190\text{-}685}$ strain had reduced weight gain when compared with unchallenged controls. Two mice challenged with $\Delta lgt_{190\text{-}685}$, including one of the mice that had failed to gain weight, also had histological disease on post mortem examination (Fig. 5D).

Virulence of *S. equi* mutants in a pony challenge study

The parent strain and both mutants were assayed for virulence in Welsh mountain ponies. The early clinical signs of strangles disease such as pyrexia, nasal discharge and swelling of the submandibular lymph nodes were apparent from day 2 post-challenge in 3/4 ponies challenged with the parental strain 4047 and from day 4 in 3/5 ponies challenged with the Δ*lgt*$_{190\text{-}685}$ deletion mutant (Fig. 6A-C). In contrast, there were no signs of disease observed in ponies challenged with the Δ*prtM*$_{138\text{-}213}$ deletion strain throughout the 17-day study period (Fig. 6A-C). There was a rise in mean rectal temperature, from day 4 post-challenge in the ponies challenged with wild-type 4047 compared to those challenged with both Δ*lgt*$_{190\text{-}685}$ and Δ*prtM*$_{138\text{-}213}$ (Fig. 6A). Moreover, pyrexia (a temperature exceeding 39.0^{o}C) was evident in 3/4 of the 4047-challenged group compared with 1/5 of the Δ*lgt*$_{190\text{-}685}$-challenged group and 0/5 of the Δ*prtM*$_{138\text{-}213}$ challenged group (Fisher's exact p = 0.048; Fig. 6B). Other clinical signs were also significantly reduced in Δ*prtM*$_{138\text{-}213}$-challenged ponies compared with the wild-type challenged group (Kruskal-Wallis test p = 0.0267; Fig. 6C). There was no significant difference in the mean clinical scores of the Δ*lgt*$_{190\text{-}685}$-challenged group compared to the 4047-challenged ponies (Figure 6C). Similarly, whereas neutrophilia (>6.5x10^{6} ml^{-1}) was observed by day 17 in both wild-type and Δ*lgt*$_{190\text{-}685}$-challenged groups, neutrophil levels in Δ*prtM*$_{138\text{-}213}$-challenged ponies remained stable (Fig. 6D).

On post mortem examination, lymph node abscesses were apparent in all 4 ponies challenged with the parental 4047 strain, 3 of 5 ponies (p = 0.44) challenged with the Δ*lgt*$_{190\text{-}685}$ strain and 0 of 5 ponies (p = 0.008) challenged with the Δ*prtM*$_{138\text{-}213}$ strain (Fig. 7A). The mean pathological scores determined at post mortem were very similar for the 4047 and Δ*lgt*$_{190\text{-}685}$ groups, whereas the low score obtained for the Δ*prtM*$_{138\text{-}213}$ group reflected low-grade pathology not indicative of strangles (Fig

7B). *S. equi* was isolated from the abscesses of ponies in the 4047 and $\Delta lgt_{190\text{-}685}$ groups in high yields (in excess of 10^9 cfu/ml of pus) and these isolates were confirmed by PCR to have the full-length or truncated *lgt* gene, respectively, thus confirming the source of infection and *in vivo* stability of the *lgt* deletion. No *S. equi* was re-isolated from any of the $\Delta prtM_{138\text{-}213}$ challenged ponies on post mortem examination, suggesting that this strain was not able to persist *in vivo* for the 3-week duration of this study.

Discussion

Comparatively little is known about the molecular basis of *S. equi* virulence (22). Greater understanding should identify rational candidates for the development of an effective vaccine. It is considered that prevention of strangles is likely to be the only effective mechanism for combating the disease as the lack of vascularity associated with abscessation prevents the delivery of effective doses of antibiotics and other drugs to the site of infection.

Bacterial lipoproteins are attractive as potential vaccine candidates since they may be exposed on the bacterial surface and thus potentially accessible by host immune molecules (28, 51). Furthermore a wide variety of functions have been attributed to bacterial lipoproteins, at least some of which are likely to be crucial to bacterial colonisation and survival within the host (46-48). Thus immune responses directed at such proteins may be highly opsonic and may also block the activity of essential proteins. In the present study we investigated the contribution of lipoproteins to *S. equi* physiology and virulence. To this end, we have characterised the consequences of disrupting both the lipoprotein biosynthetic pathway *per se* and of inactivating a specific lipoprotein, PrtM.

In order to construct a lipoprotein-deficient mutant of *S. equi* 4047 the wild-type *lgt* gene was replaced by a mutant allele that contained a 495 bp internal deletion that removed the central portion of Lgt, including the highly conserved sequence motif that defines the Lgt family of enzymes. An allelic replacement strategy was chosen so as to minimise the potential for polar effects due to the mutation. The successful construction of an Lgt-deficient mutant (*S. equi* Δ*lgt*$_{190\text{-}685}$) was confirmed by PCR and by palmitate radiolabelling which clearly showed that the mutant strain lacked labelled lipoproteins (at least 10 of which could be seen in the parent strain). The ability to generate a viable, Lgt-deficient mutant indicates that the absence of Lgt in *S. equi* is not lethal, a finding also observed for other Gram positive organisms such as *B. subtilis* (12, 29, 39), *S. pneumoniae* (34) and *S. aureus* (45).

The failure to transfer a lipid moiety to preprolipoproteins, due to Lgt inactivation, would be predicted to result in either the accumulation of signal peptide-anchored preprolipoproteins in the cell membrane or the release of the lipoprotein derivatives into the culture medium, due to either shedding or signal peptide processing at 'cryptic' signal peptidase I processing sites ('shaving'). In order to determine which of these possibilities did indeed occur for individual lipoproteins in the *S. equi* Δ*lgt*$_{190\text{-}685}$ strain, the localisation of a proven lipoprotein was investigated by Western blot analysis. Whilst it was clear that there was reduction in the level of the LppC acid phosphatase in cell extracts of the *S. equi* Δ*lgt*$_{190\text{-}685}$ mutant, there was no corresponding increase in the amount of this protein in culture supernatants. It appears, therefore, that the failure of this protein to become lipid modified may result in its degradation either before or during secretion, although it remains possible that expression of this protein is altered in the *S. equi* Δ*lgt*$_{190\text{-}685}$ mutant.

Despite the demonstration, by palmitate labelling, that the *S. equi* Δ*lgt*$_{190\text{-}685}$ mutant contained no detectable lipoproteins, a protein cross-reacting with the anti-LppC antibody was present in cell extracts of the *S. equi* Δ*lgt*$_{190\text{-}685}$ mutant albeit at significantly lower levels than in the parent strain. The cross-reacting band also appeared to have a very similar molecular weight compared to that of the wild type (mature) lipoprotein. The most likely explanation for this observation is that the LppC protein seen in *S. equi* Δ*lgt*$_{190\text{-}685}$ is not lipidated but that the LppC preprolipoprotein is proteolytically modified, yielding a product of similar size to the mature acid phosphatase in the parent strain. Comparable 'mature-like lipoproteins' have been reported previously in *B. subtilis*, *L. monocytogenes* and *S. suis* mutants lacking the Lsp signal peptiase II (18, 37, 53). Most significantly, differential processing of lipoprotein precursors has been observed in an *lsp* mutant of *M. tuberculosis*: whereas the mutant accumulated both prolipoprotein and a 'mature-like' form of a 19 kDa lipoprotein, only the prolipoprotein forms of a 27 kDa and the MPT83 lipoproteins accumulated (40).

Although the phenotype of the *S. equi* Δ*lgt*$_{190\text{-}685}$ did not correspond with that originally predicted (i.e. preprolipoprotein accumulation), convincing autroradiographic, Western blot, acid phosphatase enzyme assay and immunolocalisation data all confirmed a significant defect in lipoprotein processing in this strain.

We intended to investigate the virulence of the *S. equi* Δ*lgt*$_{190\text{-}685}$ mutant in a variety of *in vitro* and *in vivo* models of colonisation and disease and also decided to generate a mutant that was deficient in a single specific lipoprotein. For the purpose of this study we chose the putative maturase lipoprotein PrtM, one of four lipoproteins released in large amounts by an Lgt mutant of *S.aureus* (45).

Air interface respiratory organ cultures of nasal turbinate, guttural pouch and tracheal tissues were used to compare the ability of wild-type *S. equi*, the $\Delta lgt_{190\text{-}685}$ mutant and a PrtM-deficient mutant ($\Delta prtM_{138\text{-}213}$), to colonise tissues derived from a variety of anatomical sites within the equine upper respiratory tract (URT) and to assess the response of URT tissues to infection. Previous studies have shown that culturing respiratory tract tissues at an air interface provides a more physiological infection environment for bacterial pathogens than submerged culture systems (31, 59). Viable and contamination-free tissues from all three regions were successfully maintained for the duration of the experiment. Colonisation was assessed by measuring the numbers of bacteria adherent to tissues at 4 h and 24 h p.i. Wild-type *S. equi* was present in higher numbers on nasal turbinate and guttural pouch tissues than on tracheal tissues, suggesting less efficient colonisation of the trachea. The numbers of the $\Delta lgt_{190\text{-}685}$ mutant adherent to organ culture pieces were not significantly different from those of wild-type *S. equi* suggesting that this mutant was capable of colonising nasal turbinate, guttural pouch and tracheal tissues as efficiently as wild-type bacteria. In contrast, however, the $\Delta prtM_{138\text{-}213}$ mutant was present in significantly reduced numbers compared to wild-type *S. equi* in all three tissue sites at both time points measured. These data suggest that the $\Delta prtM_{138\text{-}213}$ mutant has impaired ability to colonise the equine URT.

The response of the equine URT to infection with wild-type *S. equi* and the two mutants was assessed by SEM morphometric analysis of the epithelial surface. Uninfected cultures from all three anatomical regions had a small surface area covered by mucus. Wild-type *S. equi* induced a marked mucus response that obscured the epithelial surface. It was not possible to assess the underlying

epithelium by SEM analysis although light microscopy suggested that this was intact and, furthermore, organ culture pieces infected with the wild-type bacteria continued to clear beads despite the production of mucus (data not shown). This extensive mucus response has been noted for other bacterial pathogens using air interface organ cultures of human (59) and canine (1) origin. The mutant $\Delta lgt_{190\text{-}685}$ also induced a marked mucus response that was not different from wild-type *S. equi*. Although the amount of mucus produced in these experiments was not quantifiable, the depth of the mucus layer induced by $\Delta lgt_{190\text{-}685}$ appeared thinner than that produced by wild-type infection because the epithelial surface could be discerned beneath. In contrast, $\Delta prtM_{138\text{-}213}$ did not induce a mucus response, and organ culture pieces infected with this mutant were indistinguishable from the uninfected control pieces in this regard.

Taken together, the organ culture colonisation and tissue response data suggest that colonisation of the URT (and/or possibly production of soluble factors by the bacteria) is required to induce a mucus response. Both wild-type *S. equi* and $\Delta lgt_{190\text{-}685}$ colonised the epithelium to a similar extent and induced a similar mucus response whereas $\Delta prtM_{138\text{-}213}$ was less able to colonise, was cleared from all tissue sites within 24 h p.i. and did not induce a mucus response. Since colonisation is the first step in pathogenesis these *in vitro* data suggest that $\Delta prtM_{138\text{-}213}$ is likely to have reduced virulence in the natural host. However, it should be noted that persistence and abscess formation in the horse would require evasion of the immune response, which was not assessed in the air interface organ culture models.

In the mouse *S. equi* model (9), challenge with the parental *S. equi* 4047 strain induced disease in 57% (17/30) of mice during the 5-day study period as determined by changes in weight gain, rate of sneezing and histopathological analysis. The

deletion of the *prtM* gene significantly attenuated *S. equi* in the mouse model of *S. equi* infection ($p < 0.001$). None of the mice challenged with $\Delta prtM_{138\text{-}213}$ showed signs of disease (either reduced weight gain or sneezing) throughout the study period and no disease was detected histologically. The $\Delta lgt_{190\text{-}685}$ strain was also significantly attenuated in the mouse *S. equi* infection model ($p < 0.001$). However, 3 of 30 mice challenged with $\Delta lgt_{190\text{-}685}$ had histological signs of disease and/or reduced weight gain when compared with unchallenged controls, indicating that this strain is not completely avirulent in mice.

Investigation of the *in vivo* virulence of the $\Delta lgt_{190\text{-}685}$ and $\Delta prtM_{138\text{-}213}$ deletion strains was determined in the natural host via intranasal challenge of Welsh Mountain ponies. Ponies challenged with the parental *S. equi* 4047 strain developed obvious signs of strangles during the 17-day study period as determined by pyrexia (3 of 4 ponies), clinical observations (4 of 4 ponies) and post mortem examination (4 of 4 ponies). The $\Delta prtM_{138\text{-}213}$ strain was significantly attenuated for each of these parameters and did not induce signs of pyrexia (0 of 5 ponies, $P = 0.048$), significant clinical signs of disease (0 of 5 ponies, $P = 0.027$) or obvious signs of disease on post mortem examination (0 of 5 ponies, $P = 0.008$) during the same 17-day study period. The presence of lymph node swelling in 1 of 5 ponies and follicular hyperplasia of the guttural pouch in all 5 ponies challenged with $\Delta prtM_{138\text{-}213}$ may be indicative of an immune response directed against this strain and suggests that, although not detected at 17-days post challenge, the $\Delta prtM_{138\text{-}213}$ strain may persist for a short time *in vivo*. The $\Delta lgt_{190\text{-}685}$ strain generated early clinical signs of strangles in 3 of 5 ponies challenged. *S. equi* isolated from the lesions in these ponies all contained the deleted version of the *lgt* gene indicating that the strain had not reverted *in vivo* and thus that the presence of functional Lgt is not an absolute

requirement for virulence in the natural host. However, there were no signs of strangles in 2 of the 5 ponies challenged with $\Delta lgt_{190\text{-}685}$ and there was an overall reduction in the degree of pyrexia in this cohort. This suggests that whilst this deletion mutant is not statistically significantly attenuated in the pony, there was a trend towards reduced incidence of disease compared with the parental 4047 strain, consistent with our findings in the other models. The stronger attenuation of the $\Delta lgt_{190\text{-}685}$ strain in the mouse model compared with ponies may reflect differences in either the nature of bacterial-host interactions between the two species or differences in host responses to infection. It is notable, for example, that the wild type strain causes disease in 100% of infected ponies but only 60% of infected mice. Our data demonstrate that, ultimately, it is important to evaluate virulence attenuation in the natural host.

The increased attenuation observed upon deletion of the *prtM* gene compared with deletion of the *lgt* gene suggests that lack of lipidation of PrtM does not completely eliminate its functional activity. In this context it is important to note that whilst deletion of *lgt* is not, in itself, lethal in *B. subtilis* (12, 29, 39) the PrsA lipoprotein is essential: the reduction of the cellular levels of PrsA below a critical threshold of ca. 200 molecules per cell results in cellular lysis (56). Thus it is likely that some residual functional activity may be retained by preprolipoproteins in Lgt mutants, perhaps during transient membrane association prior to shedding, shaving or proteolytic degradation. Similar conclusions have been drawn previously based on the absence of a significant growth defect in an Lgt mutant of *S. aureus* (45) and an Lsp mutant of *Lactococcus lactis* (55). In *B. subtilis* the *in vivo* function of PrsA apparently derives from the interaction of the central parvulin-like domain with the

flanking N- and C-terminal domains (57). However, a PrsA deletion mutant ($PrsA_{N+C}$), which is comparable to the $\Delta prtM_{138\text{-}213}$ mutant described herein, was unable to restore growth of PrsA-depleted cells although it did exhibit weak activity in an AmyQ secretion assay (57). Our studies suggest that deletion of the central domain of *S. equi* PrtM is sufficient to abrogate its function *in vivo*, thereby attenuating this strain. Some maturases, such as the plasmid-encoded PrtM of *L. lactis*, have clearly defined substrates (19, 20) whereas the role of the chromosomally encoded PpmA maturase has not yet been defined (15). Similarly, PrsA of *B. subtilis* may have a more pleiotropic role in protein secretion (57) and the *Bacillus anthracis* genome encodes three functional PrsA homologues that may have distinct but overlapping substrate specificities (58). Further analyses of the molecular consequences of the deletion of *prtM* in *S. equi* are now required in order to identify those virulence factors reliant on its activity and which are essential to pathogenicity in the horse.

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Table 1. Oligonucleotide primers and plasmids used in this study

Primer/plasmid	Sequence or description	Reference
Primers*		
SELGTAR 1U	5'-GGGCCCGTCATCTCCTTGAGATTCGTG-3'	
SELGTAR 2L	5'-GATTCTTGCTCCAATAATTGCC-3'	
SELGTAR 3U	5'-*GGCAATTATTGGAGCAAGAATC*GTTTCTCAATACATGTCGGTG-3'	
SELGTAR 4L	5'-CCGCGGATAATTTAGAAGCGACCTTGC-3'	
5'PRTM	5'-GGGGAATTCAAGTGTCATTACGATGAAGG-3'	
PRTM-NDEL	5'-GGGGATATCGTAATCAGCATCTGTCAGCTC-3'	
3'PRTM	5'-GGGGTCGACTTTCTGACTTAGATTTAGAAG-3'	
PRTM-CDEL	5'-GGGGATATCGAGGGTGATATTTCAGAGGTG-3'	
Plasmids		
pG+host9:IS*S1*	Replication thermosensitive derivative of pWV01 containing cloned IS*S1* sequence	Maguin et al., 1996
pAH08	pG+host9:IS*S1*† containing in-frame deleted *lgt* gene of *S. equi*	This study
pGprtMΔ	pG+host9:IS*S1*† IS*S1* containing in-frame deleted *prtM* gene of *S. equi*	This study
pGEM-T	T-A cloning vector	Promega

*Underlined sequences represent engineered restriction sites for *Apa* I (GGGCCC), *Sac* II (CCGCGG), *Eco*R I (GAATTC), *Eco*R V (GATATC) and *Sal* I (GTCGAC). The italicised sequence in SELGTAR 3U is the reverse and complementary sequence to the primer SELGTAR 2L sequence and provides the overlapping sequence for the overlap-deletion PCR strategy.

†IS*S1* sequence is completely removed during cloning strategy

Table 2: Scoring system used to quantify disease burden in ponies.

Observation	Grade	Score
Nasal *	Normal	0
	Serous	1
	Mucopurulent	2
Submandibular lymph node swelling *	Normal	0
	Slight	1
	Moderate	2
	Severe	3
Cough	Not present	0
	Present	1

* scores of left and right sides were added.

Figure 1. Labelling of lipoproteins in *S. equi* 4047 and the Δ*lgt*$_{190\text{-}685}$ mutant with [^{14}C] palmitic acid.

SDS extracts of cells grown in the presence of [^{14}C] radiolabelled palmitic acid were separated by SDS-PAGE. The dried gel was exposed to X-ray film for 24 h before developing. Lane 1, *S. equi* 4047 extract; lane 2, *S. equi* Δ*lgt*$_{190\text{-}685}$ extract. The positions of molecular weight standards (in kDa) are shown on the left.

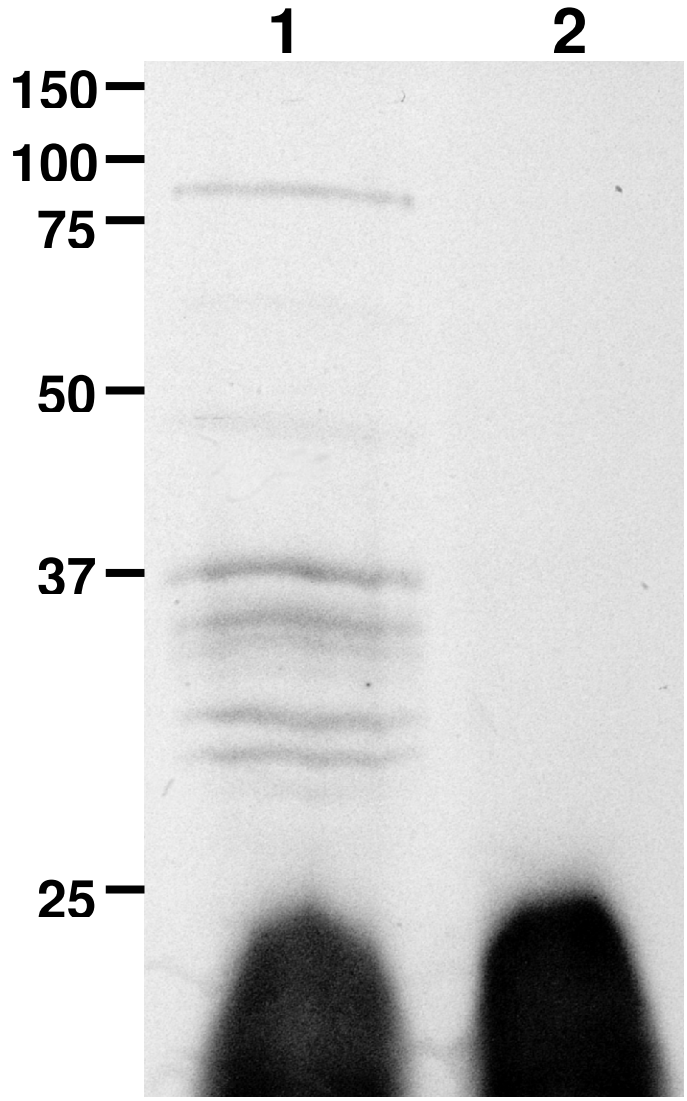
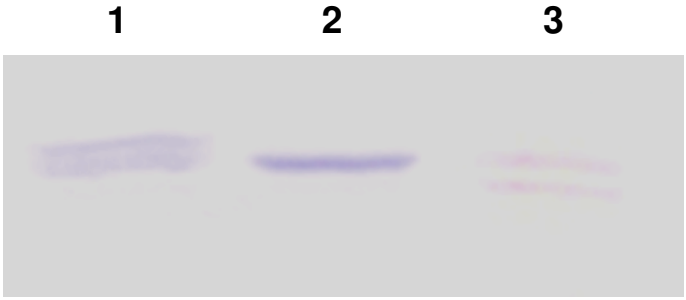


Figure 2. Changes in the nature (panel a) and the activity (panel b) of an acid phosphatase (LppC) in the *S. equi* Δ*lgt*$_{190\text{-}685}$ mutant.

A. Proteins in SDS extracts prepared from cells of the parent (4047) and mutant strain (Δ*lgt*$_{190\text{-}685}$) were separated by SDS-PAGE and transferred to nitrocellulose. Immunoblotting was carried out using a polyclonal antibody raised to the LppC acid phosphatase of *S. equisimilis*. Lane 1, globomycin–treated *S. equi* 4047; lane 2, *S. equi* 4047; lane 3, *S. equi* Δ*lgt*$_{190\text{-}685}$.

B. Whole cell acid phosphatase activity was determined for strain 4047 (♦) and the Δ*lgt*$_{190\text{-}685}$ mutant (■) across a range of pH values, by spectrophotometric changes associated with the release of p-nitrophenol from the substrate p-nitrophenol phosphate. Results are representative of three different experiments.

A



B

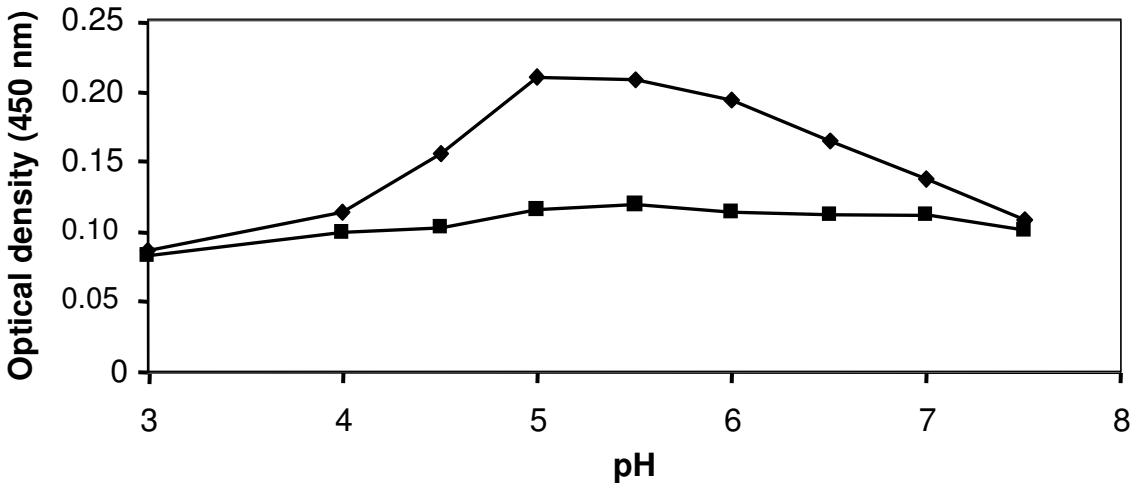
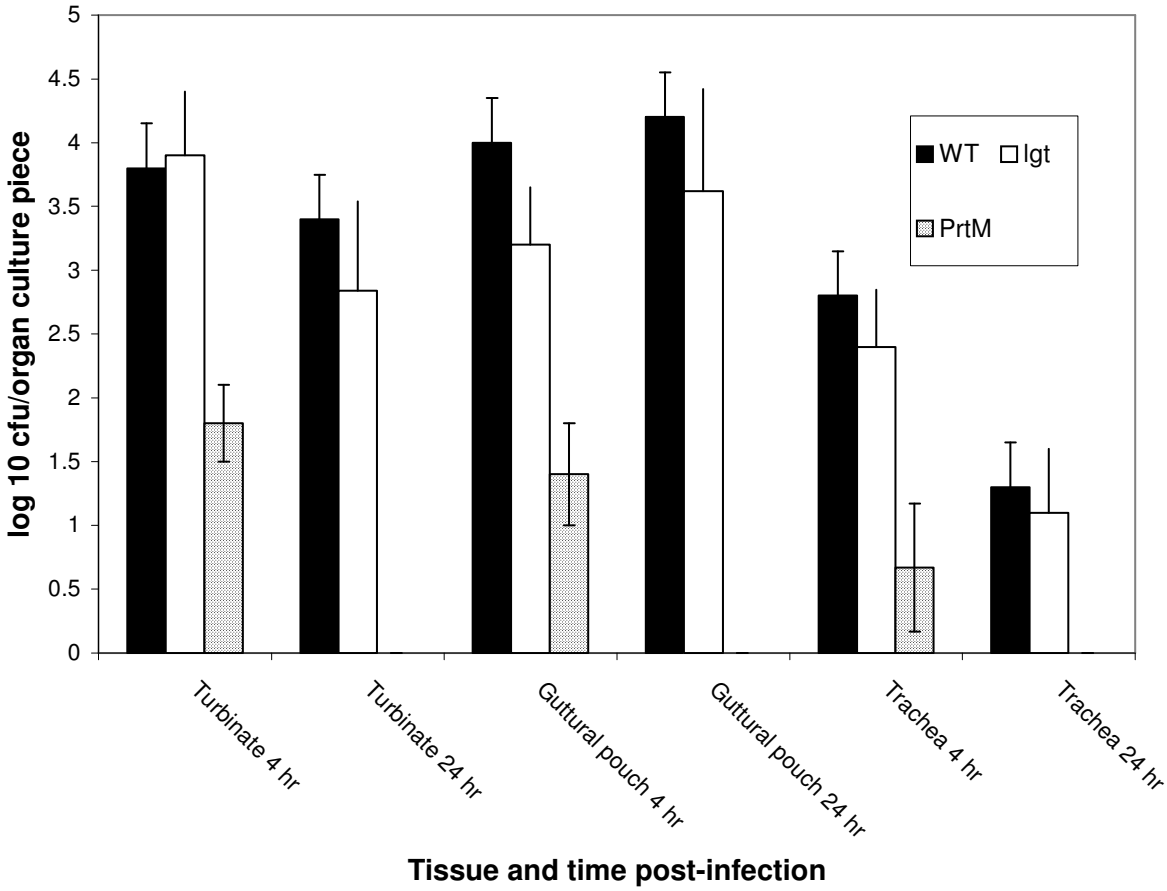


Figure 3. Colonisation and morphometric analysis of air interface organ cultures infected with *S. equi* strains

A. Recovery of viable bacteria 4 h and 24 h post-infection after infection of nasal turbinate, guttural pouch and tracheal air interface organ cultures with 1x 10^5 cfu wild-type *S. equi* or the mutants $\Delta lgt_{190\text{-}685}$ and $\Delta prtM_{138\text{-}213}$. Data bars shown the mean viable counts (± SD) from 6 independent experiments.

B. Surface morphometric analysis of nasal turbinate, guttural pouch and tracheal air interface organ cultures 24 h after infection with 5 $\log_{10}$ cfu wild-type *S. equi* or the mutants $\Delta lgt_{190\text{-}685}$ and $\Delta prtM_{138\text{-}213}$. Data bars shown the mean % surface coverage by mucus (± SD) from 6 independent experiments.

A



B

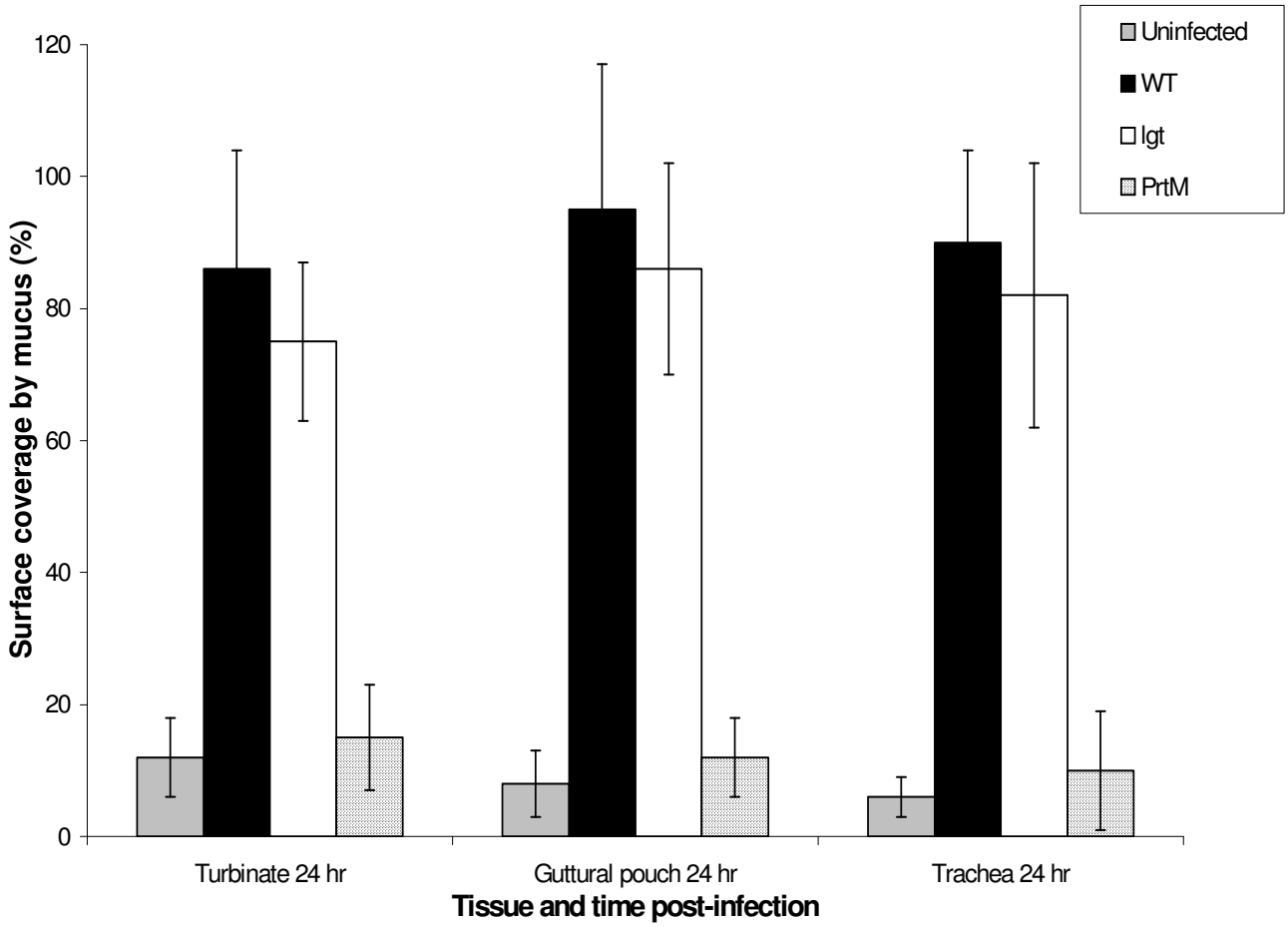

Uninfected
WT
Igt
PrtM
120
100
80
60
40
20
0
Surface coverage by mucus (%)
Turbinate 24 hr
Guttural pouch 24 hr
Trachea 24 hr
Tissue and time post-infection

Figure 4. Morphology of air interface organ cultures exposed to *S. equi* strains

Representative SEM micrographs of uninfected nasal turbinate organ culture pieces or pieces infected with 1 x 10^5 cfu wild type *S. equi* or the two mutants after 24 h in culture: A. Uninfected control; B. Wild type *S. equi* 4047; C. $\Delta lgt_{190\text{-}685}$; D. $\Delta prtM_{138\text{-}213}$. All images shown at x 2000 magnification. Scale bars = 10 micrometers.

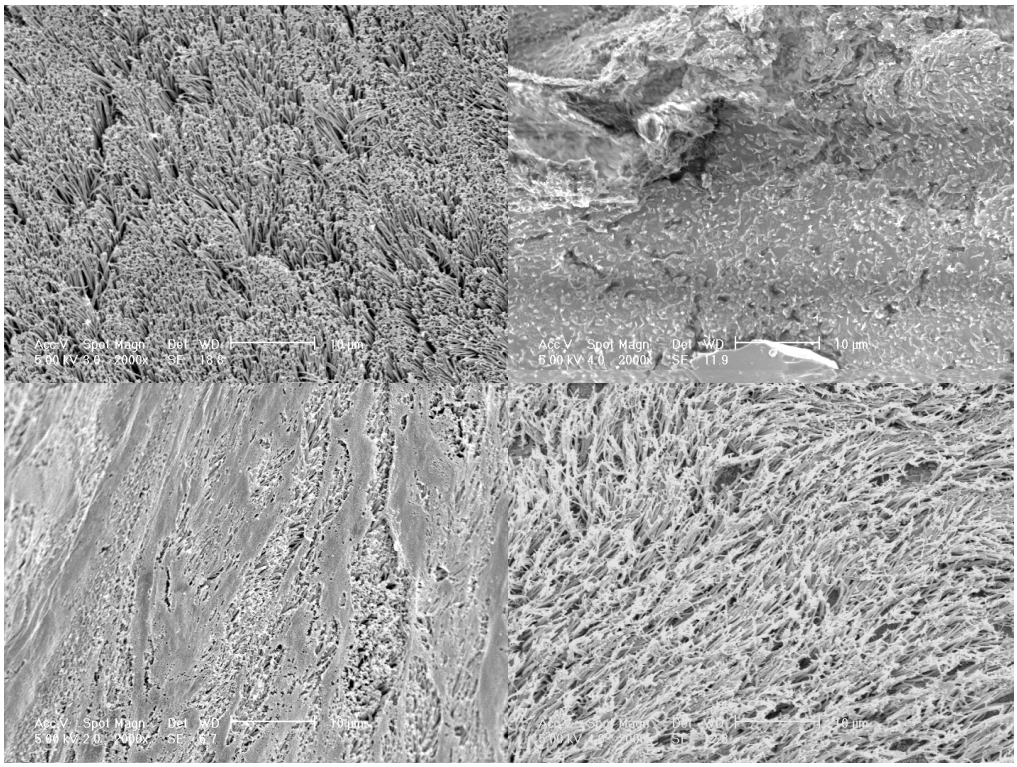


Figure 5. Challenge of mice with the $\Delta lgt_{190\text{-}685}$ and $\Delta prtM_{138\text{-}213}$ deletion strains.

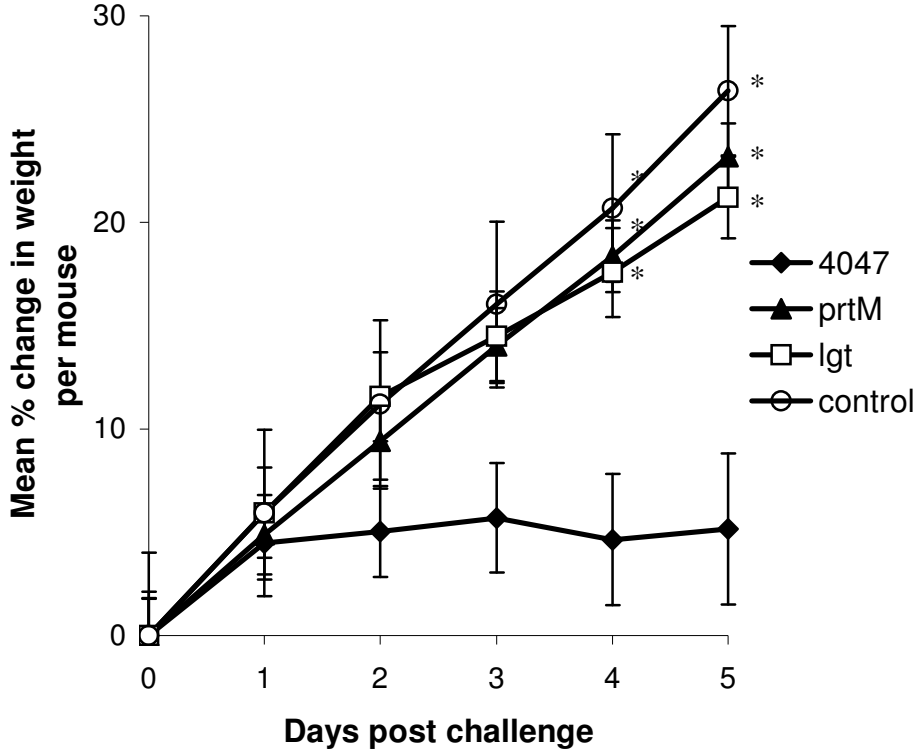
A. The mean % increase in weight per mouse was calculated for each of the challenge groups. Mice succumbing to infection with wild type *S. equi* (n=30) lost or failed to gain weight in comparison to uninfected controls (n=10). Groups of 30 mice challenged with the $\Delta lgt_{190\text{-}685}$ (lgt) and $\Delta prtM_{138\text{-}213}$ (prtM) continued to gain weight during the course of the study. Error bars indicate the standard error from the mean. * indicates a statistical significance of $P<0.05$ compared with wild type infected ponies.

B. The mean number of sneezes in 2 minutes for groups of five, co-housed mice was calculated for each of the challenge groups. Mice infected with parental *S. equi* 4047 had a significantly elevated sneezing rate when compared with uninfected and $\Delta lgt_{190\text{-}685}$ and $\Delta prtM_{138\text{-}213}$ challenged groups. Error bars indicate the standard error from the mean. * indicates a statistical significance of $P<0.05$ compared with wild type infected ponies.

C. The extent of disease on histological examination of mice was quantified according to the scoring system outlined in Materials and Methods. The mean total score per mouse was calculated. Error bars indicate the standard error from the mean. * indicates a statistical significance of $P<0.05$ compared with wild type infected ponies.

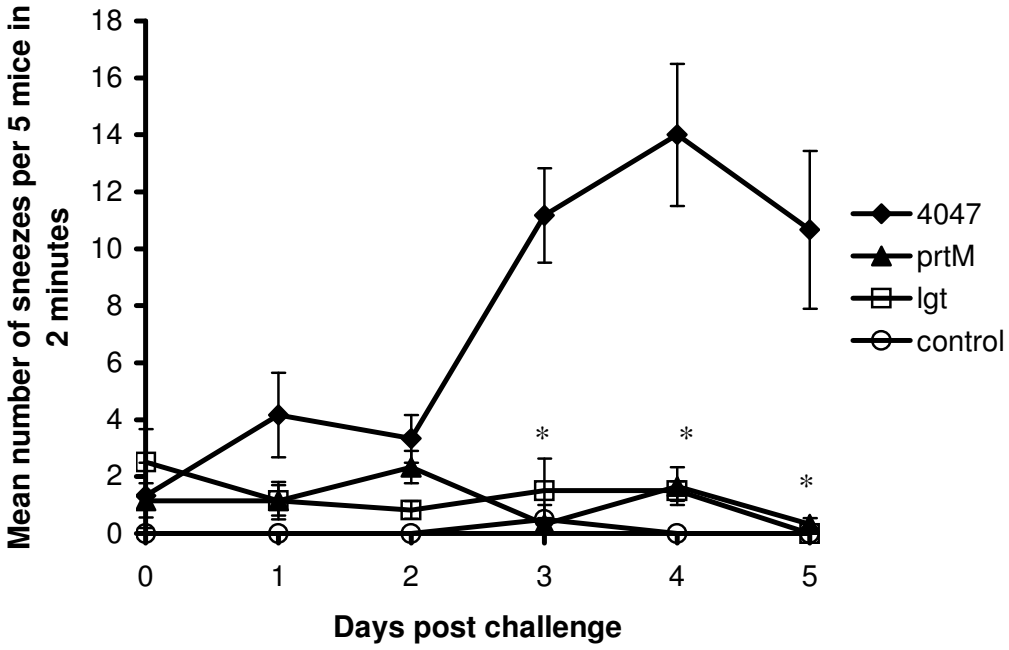
D. The number of mice with histological signs of disease attributable to *S. equi* infection following post mortem examination was compared to the number without histological signs of disease by Fisher's exact test, to determine if deletion of the *lgt* or *prtM* genes significantly attenuated *S. equi* in the mouse infection model.

A

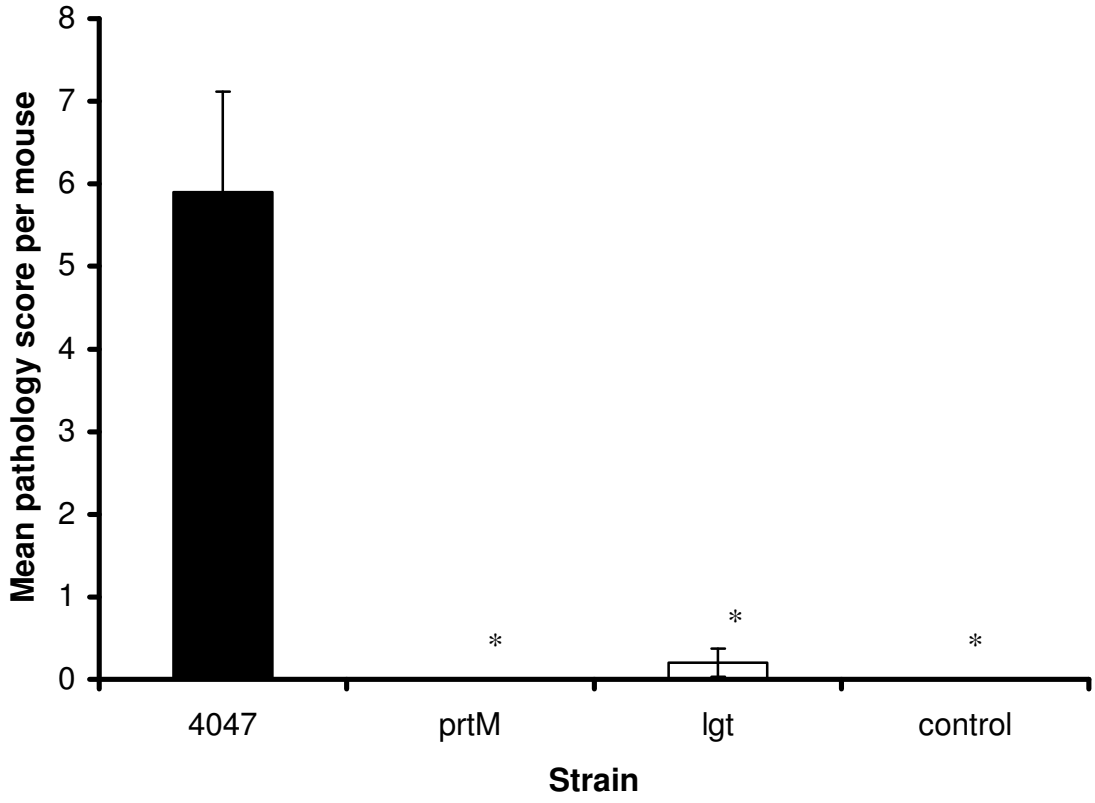

30
20
10
0
Mean % change in weight per mouse
0
1
2
3
4
5
Days post challenge
4047
prtM
lgt
control
*
*
*
*
*

1

B


Mean number of sneezes per 5 mice in 2 minutes
18
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
Days post challenge
4047
prtM
lgt
control
*
*
*

C


Mean pathology score per mouse
8
7
6
5
4
3
2
1
0
4047
prtM
lgt
control
Strain
*
*
*

D

Strain	Disease	No disease	Fisher's exact
4047	17	13	
Δ*lgt*$_{190\text{-}685}$	2	28	$p < 0.001$
Δ*prtM*$_{138\text{-}213}$	0	30	$p < 0.001$

Figure 6. Effect of intranasal challenge of ponies on rectal temperature, clinical scores and neutrophil levels.

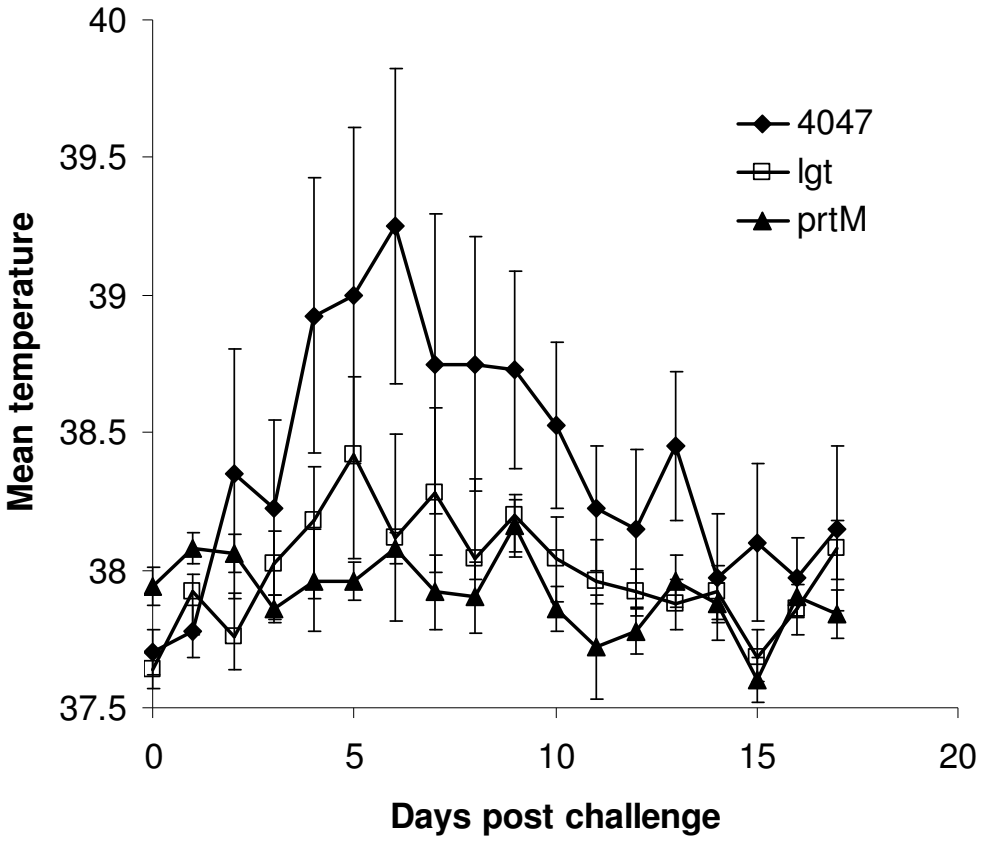
A. Rectal temperatures of ponies were taken daily from the day before to day 17 post-challenge and the mean temperature per pony for each challenge group is shown.

B. The number of ponies suffering from pyrexia in each group was compared by Fisher's exact test. Ponies were considered pyrexic when their temperature exceeded 39^{o}C. Only ponies challenged with the Δ*prtM*$_{138\text{-}213}$ strain had a significantly reduced incidence of pyrexia ($p = 0.048$).

C. The mean clinical score for each challenge group was calculated according to the scoring system presented in Table 2. Comparison of the total clinical score per pony over the study period using the Kruskal-Wallis test indicated that only the Δ*prtM*$_{138\text{-}213}$ deletion strain (prtM) was significantly attenuated $P = 0.0267$.

D. The mean number of neutrophils per ml of blood was quantified for each pony. Ponies developed signs of neutrophilia (neutrophil count >6.5×10^{6} ml^{-1}) 6 days post challenge with the parental 4047 strain, whereas ponies challenged with the Δ*lgt*$_{190\text{-}685}$ strain (lgt) developed neutrophilia 17 days post challenge and no signs of neutrophilia were observed in ponies challenged with the Δ*prtM*$_{138\text{-}213}$ strain (prtM; $p < 0.05$). Error bars indicate the standard error from the mean. * indicates a statistical significance of $P<0.05$ compared with wild type infected ponies.

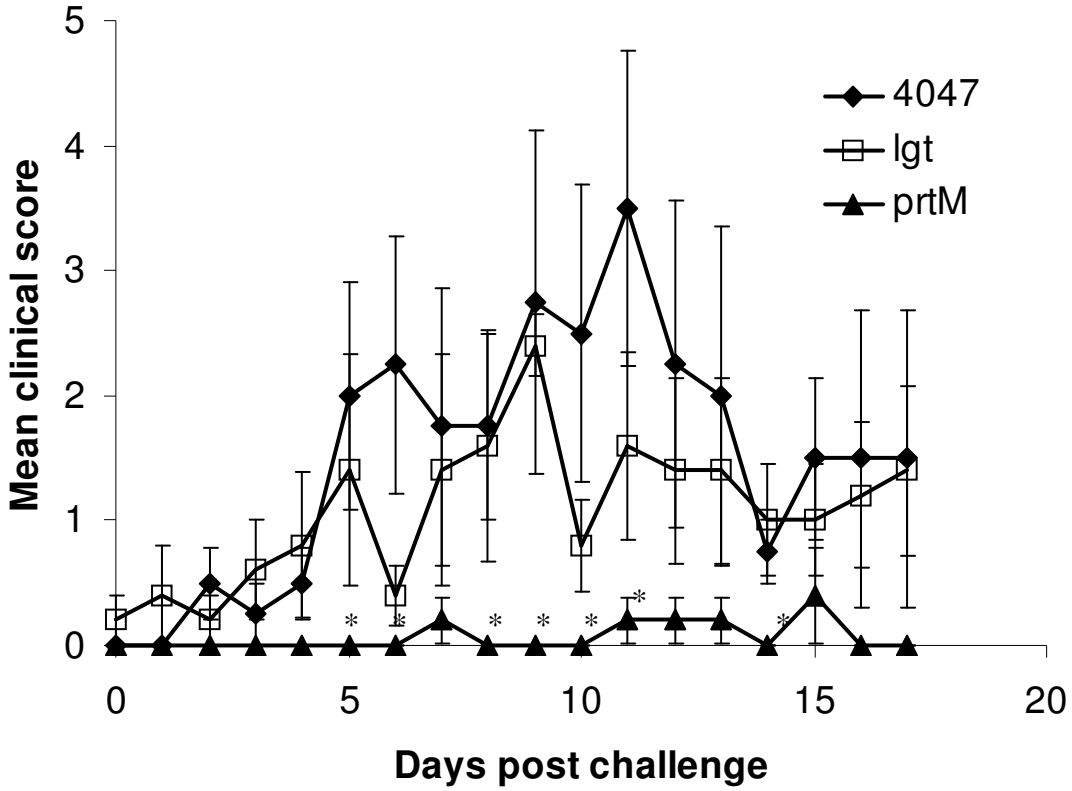
A


40
39.5
39
38.5
38
37.5
Mean temperature
0
5
10
15
20
Days post challenge
4047
lgt
prtM

B

Strain	Pyrexic	Not Pyrexic	Fisher's exact
4047	3	1	
$\Delta lgt_{190\text{-}685}$	1	4	
$\Delta prtM_{138\text{-}213}$	0	5	p = 0.048

C



D

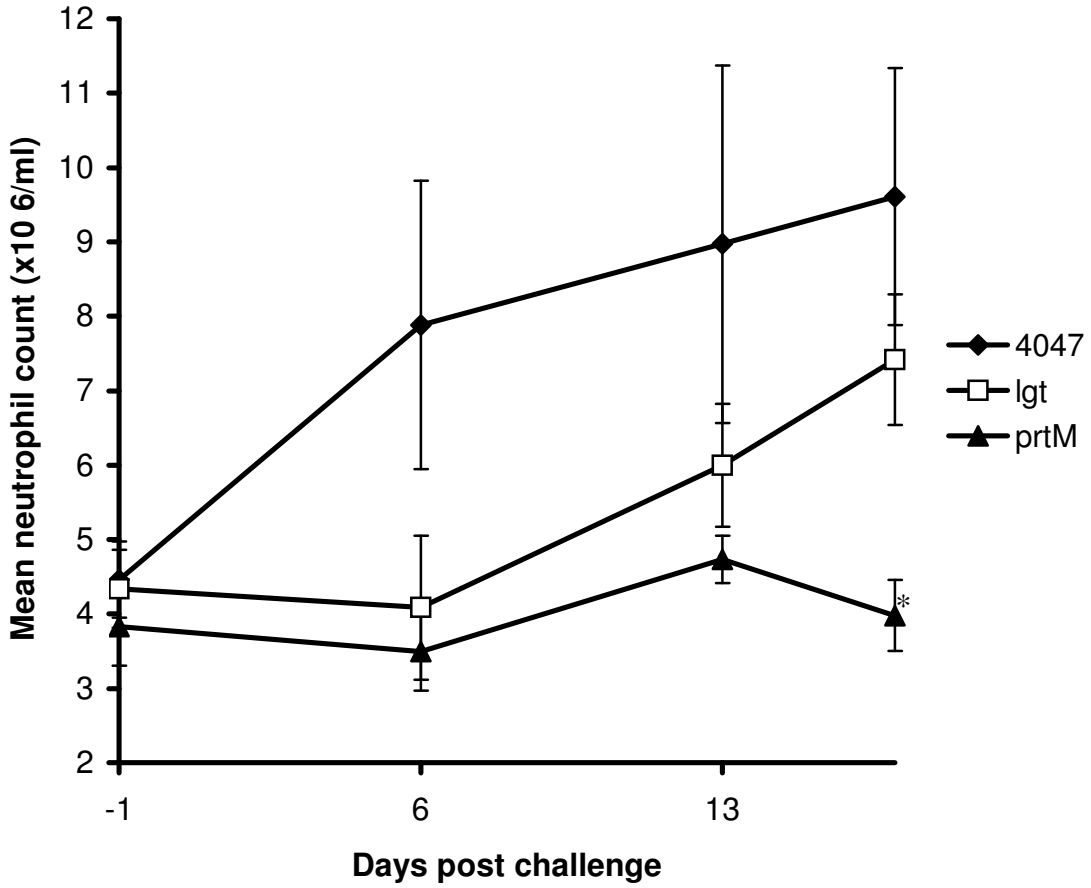

Mean neutrophil count (x10 6/ml)
12
11
10
9
8
7
6
5
4
3
2
-1
6
13
Days post challenge
4047
lgt
prtM
*

Figure 7. Effect of intranasal challenge of ponies on the disease identified on post mortem examination.

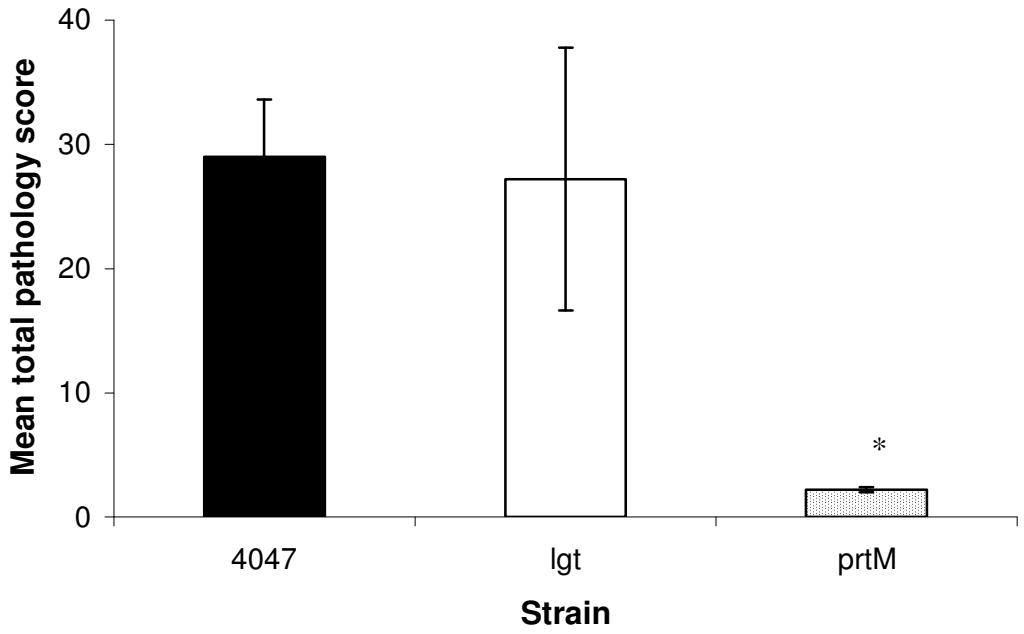
A. The number of ponies in each group with significant pathological signs of strangles attributable to infection with *S. equi* on post mortem examination were compared using Fisher's exact test. Although 2 of 5 ponies challenged with the $\Delta lgt_{190\text{-}685}$ strain had no significant signs of disease, this was not statistically significant (P = 0.44). However, ponies challenged with the $\Delta prtM_{138\text{-}213}$ strain did have significantly reduced disease on post mortem examination (P = 0.008).

B. The mean pathology score per pony was calculated for each of the challenge groups on post mortem examination using the scoring system outlined in Materials and Methods. Error bars indicate the standard error from the mean. * indicates a statistical significance of P<0.05 compared with wild type infected ponies..

A

Strain	Strangles	No strangles	Fisher's exact
4047	4	0	
$\Delta lgt_{190\text{-}685}$	3	2	p = 0.44
$\Delta prtM_{138\text{-}213}$	0	5	p = 0.008

B



1
2
3
4
5

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