Investigation of the Fim1 putative pilus locus of *Streptococcus equi* subspecies *equi*

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**Abstract**

The Gram-positive bacterium *Streptococcus equi* subspecies *equi* (*S. equi*) is the causative agent of strangles, among the most frequently diagnosed infectious diseases of horses worldwide. Genome analysis of *S. equi* strain 4047 (Se4047) identified a putative operon, Fim1, with similarity to the pilus loci of other Gram-positive bacteria. The Fim1 locus was present in all strains of *S. equi* and its close relative *S. equi* subspecies *zooepidemicus* (*S. zooepidemicus*) that have been studied to date. In this study we provide evidence that the putative structural pilus proteins, SEQ_0936 and CNE, are produced on the cell surface during *in vitro* growth and *in vivo* infection. Although the proteins encoded within the Fim1 locus are not essential for attachment or biofilm formation, over-transcription of SEQ_0936 and CNE enhanced attachment to equine tissue *in vitro*. Our data suggest that whilst the Fim1 locus does not produce a polymerized pilus structure, the products of the Fim1 locus may fulfil an adhesive function. The putative pilus-associated regulator, tetR, which contains a nonsense mutation in *S. equi*, was able to regulate transcription of the Fim1 locus following repair and over-transcription, confirming its predicted role in the operon.

**INTRODUCTION**

The interaction of bacteria with their environment is critical for bacterial attachment, colonization and persistence [1, 2]. Many bacterial species fulfil this requirement by producing adhesive proteins on their cell surface, either directly or extended away on hair-like pilus structures. The production of such proteins can help to overcome the net repulsive forces between host cell and bacterium [3], and dissipate mechanical stress [2], playing an important role in the colonization of host species. The pilus loci of Gram-positive bacteria contain specific conserved genetic features that facilitate their identification at the genome level. Pili are encoded in operons on pathogenicity islands containing or flanked by transcriptional regulators [4]. The pilus loci contain genes encoding the sortase-processed pilus backbone subunit, which includes a distinctive pilin motif and an E-box [5, 6]. Genes encoding one or more sortase-processed accessory pilins, often with an adhesive function, are also present [7, 8] along with a pilus-specific sortase [9].

**Streptococcus equi** subsp. *equi* (*S. equi*) is the causative agent of strangles, which is among the most frequently diagnosed infectious diseases of horses worldwide [10]. Strangles is characterized by pyrexia followed by abscess formation in the lymph nodes of the head and neck [11]. Rupture of abscesses formed in the retropharyngeal lymph nodes into the gullet leads to drainage of infectious pus from the nostrils. In a proportion of horses, pus drainage is incomplete and these animals become subclinical, persistently infected carriers, an event believed to be key to the global success of *S. equi* [12]. However, the processes that underlie attachment of *S. equi* to the equine mucosa and persistence in the gullet pouch remain unknown.

Analysis of the genome of *S. equi* strain 4047 (Se4047) identified just one putative pilus locus, Fim1, which contains coding sequences (CDSs) predicted to encode an accessory pilin, CNE, a backbone protein, SEQ_0936, a pilus-specific sortase, SrtC1 and a pilus-associated regulator TetR [13] (Fig. 1). CNE binds collagen [14], is immunogenic [15] and is one component of an effective subunit vaccine against...
strangles that is currently in development [16]. The TetR family of transcriptional regulators are known to have a huge diversity of binding ligands, often with broad ligand specificity [17, 18]; however, the binding ligand(s) are unknown for S. equi. After binding to a ligand, TetR would ordinarily release from its DNA operator sequence, permitting transcription of the locus and thus regulating production of the pilins in response to stimuli. However, a nonsense mutation at codon 43 of tetR was predicted to truncate the expressed TetR protein, including loss of the DNA-binding domain, leading to constitutive pilus production or the production of longer pili [13]. The Fim1 locus is present in all isolates of S. equi and S. equi subspecies zooepidemicus (S. zooepidemicus), a close relative of S. equi, studied to date, but none of the S. zooepidemicus isolates containing the nonsense mutation in tetR [13, 19]. However, electron microscopy of S. equi grown in a variety of different conditions has so far failed to identify pilus structures (D. Goulding, personal communication).

In this study we showed that the Se4047 Fim1 locus is produced during in vitro growth and in vivo infection, that increased production of the Fim1 putative pilus components enhanced attachment to equine tissues, and that engineering of the Fim1 locus influenced biofilm production in vitro.

**METHODS**

**Bacterial strains, culture conditions and extraction of genomic DNA**

All work was performed using the parental strain Se4047, originally isolated from a submandibular abscess of an infected New Forest pony in 1990. S. equi strains were cultured from single colonies in Todd–Hewitt broth (THB) (Oxoid) or on colistin-oxolinic acid blood agar (COBA) streptococcal-selective agar (bioMerieux) at 37°C in a humidified atmosphere containing 5% CO₂ unless otherwise stated. For the isolation of genomic DNA (gDNA) a single colony was cultured in THB with hyaluronidase (30 µg ml⁻¹, Sigma) and gDNA extracted using a GenElute spin column kit as per the manufacturer’s instructions (Sigma).

**Escherichia coli** E. coli strains for protein over-expression and purification (DH10B E.coli), and for generating mutant strains (TG1repA+ E.coli), were cultured from single colonies in Luria–Bertani broth (LB) (Oxoid) or on Luria–Bertani broth agar (LBA) (Oxoid) at 37°C unless otherwise stated.

**Cloning, over-expression and purification of SEQ_0936**

The Se4047 SEQ_0936 gene lacking the signal sequence and LPxtG motif was cloned, over-expressed and purified according to the method described for the purification of EqBA [20]. In brief, the SEQ_0936 fragment was amplified by PCR using the primers listed in Table S1 (available with the online Supplementary Material) and cloned into the BamHI/EcoRI sites of the pGEX-3X vector (GE Healthcare), resulting in a construct containing SEQ_0936 with a glutathione S-transferase (GST) tag fused to the N-terminus. The plasmid was transformed into E. coli DH10B and cultured on LBA containing 100 µg ml⁻¹ ampicillin (Sigma). Two l of diluted culture was induced for protein over-production by the addition of 1 mM IPTG. The culture was centrifuged at 15 344 g, the resultant cell pellet was lysed and the SEQ_0936 was purified using 3 ml of glutathione S-transferase (GST) tag fused to the N-terminus. The plasmid was transformed into E. coli DH10B and cultured on LBA containing 100 µg ml⁻¹ ampicillin (Sigma). Two l of diluted culture was induced for protein over-production by the addition of 1 mM IPTG. The culture was centrifuged at 15 344 g, the resultant cell pellet was lysed and the SEQ_0936 was purified using 3 ml of glutathione S-transferase (GST) tag fused to the N-terminus. The plasmid was transformed into E. coli DH10B and cultured on LBA containing 100 µg ml⁻¹ ampicillin (Sigma). Two l of diluted culture was induced for protein over-production by the addition of 1 mM IPTG. The culture was centrifuged at 15 344 g, the resultant cell pellet was lysed and the SEQ_0936 was purified using 3 ml of glutathione S-transferase (GST) tag fused to the N-terminus. The plasmid was transformed into E. coli DH10B and cultured on LBA containing 100 µg ml⁻¹ ampicillin (Sigma). Two l of diluted culture was induced for protein over-production by the addition of 1 mM IPTG. The culture was centrifuged at 15 344 g, the resultant cell pellet was lysed and the SEQ_0936 was purified using 3 ml of glutathione S-transferase (GST) tag fused to the N-terminus. The plasmid was transformed into E. coli DH10B and cultured on LBA containing 100 µg ml⁻¹ ampicillin (Sigma). Two l of diluted culture was induced for protein over-production by the addition of 1 mM IPTG. The culture was centrifuged at 15 344 g, the resultant cell pellet was lysed and the SEQ_0936 was purified using 3 ml of glutathione S-transferase (GST) tag fused to the N-terminus. The plasmid was transformed into E. coli DH10B and cultured on LBA containing 100 µg ml⁻¹ ampicillin (Sigma). Two l of diluted culture was induced for protein over-production by the addition of 1 mM IPTG. The culture was centrifuged at 15 344 g, the resultant cell pellet was lysed and the SEQ_0936 was purified using 3 ml of glutathione S-transferase (GST) tag fused to the N-terminus.

Rabbit polyclonal sera to the purified recombinant SEQ_0936 protein were produced by Yorkshire Biosciences Ltd.

**Deletion of Fim1 genes**

The individual Fim1 genes cne, SEQ_0936 and srtC1, and the whole Fim1 locus, including tetR, were deleted from Se4047 using the allelic replacement mutagenesis system previously described for deletion of prtM [21]. The regions flanking each sequence for deletion were amplified by PCR (the primers are listed in Table S1) and cloned into the multiple cloning site of pGHOST9 using EcoRI/SalI. Correct
constructs were transformed into Se4047, integrated into the chromosome and then excised, generating the strains Δcne, ΔSEQ_0936, ΔsrtC1 and ΔFim1 (Table S2). Gene deletions were confirmed by PCR and capillary sequencing of the target site.

Construction of Fim1 over-transcription (+) mutants

Full copies of the Fim1 genes cne, SEQ_0936 and srtC1, each with the Fim1 promoter region, were cloned individually into a fragment of the SEQ_0495 pseudogene of Se4047 using SalI/XhoI sites in pGHost9. The primers used are listed in Table S1. Three putative promoters were predicted for the Fim1 locus (Fig. S1) and therefore a fragment containing all three was used for the promoter region. The allelic replacement mutagenesis system described for the deletion of prtM [21] was used to introduce these constructs into SEQ_0495 of strains in which the corresponding gene had previously been deleted, generating the strains cne+, SEQ_0936+ and srtC1+. Gene insertions were confirmed by PCR and capillary sequencing of the target site. Generation of Fim1+ was not possible, as cloning of the whole locus into E. coli could not be achieved. All of the strains are listed in Table S2.

Formic acid extraction of cell surface proteins

Colony material from strains Se4047 wild-type (WT), ΔFim1, ΔSEQ_0936, SEQ_0936+, ΔCNE and CNE+ grown on COBA plates were resuspended in PBS and then incubated with hyaluronidase (30 µg ml⁻¹) for 10 min at 37 °C. Bacteria were collected by centrifugation and the cell pellet was washed twice with PBS. Cell pellets were resuspended in 70% formic acid and incubated at 65 °C for 30 min. The solution was centrifuged at 16 000 g for 5 min and the supernatant containing solubilized proteins removed and retained. Proteins were then purified by trichloroacetic acid precipitation and acetone washing. Protein pellets were resuspended in 50 µl TBS and stored at −20 °C.

Western blot analysis of Se4047

Formic acid-extracted proteins of Se4047 WT, ΔFim1, ΔSEQ_0936, SEQ_0936+, ΔCNE and CNE+ were analysed by Western blot along with purified SEQ_0936 (46.7 kDa) and CNE (66.7 kDa) (the latter supplied by Bengt Guss [14]) protein controls with primary antibodies to SEQ_0936 and CNE (66.7 kDa) (the latter supplied by Bengt Guss [22]). For each sample, 10 µg of total protein, or 0.5 ng and 0.25 ng of purified SEQ_0936 and CNE proteins, respectively, were separated on 12% polyacrylamide gels by SDS-PAGE and then transferred to nitrocellulose membrane (Whatman). Membranes were blocked overnight at 4 °C in 5% skimmed milk (Marvel) made with PBS. Blocked membranes were rinsed twice with PBS containing 0.1% Tween20 (Sigma) (PBST), washed with gentle agitation for 30 min in PBST, and then incubated for 1 h at room temperature with gentle agitation in block solution containing 1/1000 polyclonal swine anti-rabbit immunoglobulins/HRP secondary antibody (Dako). Membranes were rinsed twice in PBST, washed with gentle agitation for 1 h in PBST, and then visualized using Western blotting detection reagents (GE Healthcare) on chemiluminescent film (GE Healthcare). A Precision Plus Protein WesternC Protein Standard ladder (Bio Rad) was used to determine band sizes.

Measurement of antibodies towards SEQ_0936 and CNE by iELISA

Antibody titres towards SEQ_0936 and CNE were measured in archived serum samples from Welsh mountain ponies challenged with Se4047 taken prior to challenge and then 7 and 14 days post-challenge. The indirect ELISA (iELISA) method previously described for the detection of exposure to S. equi was utilized, substituting recombinant SEQ_0936 and CNE proteins, provided by Colin Moncrieff from the University of Newcastle, for antigens A and C [23]. The fold changes in titre for 7 and 14 days post-challenge were calculated compared to pre-challenge samples for each pony.

Quantification of Fim1 gene transcription in Fim1 allelic replacement mutants

Total RNA was extracted from 4 ml cultures grown to optical density (OD) 0.6 at λ 600 nm from strains Se4047 WT, ΔFim1, Δcne, cne+, ΔSEQ_0936, SEQ_0936+, ΔsrtC1 and srtC1+ using RNA protect, RNase and DNase kits (all Qiagen) as per the manufacturer’s instructions, and employing the strategy previously described in [20]. RNA purity and quantity were determined using a Nanodrop ND1000 spectrophotometer (Nanodrop Technologies). One hundred ng of RNA per sample was used to synthesize cDNA in 20 µl reverse transcription (RT) reactions using a Verso cDNA kit (Thermo Scientific) as per the manufacturer’s instructions, and primed using random hexamers. Transcription of cne, SEQ_0936 and srtC1, and the housekeeping gene gyrA, were quantified by quantitative PCR (qPCR) in 20 µl reactions comprising 1× Kapa SYBR fast qPCR mix (Anachem), 0.3 µM forward and reverse primers (Table S1) and 6 µl of a 1/10 dilution of RT reaction, and thermocycled at 95 °C for 3 min, with 40 cycles of 95 °C for 3 s, 60 °C for 10 s and then 95 °C for 15 s. A ramp step from 60 °C to 95 °C with SYBR reads every 0.3 °C was performed to calculate the dissociation curves for products, and no template or RT controls were included to confirm the absence of contaminating DNA and RNA in the samples. Copy numbers were calculated from standard curves and normalized to gyrA to demonstrate the cessation and restoration of transcription for each Fim1 gene in the corresponding deletion and + mutants. Transcriptional values were compared to Se4047 WT using Student’s unpaired t-tests.

Growth curve analysis of Se4047 WT and Fim1 mutants

Overnight cultures of Se4047 WT and the Fim1 mutant strains were diluted to OD 0.05 at λ 600 nm in fresh pre-
warmed and pre-gassed THB and the OD at λ 600 nm was measured every 30 min until all of the cultures were in the stationary phase. This was repeated on four independent occasions. Mean data were plotted on a scatter plot, the doubling time was calculated from log phase data using an online calculation tool (http://www.doubling-time.com/compute.php), and the statistical significance of the variation in doubling time was tested using the Mann–Whitney U test. Where the end-point OD varied between strains, cultures were diluted 1×10⁻⁶ in PBS and cultured on COBA plates, and the bacterial colonies were enumerated to determine whether the differences in stationary-phase OD were due to the numbers of viable bacteria present.

Air-interface organ culture

The S. equi strains Se4047 WT, ΔFim1, Δcne, cne⁺, ΔSEQ_0936, SEQ_0936⁺, ΔsrtC1 and srtC1⁺ were used to infect tracheal cannalized epithelium using the air-interface organ culture system previously described for the quantification of lgt and prtM mutant attachment [21] with some modifications. Whole tracheas were recovered within an hour of the post-mortem examination of Welsh mountain ponies that were euthanized for reasons unrelated to this project. Tissues were treated with antimicrobials [1×penstrept (PAA), 2.5 µg ml⁻¹ amphotericin-B (Sigma) and 100 µg ml⁻¹ gentamicin (Sigma)] in Dulbecco’s modified Eagle’s medium (DMEM) (PAA) supplemented with 2 mM l-glutamine (Sigma) for 2 h with gentle agitation to remove microbial flora. Fifteen cm sections were then washed for 10 min eight times with fresh DMEM supplemented with 2 mM l-glutamine to remove antimicrobials. Tissue pieces were prepared as previously described [21] and infected with ~1×10⁶ c.f.u. of log-phase culture, which was confirmed by enumeration of the inoculum following overnight growth on COBA at 37 °C. Following 2 h incubation at 37 °C, non-adherent bacteria were removed and the amount of S. equi attached to the tissue pieces was enumerated following homogenization. Six tissue pieces were used per strain per trachea and the mean bacterial attachment was calculated, correcting for slight differences in the original infectious dose. This was repeated using at least six different tracheas per strain and the variation between tracheas was standardized using infection with WT as a benchmark. The significances of differences in attachment resulting from pilus mutations was determined by the two-sided Mann–Whitney U test.

Biofilm assay

Biofilm assays were performed on strains Se4047 WT, ΔFim1, Δcne, cne⁺, ΔSEQ_0936, SEQ_0936⁺, ΔsrtC1 and srtC1⁺ to quantify biofilm formation for the Fim1 mutants compared to WT. Biofilm formation was tested in THB and tryptone soya broth (TSB) (Oxoid) supplemented with 0.5 % glucose (Sigma), both of which reliably allow biofilm development in Se4047 WT. Cells from overnight cultures of the test strains were harvested by centrifugation, washed with PBS and diluted to OD 0.05 at λ 600 nm in fresh broth. Duplicate sets of CellBIND 96-well clear polystyrene flat-bottomed plates (Corning) were prepared (one for crystal violet staining and one for CellTiter-Blue assay) containing 200 µl of culture or PBS control per well. In each set of plates, 12 wells per strain were filled with OD 0.05 at λ 600 nm culture and 12 wells were left empty as blank controls. On each plate, 12 wells were filled with PBS as a negative control. Plates were incubated for 24 h at 37 °C, 5 % CO₂ with gentle agitation, emptied and washed twice with the corresponding pre-warmed media, and then 200 µl well⁻¹ of fresh pre-warmed media was added and the plates incubated for another 24 h. Plates were then emptied, washed with PBS and air-dried.

One set of plates was stained with 0.2 % crystal violet. Following three washes, bound crystal violet was resuspended by the addition of 100 µl well⁻¹ of 30 % glacial acetic acid, and the absorbance was read at λ 490 nm. Mean values were calculated for each set of 12 wells and corrected to the mean blank and PBS control values.

With the second set of plates, 100 µl well⁻¹ CellTiter-Blue reagent (Promega), a metabolic dye, diluted in PBS as per the manufacturer’s instructions, was added to 8 out of the 12 wells per sample. PBS was added to the remaining four wells per sample as a negative control. The plates were incubated for a further 5 h and the fluorescence at λ 560/590 nm was measured to quantify the cell content. Mean values were calculated for each set of eight wells and corrected to the mean blank and PBS control wells.

The biofilm assays were repeated four times and the values standardized between experiments using Se4047 WT as a benchmark. Significant differences in biofilm development were determined by the two-sided Mann–Whitney U test.

Point mutation in the tetR-like regulator

A repair was made to the terminal nonsense mutation at codon 43 of tetR in Se4047, changing TAA to TAC, the equivalent residue in its close relative S. zooepidemicus strain H70 [13], by recombinant PCR and the allelic replacement method described for the deletion of prtM [21]. Overlapping fragments with the mutated base in the central primer were separately amplified by PCR (primers in Table S1). Both PCR products were pooled and a second PCR using the two external primers was performed to generate a single product containing the mutated base. The recombinant fragment was cloned into the multiple cloning site of pGHost9 at EcoRI/SalI and the plasmid transformed into Se4047. The plasmid was integrated into the chromosome and then excised, generating the strain tetR repaired (Table S2), which was confirmed by PCR and capillary sequencing of the target site.

Construction of an Se4047 mutant over-transcribing the repaired tetR-like regulator
tetR and the eqbB iron-responsive promoter (to enable control over transcription levels) from the strain Se4047 tetR repaired were cloned into a fragment of the SEQ_0495 pseudogene of Se4047 using Sall/XhoI sites in pGHost9. The
primers used are listed in Table S1. The allelic replacement mutagenesis system described for the deletion of *prtM* [21] was again used to introduce this construct into Se4047, generating the strain *tetR* (Table S2). Successful fragment insertion was confirmed by PCR and capillary sequencing.

**Quantification of *tetR*-like regulator and downstream Fim1 gene transcription in *tetR* repaired and *tetR*+ mutants**

RNA was extracted, reverse-transcribed and analysed by qPCR from strains Se4047 WT, *tetR* repaired and *tetR*+ as described for the quantification of Fim1 gene transcription above. Transcription of *cne*, SEQ_0936, srtC1, *tetR* and the housekeeping gene *gyrA* was quantified (primers Table S1). The process was repeated three times in total, transcription was normalized to *gyrA*, and data were standardized using WT transcription as a baseline. Mean values were calculated and the statistical significance of transcription variation between the strains was tested using the two-sided Mann–Whitney U test.

**RESULTS AND DISCUSSION**

**The Fim1 proteins, SEQ_0936 and CNE, are expressed by Se4047 during in vitro growth**

We aimed to ascertain whether the SEQ_0936 (putative pilus backbone) and CNE (putative accessory pilin) proteins of the Fim1 locus were being translated and expressed on the cell surface by Se4047 WT during in vitro growth. To achieve this, we performed Western blot analysis with polyclonal rabbit sera to SEQ_0936 and CNE to detect both proteins in a formic acid-extracted surface protein preparation. Blots revealed the presence of both SEQ_0936 (46.7 kDa) (Fig. 2a) and CNE (66.7 kDa) (Fig. 2b) monomers in the cell-surface protein preparation when compared to the purified proteins, which were absent from strain ΔFim1, from which the whole Fim1 locus had been deleted. Whilst this demonstrates that the proteins are being produced *in vitro*, Western blots of pili from other Gram-positive bacteria, where pilus structures on the cell surface have been seen under electron microscopy, show polymers as well as monomers of the backbone subunit [24]. Culture media, cell membrane and cell wall fractions were also analysed by Western blot (data not shown), but failed to identify pilus polymers, and no monomers were identified in the culture media. This suggests that although the proteins are being produced, they may not be forming overt pilus structures during *in vitro* culture, potentially being surface-bound as monomers. In *Streptococcus pyogenes* (*S. pyogenes*) and *Streptococcus pneumoniae* (*S. pneumoniae*), the production of pili on the cell surface has been demonstrated to be condition-dependent [8, 25–27]. However, as pilin monomers are observed in *S. equi*, indicating that expression of the pilins is occurring, it is more likely that the pilus-associated sortase is deficient in its polymerization function, as observed when the pilus-associated sortase is deleted in *Corynebacterium diphtheriae* [6].

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Western blot analysis of Se4047 WT, ΔFim1, ΔSEQ_0936, SEQ_0936+, ΔCNE and CNE+ formic acid extracts with primary antibodies to (a) SEQ_0936 and (b) CNE proteins. The samples are indicated above each lane and purified proteins are included as positive controls. Each lane contains 10 µg of formic acid-extracted protein or 0.5 and 0.25 ng of purified recombinant SEQ_0936 and CNE control proteins, respectively. Arrows indicate the molecular mass of the cross-reactive bands.
The Fim1 proteins, SEQ_0936 and CNE, are expressed by Se4047 during in vivo infection

To determine whether there is an equine immune response to the Fim1 putative structural proteins, SEQ_0936 and CNE (indicating that they are expressed during in vivo infection), we screened archived sera from experimentally infected animals by iELISA, and antibody titres were compared to pre-infection levels. The data showed that 12/12 and 3/12 ponies had a greater than twofold increase in antibody titre to SEQ_0936 (Fig. 3a) and CNE (Fig. 3b), respectively in the 2 weeks following challenge, demonstrating that both proteins are produced during in vivo infection.

**Generation of Se4047 Fim1 gene deletion and + mutants**

In order to evaluate the importance of Fim1 to bacterial attachment and biofilm formation by S. equi, deletions of cne, SEQ_0936, srtC1 and the whole Fim1 locus were made in Se4047. Full copies of each of the genes preceded by the Fim1 promoter were then inserted into the pseudogene SEQ_0495 of their respective deletion strains to produce + strains over-transcribing the corresponding gene (Table S2).

qPCR of cDNA from Se4047 WT and the mutant strains confirmed that transcription of cne, SEQ_0936 and srtC1 in the strain, and transcription of each gene in the appropriate individual deletion mutants, had been abolished (Fig. 4). Over-transcription of the desired Fim1 gene in each appropriate strain was also confirmed with 51-fold ($P<0.0001$), 23-fold ($P<0.0001$) and 38-fold higher ($P<0.0001$) levels of transcription in the cne, SEQ_0936 and srtC1 + strains, respectively, than was present in Se4047 WT. Western blots of formic acid extracts from the Se4047 WT, ΔFim1, ΔSEQ_0936, ΔSEQ_0936+, ΔCNE and CNE+ strains confirmed the absence of bands corresponding to SEQ_0936 (Fig. 2a) and CNE (Fig. 2b) in the corresponding deletion strains and bands of greater intensity than Se4047 WT in the appropriate + mutant strains.

To determine whether the engineering of the Fim1 locus affected strain growth rates, we plotted growth curves for Se4047 WT and the Fim1 mutants from the lag into the stationary phase (Fig. 5) and calculated doubling times. The mean doubling time of Se4047 WT was 185 min. cne+ had a significantly reduced doubling time of 61 min ($P=0.0209$), and SEQ_0936+ had a significantly increased doubling time of 381 min ($P=0.0209$). No other strains were significantly different from Se4047 WT (Fig. 5). Enumeration of cultures from the growth curve end point revealed that differences in the final OD at λ 600 nm for cne+ (OD=1.196) and SEQ_0936+ (OD=0.652) compared to Se4047 WT (OD=0.721) corresponded to real differences in the number of viable bacteria present ($9.6 \times 10^8$, $5.8 \times 10^8$ and $6.5 \times 10^8$ c.f.u. ml$^1$, respectively). The reduced growth rate of SEQ_0936+ might be explained by the increased energetic cost of overproducing the SEQ_0936 surface protein, although the reasons for the increased growth rate of cne+ are unclear.

**Over-transcription of SEQ_0936 and cne enhance the attachment of Se4047 to equine ciliated columnar epithelium**

Gram-positive pili are known to have an important role in bacterial attachment [1, 8]. To determine whether the products of the Fim1 locus might fulfil this role, we used an air-interface organ culture system to quantify the attachment of Se4047 WT and the Fim1 mutants to equine tracheal columnar ciliated epithelium. The nasopharynx, nasal turbinates and palatine tonsils are considered to be the likely sites of attachment for S. equi infection. However, trachea was used in this study for reliability and logistical reasons. As with the nasopharynx, equine trachea is composed of columnar ciliated epithelium and has previously been utilized as an in vitro model to successfully predict the importance of lgt and prtM for virulence in a susceptible natural host [21], suggesting that it provides a suitable ex vivo model. Deletion of the Fim1 pilus genes did not affect adhesion, but over-transcription of the structural pilins, SEQ_0936 and CNE, increased adhesion compared to WT, $P=0.005$ and $P=0.004$, respectively (Fig. 6). This indicates that the Fim1 pilins are not required for the attachment of Se4047 to equine tracheal epithelium.
ciliated columnar epithelium in this model. However, over-transcription of the structural pilins significantly increased attachment to explants of equine trachea, providing evidence that the putative pilin proteins could play a role in the attachment of *S. equi* to host tissues under conditions where their expression is enhanced. The increase in attachment cannot be attributed to increased strain growth rate, as growth curve analysis of the mutant strains revealed that whilst *cne*+ grew significantly faster than *Se4047 WT* (*P*=0.0209), SEQ_0936+ grew significantly more slowly.

**Fig. 4.** Graph of Fim1 gene transcription measured by qPCR of cDNA from *Se4047 WT* and Fim1 mutant strains. qPCR values are normalized to transcription of the housekeeping gene *gyrA*. Strains from which RNA was isolated are indicated on the x-axis and qPCR targets are indicated to the right of the figure, *n*=3. Significant increases in transcription in over-transcription mutants compared to *Se4047 WT* are marked *P*≤0.05, **P*≤0.01, ***P*≤0.001 and ****P*≤0.0001, and the data being compared are indicated with a dashed line.

**Fig. 5.** Growth curves for *Se4047 WT* and Fim1 mutants. Mean OD values from growth of *Se4047 WT* and Fim1 mutants measured at 30 min intervals from the lag into the stationary phase. The strains are indicated to the right of the figure. The error bars show the standard error from the mean, *n*=4.
There is a lack of detectable pilus structures during in vitro culture, which may explain why deletion had no effect on attachment. However, the increased attachment seen for the over-transcription mutants suggests that the pilin monomers still fulfil an adhesive role. Whilst the increased production of SEQ_0936 and CNE observed for the over-transcription strains is artificially engineered, the levels of expression of the pilin proteins within natural infection in the natural host are unknown. It is therefore possible that conditions during infection could naturally result in high expression levels. In S. pyogenes, the involvement of pili in attachment has been shown to vary between tissues [28]; it is therefore also possible that the Fim1 pili are more important for adhesion to tissues that were not studied here.

**Fim1 is not required for biofilm formation by Se4047 in vitro**

Another known function of Gram-positive pili is in biofilm formation [1, 8]. To ascertain whether the Fim1 gene products were involved in this function, we quantified biofilm formation by Se4047 and the Fim1 mutants cultured in 96-well plates in THB and TSB supplemented with 0.5% glucose. Quantification of the cell content (CellTiter-Blue assay) and total biofilm formation (crystal violet staining) by Se4047 WT, the Fim1 deletion and + mutants on polystyrene revealed differences between the strains that varied depending on the culture medium. However, none of the deletion strains showed statistically significant reductions in biofilm formation or cell content in either growth medium. In THB, strain ΔSEQ_0936, which does not produce the
putative Fim1 pilus backbone subunits, had significantly greater fluorescence than Se4047 WT (P=0.029), but did not have significantly greater absorption than Se4047 WT (P=0.686) (Fig. 7). This corresponds to an increase in the mean cell content within the wells, without an increase in the amount of total biofilm produced. These results imply that there must have been a corresponding decrease in matrix content. In Streptococcus agalactiae (S. agalactiae) strain NEM316, deletion of the pilus backbone was proposed to result in surface mounting of accessory pilins rather than presentation on extended pili [7], and this was confirmed in the E. coli Pap pilus model system [29]. This may also be true in S. equi, explaining the increased cell density, as bacterial cells would be able to associate more closely without interference from surface appendages. The effect is not observed when the whole pilus locus is deleted, possibly because the accessory pilins are deleted in addition to the backbone and are therefore not influencing attachment. The deletion of srtC1 appears to have no effect on biofilm formation. Together with the absence of pilus polymers, this might suggest that SrtC1 is not functional on the Fim1 locus in Se4047. There is evidence in S. agalactiae strain NEM316 that alternative srtases, such as the housekeeping sortase, SrtA, are responsible for anchoring pilus proteins to the cell wall [30] and could therefore account for the presence of pilin monomers on the cell surface.

When grown in TSB supplemented with 0.5 % glucose, deletion of the Fim1 genes had no effect on biofilm development. However, over-transcription of the major pilin (strain SEQ_0936+) resulted in statistically significant increases in both fluorescence (P=0.029) and absorption (P=0.029) compared to Se4047 WT, corresponding to increased total biofilm formation and increased cell content (Fig. 7). It is interesting that this strain also exhibits increased attachment in the air-interface model, despite having a significantly reduced growth rate. Over-transcription of the major pilin has been shown to result in longer pili in S. agalactiae and a role in extending accessory pilins beyond the capsule has been proposed for it [7, 30, 31]. As pilus polymers have not been identified in S. equi, this is unlikely to explain the results seen in this study. However, an increased density of SEQ_0936 on the cell surface may enable greater interaction between bacterial cells, enhancing biofilm formation.

Therefore, we propose that although Fim1 is not essential for biofilm formation by S. equi, the differences seen in response to engineering the Fim1 locus suggest that it does
have a role in biofilm formation in vitro. In S. pyogenes, other surface proteins have been shown to be required for biofilm formation [8, 32] and the presence of different binding ligands on the culture surface have been shown to influence biofilm development [33]. The results of the biofilm assays in the two media are markedly different, which suggests that nutrition is also an important factor in the development of biofilms by S. equi, a phenomenon previously seen in S. agalactiae [30, 34]. Here we have demonstrated the ability of S. equi to form a biofilm experimentally, a mode of growth known to be important for survival in other bacterial species. In animals that become carriers but lack overt chondroids, the development of a biofilm on the internal surface of the guttural pouch may be one mechanism by which S. equi is able to persist with periodic shedding of free bacteria that can infect naïve animals.

**Se4047 tetR represses Fim1 transcription following repair and over-transcription**

TetR is proposed to regulate the Fim1 pilus locus. However, in Se4047 and all other S. equi strains surveyed [19], there is a nonsense mutation, which is likely to render it non-functional and have implications for pilin production and subsequent function [13, 35].

Mutants in which the nonsense mutation in tetR of Se4047 had been repaired (tetR repaired) and then subsequently inserted into a pseudogene with the eqbB promoter (tetR+) were successfully constructed. qPCR of cDNA from the Se4047 tetR mutants confirmed that repair of the tetR gene gave increased tetR transcription (1.54 times greater than Se4047 WT), although this was not significant (P=0.127). Significantly greater tetR transcription (23-fold greater than Se4047 WT) was seen in the tetR+ mutant compared to Se4047 WT (P=0.0495) (Fig. 8a).

Transcription of the Fim1 genes cne, SEQ_0936 and srtC1 in Se4047 WT, tetR repaired and tetR+ was quantified by qPCR. The data showed that repairing tetR in the Fim1 locus alone was insufficient to cause a statistically significant reduction in Fim1 gene transcription when cells were grown in vitro (Fig. 8b). TetR’s regulatory behaviour is modulated by the presence of binding ligands [17]. If the proportion of its binding ligands compared to the amount of TetR present was sufficiently high, then it would prevent TetR from inducing a significant repression of the locus. However, the much greater increase in transcription of the repaired tetR in the tetR+ mutant led to a statistically significant reduction in the transcription of cne (P=0.0495) and SEQ_0936 (P=0.0495). In this situation, the proportion of TetR present may have been high enough to saturate the binding ligand, allowing TetR to overcome the threshold and induce repression of the Fim1 locus. There was no statistically significant

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**Fig. 8.** Fim1 gene transcription measured by qPCR of cDNA from Se4047 WT, tetR repaired and tetR+ mutant strains. Transcription of tetR is shown in (a) and transcription of cne, SEQ_0936 and srtC1 are shown in (b). qPCR values are normalized to transcription of the housekeeping gene gyrA. Strains from which RNA was isolated are indicated on the x-axis and qPCR targets are indicated to the right of the figure, n=3. The P-values for differences in transcription to Se4047 WT are shown above, significant values marked * P≤0.05, ** P≤0.01, *** P≤0.001 and **** P≤0.0001, and the data being compared are indicated with a dashed line. Fold change values compared to Se4047 WT are indicated below the bars.
reduction in srtC1 transcription in either mutant. srtC1 is the gene furthest from the promoter region of Fim1, providing one explanation as to why it was affected the least by changes in the transcription of the repaired tetR. Pilus loci in S. pneumoniae and S. agalactiae have multiple levels of regulation [36, 37]. The S. equi tetR regulator may also be regulated by another regulator in addition to modulation by its binding ligands, keeping the locus under tight control.

In conclusion, we have demonstrated that the structural proteins of the Fim1 locus are expressed during in vitro and in vivo growth. These may, however, only be present on the cell surface as monomers rather than in a pilus structure under the in vitro culture conditions tested, possibly as a result of a deficiency of the associated sortase. The lack of an effect on attachment and biofilm development following deletion of the Fim1 genes suggests that Fim1 is not necessary for either function in vitro, both of which are associated with pili in other Gram-positive bacteria. However, engineering of the Fim1 genes affected the biofilm production and attachment of S. equi, suggesting that these genes could contribute to the ability of S. equi to attach to and persist on equine tissues under conditions where their expression is enhanced. The domains and residues known to be important to pilus formation in other species are intact, so it is possible that these monomers could form functional pili under the right conditions or during in vivo growth. We demonstrated that the Fim1 locus is regulated by tetR following repair and over-production, but the regulatory binding ligands modulating its regulatory behaviour remain to be identified.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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