Characterization of the haem-uptake system of the equine pathogen *Streptococcus equi* subsp. *equi*

Mary Meehan, Fiona M. Burke, Susan Macken and Peter Owen

Correspondence

Mary Meehan

meehanmary@yahoo.co.uk

Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College Dublin, Dublin 2, Ireland

Streptococcus equi possesses a haem-uptake system homologous to that of *Streptococcus pyogenes* and *Streptococcus zooepidemicus*. The system consists of two ligand-binding proteins (Shr and Shp) and proteins (HtsA–C) with homology to an ABC transporter. The haem-uptake system of *S. equi* differs from that of *S. pyogenes* and *S. zooepidemicus* in that Shr is truncated by two-thirds. This study focused on the SeShr, SeShp and SeHtsA proteins of *S. equi*. Analysis of *shr*, *shp* and *shphtsA* knockout mutants showed that all three proteins were expressed *in vitro* and that expression was upregulated under conditions of iron limitation. SeShr possesses no membrane-/cell wall-spanning sequences and was shown to be secreted. Both SeShp and SeHtsA were confirmed to be envelope-associated. Recombinant SeShp and SeHtsA proteins have been previously shown to bind haem and SeHtsA could capture haem from SeShp. This report extends these studies and shows that both SeShp and SeHtsA can sequester haem from haemoglobin but not from haemoglobin–haptoglobin complexes. Like full-length Shr, SeShr possesses haemoglobin and haemoglobin–haptoglobin binding ability but unlike full-length Shr, it lacks haem- or fibronectin-binding capabilities. Analysis of SeShr truncates showed that residues within and upstream of the near transporter (NEAT) domain are required for this ligand binding. Structural predictions suggest that truncation of NEAT1 in SeShr accounts for its impaired ability to bind haem. Haem and haemoglobin restored to almost normal the impaired growth rates of wild-type *S. equi* cultured under iron-limiting conditions. However, no difference in the growth rates of wild-type and mutants could be detected under the *in vitro* growth conditions tested.

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INTRODUCTION

Limitation of iron is an important innate host defence mechanism and strategies to overcome this limitation and compete with the host are crucial for the survival of pathogenic bacteria. The majority of iron in the host is in the form of haem (Fe–protoporphyrin complex) bound by several host proteins, the principal one being haemoglobin (Hb). Not surprisingly, pathogenic bacteria have evolved numerous acquisition systems to take advantage of this iron-rich reservoir. Common to both Gram-positive and Gram-negative pathogens are ABC transporters, which

transport haem through the plasma membrane. Gram-negative bacteria have outer membrane receptors that acquire haem from haemoproteins/haemophores and transfer it to the ABC transporters via the periplasm. Gram-positive bacteria possess receptors that transfer haem to the ABC transporters (reviewed by Genco & Dixon, 2001; Wandersman & Delepelaire, 2004; Wilks & Burkhard, 2007). In the *Staphylococcus aureus* Isd system, nine proteins (IsdA–I) constitute the capture and transport pathway (Mazmanian *et al.*, 2003). IsdB and IsdH appear to remove haem from Hb and transfer it directly (or via IsdA) to IsdC. IsdC then transfers bound haem to the ABC transporter (IsdD–F). Once in the cytoplasm, haem is degraded by monooxygenases, IsdG and IsdI (Maresso & Schneewind, 2006; Muryoi *et al.*, 2008; Reniere *et al.*, 2007; Zhu *et al.*, 2008b). The haem-acquisition machinery of *Streptococcus pyogenes* parallels that of *Staph. aureus* and consists of Shr which binds Hb, haemoglobin–haptoglobin (Hb–Hp) complexes and haem. Shr can transfer haem to a second protein, Shp which in turn transfers it to HtsA, the lipoprotein component of the ABC transporter (HtsA–C also termed SiaA–C; Bates *et al.*, 2003; Lei *et al.*, 2002, 2003; Zhu *et al.*, 2008a). The four Isd receptor proteins, Shr and Shp possess one or more copies of the structurally

Abbreviations: DIP, 2,2' dipyridyl; Fn, fibronectin; GST, glutathione S-transferase; Hb, haemoglobin; Hp, haptoglobin; HRP, horseradish peroxidase; LB, Luria–Bertani; LRR, leucine-rich repeat; NEAT, near transporter; THYE, Todd–Hewitt yeast extract.

Supplementary material, describing detailed construction of *shr*, *shp* and *htsA* insertion mutations, a supplementary figure, showing schematic representations of the secondary structures of the NEAT domains of IsdC–haem, IsdH–NEAT3 and IsdA and the predicted structures of the NEAT1 domains of SzShr and SeShr modelled on the structures of the three Isd proteins utilizing the Phyre server, and two supplementary tables, listing strains and plasmids used to construct *S. equi* mutants and PCR primers used to construct *S. equi* mutants and recombinant proteins, are available with the online version of this paper.

conserved but sequence divergent near iron transporter (NEAT) domain (Andrade *et al.*, 2002). Three-dimensional and other studies of several NEAT domains have shown that they possess a common β -sandwich immunoglobulin-like fold (Aranda *et al.*, 2007; Dryla *et al.*, 2007; Grigg *et al.*, 2007a; Pilpa *et al.*, 2006; Sharp *et al.*, 2007; Villareal *et al.*, 2008; Watanabe *et al.*, 2008) and can be categorized on the basis of their sequence similarities and ligand-binding profiles. Some iron-regulated NEAT-containing proteins such as IsdA and Shr are multifunctional and possess adhesive/survival functions in addition to haem acquisition (Clarke *et al.*, 2004, 2009; Fisher *et al.*, 2008).

Streptococcus equi subsp. *equi* (herein referred to as *S. equi*) is the causative agent of strangles, one of the most feared diseases of horses. Strangles is a highly contagious disease of the upper respiratory tract characterized by abscessation of lymph nodes in the head and neck (Timoney, 1993, 2004). *S. equi* is considered to have evolved from *S. equi* subsp. *zooepidemicus* (herein referred to as *S. zooepidemicus*; Chanter *et al.*, 1997), an opportunistic mucosal commensal which can cause serious diseases in a range of animals and, occasionally, humans (Timoney, 2004).

Several putative virulence factors and protective antigens of *S. equi* have been identified. These include numerous ligand-binding surface-associated and secreted proteins, several superantigens and the hyaluronic acid capsule (Harrington *et al.*, 2002; Holden *et al.*, 2009; Timoney, 2004). Recently, *S. equi* homologues of the *S. pyogenes* Shp (designated SeShp) and HtsA (designated SeHtsA) have been described. Recombinant SeShp and SeHtsA proteins were shown to bind haem and SeHtsA to capture haem from SeShp (Nygaard *et al.*, 2006b). Here, we demonstrate that *S. equi* expresses these two proteins and also a truncated Shr homologue (designated SeShr) under conditions of iron-limitation. We describe the cloning and overexpression in *Escherichia coli* of SeShr, SeShp and SeHtsA recombinant fusion proteins and use these proteins to extend studies of their ligand-binding capabilities.

METHODS

Growth conditions, bacterial strains and plasmids. *S. equi* was grown on Todd-Hewitt Yeast Extract (THYE) supplemented where appropriate with 100–600 μM 2,2'-dipyridyl (DIP), 10 mM NTA, 5 mM ferric citrate or streptonigrin (0.05 μM to 1.6 μM). THYE with NTA (THYE + NTA; Eichenbaum *et al.*, 1996) was supplemented with 500 μM MgCl_2 , MnCl_2 and CaCl_2 and 125 μM ZnCl_2 . *E. coli* strains BL21 (DE3) and TB1, used for protein expression, were grown in Luria-Bertani (LB) broth or M9 medium. Bacterial strains and plasmids used in construction of *S. equi* mutants are shown in Supplementary Table S1, available with the online version of this paper.

Expression and purification of recombinant SeShr, SeShp and SeHtsA proteins. Appropriate DNA fragments were amplified using specific primers (see Supplementary Table S2, available with the online version of this paper) and cloned into pET16b (Novagen), pGEX-KG (T. J. Foster, Trinity College Dublin) or pMAL-c2X (New England Biolabs) plasmids. His-tagged proteins, glutathione S-transferase (GST)-fusion proteins and MBP-fusion proteins were purified from

E. coli by Ni-affinity chromatography, GSTrap FF 5 ml columns and amylose agarose (GE Healthcare and New England Biolabs), respectively. Proteins were dialysed against 20 mM Tris/HCl, pH 8.0, concentrated by ultrafiltration and stored at -70°C . Where appropriate, His-tags were removed with Factor Xa.

Construction of *S. equi* mutants. Utilizing the temperature-sensitive broad-host-range plasmid pG + host9, insertion mutants of *shr*, *shp* and *htsA* were generated by replacing *S. equi* DNA with the ΩKm2 interposon (Maguin *et al.*, 1996; Meehan *et al.*, 2001; Perez-Casal *et al.*, 1991; Prentki & Krisch, 1984). For *S. equi shr::\Omega\text{Km2}*, a central 127 bp fragment (nucleotides 619–745) was replaced. For *S. equi shp::\Omega\text{Km2}*, the N-terminal 791 bp of *shp* and 232 bp upstream of *shp* was replaced and for *S. equi shphtsA::\Omega\text{Km2}*, an additional 894 bp of *htsA* was replaced (see Supplementary Tables S1 and S2 and supplementary material for full methods).

Biochemical procedures. UV-visible absorbance spectra of proteins in 20 mM Tris/HCl, pH 8.0, were recorded on a Shimadzu UV-160PC spectrophotometer. The haem content of proteins was estimated from reduced pyridine haemochrome spectra using an $\epsilon_{418.5}$ of $191.5\text{ mM}^{-1}\text{ cm}^{-1}$ (Smith, 1975). Haem transfer was examined by recording the spectral shifts following co-incubation of proteins at 24°C . Protein concentrations were estimated by a modification (Dulley & Grieve, 1975) of the Lowry method using BSA as a standard. Bacterial envelopes from *S. equi* strains, grown under iron-rich and iron-restricted conditions, were isolated as previously described and exhibited very similar protein profiles on SDS-PAGE gels to those previously observed (Meehan *et al.*, 1998). For *in vitro* expression studies, equal protein loadings of envelope preparations were analysed by Western immunoblotting.

In silico sequence and 3D homology modelling of NEAT1 domains of Shr, SeShr and SzShr. Amino acid sequence alignments and 3D modelling were performed following submission of NEAT1 domain sequences of Shr, SeShr and SzShr proteins to the Phyre webserver (Kelley & Sternberg, 2009).

Hb-agarose binding experiments. Binding of SeShr and SzShr to Hb-agarose was performed as follows. Triton X-100-solubilized cell envelopes (150 μg), purified SeShr (20 ng) or culture supernatants (obtained from cultures normalized to OD_{600} 0.8) in THYE were incubated for 1.5 h with 45 μl Hb-agarose (Sigma Aldrich) in 0.5 M NaCl and 0.05 % (v/v) Tween 20. The agarose was then washed in PBS (Dulbecco A; Oxoid) containing 0.05 % (v/v) Tween-20 (PBS-T), boiled in Laemmli sample buffer (Laemmli, 1970) and supernatants were analysed by Western immunoblotting. Haem capture from Hb agarose was performed by incubating 10 μM GST-SeShp, GST-SeHtsA (exhibiting 1.2–1.3 % haem saturation) or GST in PBS containing 0.5 M NaCl with Hb agarose at 37°C for 2 h and then recording the UV-visible spectra of the supernatants.

GST pull-down assays. Glutathione-agarose (10 μl ; GE Healthcare) was incubated at room temperature for 45 min with 17 μM GST or GST fusion proteins, washed three times in PBS and finally resuspended in PBS-T. His-tagged or untagged proteins were then added to a final concentration of 0.8 μM and incubation continued for 2 h. Agarose beads were washed three times with PBS-T, resuspended in Laemmli sample buffer and analysed by SDS-PAGE and Western immunoblotting.

ELISA. To examine Hb, Hp and Hb–Hp binding, 96-well plates were coated with recombinant SeShr, SeShp, SeHtsA and IsdH proteins ($10\text{ }\mu\text{g ml}^{-1}$). Following blocking with 5 % (w/v) BSA, plates were probed with doubling dilutions of either human Hb, Hp (Sigma) or Hb–Hp complexes (prepared by mixing equimolar amounts of Hb and Hp for 1 h at room temperature). Molarities of haemoglobin

were calculated from the molecular mass (34 000) of an $\alpha_1\beta_1$ dimer. Ligand binding was detected using anti-Hb (1:2000; Biogenesis) or anti-Hp (1:20 000; Sigma) monoclonal antibodies followed by 1:2000 horseradish peroxidase (HRP)-labelled anti-mouse IgG (Sigma). Staphylococcal IsdH/HarA which binds Hb, Hp and Hb-Hp complexes was a gift from Dr Naoko Yanagisawa (Trinity College Dublin) and was used as positive control. Recombinant fibrinogen-binding protein (FgBP1) of *S. equi* was used as a negative control (Meehan *et al.*, 1998).

To examine binding to immobilized Hb, plates were coated with Hb (10 $\mu\text{g ml}^{-1}$) and then probed with doubling dilutions of recombinant proteins. Binding was detected with anti-SeShr (1:80 000), anti-SeShp (1:4000) and anti-SeHtsA (1:16 000) followed by HRP-labelled goat anti-rabbit IgG (1:2000; MP Biomedicals). To examine binding to fibronectin (Fn), plates were coated overnight with GST-SeShr, GST or FnBPA₄₇₆₋₈₃₈ of *Staph. aureus* (20 $\mu\text{g ml}^{-1}$) followed by blocking with 5% (w/v) dried skimmed milk and probing with doubling dilutions of Fn (100–0 $\mu\text{g ml}^{-1}$). Binding was detected with rabbit anti-Fn antibodies (Sigma) followed by HRP-labelled goat anti-rabbit IgG. ELISA tests were developed using 3,3',5,5'-tetramethylbenzidine and A₄₅₀ was measured. Dissociation constants (K_d s) of binding curves were calculated using Graphpad Prism software.

Western immunoblotting and affinity blotting experiments.

Antibodies to His-tagged recombinant SeShr, SeShp and SeHtsA were raised and partially purified as described previously (Owen, 1985). For Western immunoblotting, membranes were blocked with 5% (w/v) dried skimmed milk, probed with anti-SeShr, anti-SeHtsA or anti-SeShp as appropriate followed by incubation with HRP-labelled goat anti-rabbit IgG (Dako). For dot blots, doubling dilutions of purified proteins were dotted onto nitrocellulose and then probed with 63 nM Hb or Hb-Hp followed by either 1:5000 anti-Hp or 1:3000 anti-Hb mouse monoclonal antibodies and 1:2000 HRP-labelled anti-mouse IgG. Probes were detected by chemiluminescence using ChemiGLO (Cell Signalling Technology) and visualized by exposure to X-ray film.

RESULTS

S. equi haem-uptake system

The *S. equi* and *S. zooepidemicus* genomes (Holden *et al.*, 2009) possess a highly homologous 10 gene cluster, the first five genes of which resemble the Hts haem-uptake system of *S. pyogenes* (Fig. 1a; Bates *et al.*, 2003; Lei *et al.*, 2002). *shr*, the first gene in the cluster, is truncated in *S. equi* compared with its *S. pyogenes* and *S. zooepidemicus* homologues due to a single base (A/T) pair deletion at position 1327 of the *shr* gene, which results in a frame-shift and a nonsense codon 54 bp downstream. Shr (M_r 142 704) in *S. pyogenes* and its homologue SzShr (M_r 138 516) in *S. zooepidemicus* are large proteins, both possessing a predicted N-terminal signal sequence followed by two NEAT domains encompassing a leucine-rich repeat (LRR) region and terminating in a stretch of 19 hydrophobic aa likely to be involved in membrane spanning followed by a short charged tail (Fig. 1b). In contrast, the *S. equi* homologue (termed SeShr) possesses only the N-terminal 460 aa (M_r 51 439). Although there is strong homology (96%) throughout SeShr and SzShr, the final 18 aa show considerable divergence in sequence due to the frame shift mutation. SeShr terminates in a cysteine residue and a truncated NEAT

domain (Fig. 1c). The NEAT domains of Shr, SzShr and SeShr exhibit strong homology. Thus, the NEAT1 domain of Shr exhibits 80% and 82% identity with SeShr (excluding the final divergent 18 aa) and SzShr, respectively. The NEAT2 domains of Shr and SzShr exhibit 73% identity.

Shr is followed by ORFs encoding the 293 residue haem-binding protein SeShp and three components of an ABC transporter, viz. a ligand-binding lipoprotein (SeHtsA), a permease (SeHtsB) and an ATPase (SeHtsC). The sequences of SeShp (residues 30–261) and SeHtsA (residues 21–294), determined in this study for strain TW, are identical to those determined for strain SEM1 (Nygaard *et al.*, 2006b) and strain 4047 (Holden *et al.*, 2009). The N-termini of Shp, SeShp and SzShp show most homology and are likely to possess the same immunoglobulin-like fold as that of Shp (Aranda *et al.*, 2007; Holden *et al.*, 2009; Nygaard *et al.*, 2006b). The HtsA proteins exhibit strong (40%) identity with the staphylococcal IsdE protein which has been shown to possess a bi-lobed structure characteristic of class III periplasmic binding proteins (Grigg *et al.*, 2007b). Other genes within the cluster encode proteins with homology to permease/ATP-binding export proteins and proteins involved in cobalt transport (Bates *et al.*, 2003).

In silico sequence and structural homology modelling of SzShr and SeShr NEAT domains

Shr binds a broad spectrum of ligands (Bates *et al.*, 2003; Fisher *et al.*, 2008; Zhu *et al.*, 2008a). However, the binding domains of these ligands have not been localized. Sequence comparisons and *in silico* structural homology modelling was performed using the PHYRE website (Fig. 1c and Supplementary Fig. S1, available with the online version of this paper; Kelley & Sternberg, 2009) to determine the likely effect of the truncated NEAT on structure and function. Results showed that SzShr, SeShr and Shr modelled best (100% precision) on the structures of the haem-binding proteins IsdA-NEAT, IsdC-NEAT and IsdH-NEAT3, producing a similar model regardless of the template protein; these were predicted to possess an immunoglobulin-like β -sandwich fold (Fig. 1c and Supplementary Fig. S1). The predicted structural models of the two full-length Shr-NEAT1 domains showed that they possess several identical or similar residues to those that constitute the haem-binding pocket of the Isd proteins. These include the key metal-co-ordinating Tyr residue in β 8 and a Ser residue in the 3_{10} helix (linking β 1b and β 2), conserved in all Isd proteins, and hydrophobic residues in β 4 and β 7 (Fig. 1c and Supplementary Fig. S1). A lower precision was obtained when Shp (95 and 40% precision for SzShr and SeShr, respectively) and IsdH-NEAT1 (80 and 15% precision for SzShr and SeShr, respectively) were used as templates. Shp binds haem by bis-methionyl co-ordination of iron, contrasting with the Tyr co-ordination of the Isd proteins (Aranda *et al.*, 2007). IsdH-NEAT1 binds Hb and Hb-Hp through an YYHFF motif in the β 1– β 2 loop, a motif not present in the haem-binding Isd proteins or Shr (Pilpa *et al.*, 2009).

Fig. 1. Schematic representations of the haem-uptake gene cluster of *S. equi* and the SeShr protein and alignment of Shr and Lsd NEAT domains. (a) The haem-uptake gene cluster of *S. equi*. The percentage identities between proteins encoded by the *S. equi*, *S. pyogenes* and *S. zooepidemicus* genes are shown. *shr** indicates the pseudogene generated as a result of a base pair deletion (arrow). (b) Comparisons of Shr of *S. pyogenes* and SeShr of *S. equi*. The positions of the NEAT domains, leucine-rich repeat (LRR) region, signal sequence (S) and membrane-spanning region (M) are indicated. Below the diagram are the positions of recombinant SeShr proteins used in this study. (c) Alignment of the NEAT1 domains of SeShr (*S. equi*), SzShr (*S. zooepidemicus*) and Shr (*S. pyogenes*) with the NEAT domains of LsdA, LsdC and LsdH-NEAT3 using structural predictions from Phyre. Tyr and Ser residues conserved among all proteins are highlighted in grey. In the Lsd proteins, residues in bold type have been shown to interact with haem. The predicted positions of the eight β sheets for SzShr are indicated by unbroken arrows. Broken arrows indicate the predicted positions of $\beta 6$ and $\beta 7$ of SeShr.

Significantly, due to truncation and a lack of conservation in the final 18 aa, the region of SeShr encompassing $\beta 6$ to $\beta 8$ differs from that of Shr/SzShr. SeShr lacks all of the residues which constitute $\beta 8$, including the co-ordinating Tyr. Furthermore, residues constituting $\beta 6$ and $\beta 7$ are predicted to be shifted in SeShr (see Fig. 1c). Key Val/Ile residues which form the binding pocket are in the $\beta 7$ sheet of the Lsd proteins and are conserved in Shr/SzShr (Fig. 1c). SeShr possesses a shorter $\beta 7$ sheet containing two bulkier hydrophobic residues Trp and Phe (Fig. 1c).

These data suggest that the NEAT1 domains of SzShr/Shr are responsible for the haem-binding function and, given the significant difference in the C terminus of SeShr, that

this protein has impaired haem-binding ability. Certainly, mutation of the co-ordinating Tyr resulted in almost complete abolition of haem binding in LsdA and significant spectral changes in LsdH-NEAT3 (Grigg *et al.*, 2007a; Watanabe *et al.*, 2008).

Expression, purification and haem-binding properties of recombinant SeShr, SeShp and SeHtsA proteins

Recombinant decaHis and GST fusion proteins, lacking predicted signal sequences and putative transmembrane region (SeShp only), were purified to homogeneity.

SDS-PAGE analysis revealed that all purified recombinant proteins migrated at their predicted M_r s, except His-tagged SeShr which migrated predominantly as a dimer under non-reducing conditions (Fig. 2a).

UV-visible spectral analysis revealed that SeShp and SeHtsA proteins possessed absorption spectra characteristic of

Fig. 2. SDS-PAGE of purified His-tagged recombinant proteins (a) and analysis of GST fusion proteins binding to SeShp during *in vitro* pull-down assays (b, c). (a) His₁₀-tagged recombinant proteins SeShr (SeShr+, lanes 1 and 5), SeShr NEAT⁻ (SeShr-, lanes 2 and 6), SeShp (lanes 3 and 7) and SeHtsA (lanes 4 and 8). Samples were boiled in Laemmli sample buffer with (+mer; lanes 1–4) or without (–mer; lanes 5–8) 2-mercaptoethanol. The positions of the His-tagged recombinant proteins are indicated to the left and the positions and molecular masses (in kDa) of marker proteins are indicated to the right. (b, c) GST-SeHtsA (lanes 1 and 4), GST-SeShr (lanes 2 and 5) or GST (lanes 3 and 6) were firstly immobilized on glutathione agarose. The agarose beads were then incubated with either control PBS (lanes 1–3) or with soluble His₁₀-SeShp (lanes 4–6), washed, boiled in Laemmli sample buffer and analysed by SDS-PAGE (b). Bound SeShp was detected by Western immunoblotting using anti-SeShp antibodies (c). Similar results were obtained when SeShp without a histidine tag was used (data not shown). Lane 7, purified His₁₀-SeShp. The positions of the GST fusion proteins are indicated to the left and that of His₁₀-SeShp is indicated to the right.

haemoproteins with Soret peaks of 427 nm and 406 nm, respectively (Fig. 3a; see also Nygaard *et al.*, 2006b). The level of haem saturation, determined from spectra of their pyridine haemochromes, was 7–11 % and 1.2–1.3 % when purified from *E. coli* cultured in enriched LB medium and minimal medium, respectively. The difference probably reflects a reduced level of accumulated haem when grown in minimal media. In marked contrast with analysis of recombinant full-length Shr (Zhu *et al.*, 2008a) and with the above proteins, *E. coli* cells expressing His-SeShr, GST-SeShr or GST were not red in colour and purified proteins did not exhibit spectra characteristic of haemoproteins, suggesting that SeShr binds negligible levels of haem (Fig. 3a).

Ligand-binding properties of SeShr, SeShp and SeHts

Both ELISA tests and affinity dot blots were performed to investigate the Hb-binding properties of the (truncated) SeShr (Fig. 4). ELISA tests showed that SeShr could bind both Hb and Hb–Hp complexes (but not human Hp) in a dose-dependent manner and that Hb–Hp (K_d 0.02 ± 0.004 μ M) was bound more efficiently than Hb (K_d 0.11 ± 0.04 μ M) alone (Fig. 4a). Similar ELISA tests showed that SeShp and SeHtsA bound soluble Hb (K_d s of 2.3 ± 0.3 μ M and 1.5 ± 0.5 μ M, respectively) to a significantly weaker extent than SeShr (Fig. 4b) and binding to Hb was inhibited by Hp (data not shown). Binding to Hb was not affected by mammalian source, since each bacterial protein bound immobilized human, equine, bovine and porcine Hb with equal efficiency (data not shown).

The above results provide evidence that the N-terminal region encompassing the NEAT1 domain of Shr proteins possesses Hb and Hb–Hp binding abilities. To investigate whether the (truncated) NEAT domain of SeShr was involved in Hb and/or Hb–Hp binding, a recombinant truncated SeShr protein (residues 19–358) lacking the predicted NEAT domain (termed Shr NEAT⁻) and a recombinant MBP–NEAT domain fusion protein were constructed. The construction of the latter was necessary since recombinant (untagged) NEAT domain (residues 359–460) was insoluble. Semiquantitative dot blot assays revealed that (i) deletion of the predicted NEAT domain reduced Hb and Hb–Hp binding by about 16-fold but did not totally eliminate binding and (ii) the MBP–NEAT fusion protein did not bind either ligand (Fig. 4c). ELISA tests confirmed that SeShr NEAT⁻ possesses reduced binding to Hb–Hp and Hb, and possessed K_d s of 0.2 ± 0.05 μ M and 0.18 ± 0.03 μ M for Hb and Hb–Hp, respectively. Together, these results suggest that residues both within and upstream of the NEAT domain of SeShr are required for full wild-type Hb binding.

Fisher *et al.* (2008) recently showed that Shr additionally binds to Fn and laminin. To investigate the Fn-binding ability of SeShr, ELISA tests were performed. These tests showed that recombinant GST-SeShr did not bind to Fn significantly better than the non-Fn-binding GST

Fig. 3. UV-visible absorption spectra of purified GST-fusion proteins and the spectral shifts observed following incubation of GST-SeShp and GST-SeHtsA with Hb agarose, Hb and Hb-Hp complexes. (a) GST fusion proteins (25 μ M; possessing 7–11 % haem) and GST. (b) Spectra of supernatants obtained following incubation of 10 μ M GST, GST-SeShp and GST-SeHtsA with Hb agarose (Hb). (c, d) Spectra of purified Hb and spectral shifts obtained following an 8 h incubation of 25 μ M GST-SeShp (c) and GST-SeHtsA (d) with soluble Hb or Hb-Hp. GST fusion proteins (possessing 1.2 % haem) purified from *E. coli* grown in minimal media were used for spectral shift experiments in (b)–(d).

protein. The control staphylococcal recombinant protein FnBPA_{476–838} exhibited significant Fn binding (Fig. 4d; Fitzgerald *et al.*, 2006).

SeShp and SeHtsA can capture haem from Hb but not from Hb-Hp complexes

Previous studies have shown that Shp of *S. pyogenes* can scavenge haem when incubated with Hb (Liu & Lei, 2005). The ability of HtsA to capture haem from Hb has not been reported to date. Two lines of experimentation were used to convincingly show that both SeShp and SeHtsA can capture haem from Hb. The first involved spectral analysis of supernatant fractions obtained following co-incubation of GST-fusion proteins and Hb agarose (Fig. 3b). It should be noted that only a very small absorbance peak at 406 nm was observed for control supernatants when Hb agarose was incubated alone or with GST and that neither (apo) SeShp nor (apo) SeHtsA exhibits significant absorbance in the 400–600 nm range. Significantly, following incubation with Hb agarose, SeShp and SeHtsA gained major Soret peaks at 421 and 414.5, respectively, plus minor peaks at 530–560 nm (Fig. 3b). Notably, however, unlike the ability of Shr to bind Hb-agarose (Fig. 6), no binding of SeShp or SeHtsA to Hb agarose could be detected (data not shown). Analysis of spectral shifts obtained following co-incubation of soluble Hb with the GST fusion proteins for several hours gave similar spectral shifts to those obtained with the Hb agarose (compare Fig. 3b with Fig. 3c and d). The 406 nm Soret peak of soluble Hb exhibited a significant shift to give major Soret peaks at 428 nm and 411 nm when incubated with SeShp or SeHtsA, respec-

tively (Fig. 3c and d). However, co-incubation of soluble Hb with either GST or GST-SeShr resulted in a Soret band that remained unaltered in position (406 nm) or intensity (data not shown).

To extend these studies, the effect of Hp on haem transfer from Hb to either SeShp or SeHtsA was tested in spectral-shift assays utilizing Hb-Hp as haem donor. The results of these studies showed that in contrast with the significant spectral shifts observed following incubation with Hb, only a relatively slight spectral shift occurred (Fig. 3c and d) when either SeShp or SeHtsA was incubated with Hb-Hp, suggesting that SeShp and SeHtsA can efficiently sequester haem from Hb but not from Hb-Hp complexes. The inability to bind to or scavenge haem from Hb-Hp, the slow rate of haem transfer from Hb to the SeShp and SeHtsA and the low efficiency of binding of either protein to Hb lends further support for the prediction (Zhu *et al.*, 2008a) that haem transfer from Hb to Shp is not by direct acquisition but occurs indirectly following diffusion of haem from Hb.

Recombinant Shr can acquire haem from *E. coli* haemoproteins and directly transfers bound haem to Shp (Zhu *et al.*, 2008a). However, haem transfer from Hb to full-length Shr has yet to be documented. Given the ability of full-length Shr to bind haem, Hb and Hb-Hp and transfer bound haem to Shp, the proposed role for Shr in haem acquisition would be to accelerate the rate of haem transfer from Hb/Hb-Hp to Shp (Zhu *et al.*, 2008a). Despite repeated efforts, no *in vitro* SeShr-dependent promotion of haem transfer could be detected following incubation of SeShr with SeShp or SeHtsA together with MetHb or

Fig. 4. ELISA and dot blot analysis of SeShp, SeHtsA, SeShr, GST and FnBPA_{476–838} proteins binding to Hb, Hp, Hp–Hb and Fn. Binding of Hb, Hp and Hb–Hp complexes to immobilized untagged SeShr (a) and binding of Hb and Hp to immobilized untagged SeShp and SeHtsA (b) was detected using anti-Hb and anti-Hp monoclonal antibodies. (c) Hb–Hp-affinity dot blot of recombinant SeShr, SeShr NEAT[–], MBP–NEAT domain fusion (MBP+NEAT) and MBP alone. The first well in each row of doubling dilutions contains 180 pmol recombinant protein. (d) Binding of immobilized GST–SeShr, GST and FnBPA_{476–838} to solubilized Fn. Binding was detected using anti-Fn antibody. The means $\pm$ SD of three experiments are shown.

Hb–Hp complexes (data not shown). Furthermore, pull-down assays confirmed, as expected, a direct interaction between SeShp and GST–SeHtsA. However, a similar interaction between SeShp and GST–SeShr or control GST protein was not evident (Fig. 2b and c).

SeShr, SeShp and SeHtsA are expressed *in vitro* and growth of iron-restricted *S. equi* is enhanced by haemoglobin

To facilitate *in vitro* expression studies, insertion knockout mutants of *shr*, *shp* and *shphtsA* were generated. Western immunoblotting of cell envelopes showed that expression of SeShp and SeHtsA was significantly increased in *S. equi* grown under iron-limiting conditions (THYE supplemented with either DIP or NTA) when compared with *S. equi* grown under iron-rich conditions (Fig. 5a, lanes 1, 4 and 7 and Fig. 5b, lanes 1 and 4). Importantly, neither SeShp nor SeHtsA was expressed in *shp*:: ΩKm2 or *shphtsA*:: ΩKm2 mutants (Fig. 5a, lanes 2–3, 5–6 and 8–9 and Fig. 5b, lanes 2–3 and 5–6). These results thus validate the knockout mutations, show that SeShp and SeHtsA expression is iron-regulated in wild-type cells and also provide evidence that *shp* and *htsA* genes are co-transcribed, since inactivation of *shp* abolished expression not only of SeShp but also of SeHtsA (Fig. 5a, lanes 3, 6 and 9 and Fig. 5b, lanes 3 and 6). The *S. equi shr* mutant showed the same low level

expression of SeHtsA when cultured in THYE as wild-type *S. equi* (data not shown) and SeHtsA expression was enhanced in the *shr* mutant grown under iron-limiting conditions (Fig. 5a, lane 10).

Shr/SzShr possesses a predicted membrane-spanning domain, and localization studies have recently shown that Shr is cell-membrane-associated and is also found in culture supernatants (Fisher *et al.*, 2008; Bates *et al.*, 2003). Sequence analysis of pre-SeShr indicates that the protein possesses an N-terminal signal sequence but, unlike pre-Shr/SzShr, that it lacks a C-terminal membrane-spanning sequence and is therefore predicted to be secreted. To confirm this prediction, expression of SeShr in envelope and supernatant fractions was assessed by a sensitive Hb-agarose pull-down assay (Fig. 6b, lane 6). Results showed that (i) SeShr is present in culture supernatants (Fig. 6a, lanes 5, 8, 11) but cannot be detected in cell envelopes (Fig. 6b, lanes 3–4) of wild-type *S. equi* even at loadings 10 times higher than the equivalent loading of culture supernatants; (ii) SeShr is expressed at low levels following culture in iron-rich conditions but that expression increases following iron restriction (Fig. 6a, compare lane 2 with lanes 5, 8 and 11); and (iii) SeShr is not expressed in *shr*:: ΩKm2 (Fig. 6a, lanes 3, 7, 10 and 13) but is expressed in the *shphtsA* double mutant (Fig. 6a, lanes 6, 9 and 12). Sequence analysis predicts that SzShr of *S. zooepidemicus* is

Fig. 5. *In vitro* expression of SeHtsA and SeShp. (a) Cell envelopes (30 µg) prepared from *S. equi* wild-type (WT, lanes 1, 4 and 7), *S. equi shp::ΩKm* (lanes 2, 5 and 8), *S. equi shphtsA::ΩKm* (lanes 3, 6 and 9) and *S. equi shr::ΩKm* (lane 10) grown in THYE (lanes 1–3), THYE+400 µM DIP (lanes 4–6) and THYE+NTA (lanes 7–10). Lane 11, purified His-tagged SeHtsA. (b) Cell envelopes (15 µg) were prepared from *S. equi* wild-type (lanes 1 and 4), *S. equi shp::ΩKm* (lanes 2 and 5) and *S. equi shphtsA::ΩKm* (lanes 3 and 6) grown in THYE (lanes 1–3) and THYE+NTA (lanes 4–6). Lane 7, purified His₁₀-tagged SeShp. Samples were analysed by SDS-PAGE and Western immunoblotting using anti-SeHtsA (a) or anti-SeShp (b) antiserum. The positions of SeHtsA, His₁₀-tagged SeHtsA, SeShp and His₁₀-tagged SeShp are indicated.

not truncated (Fig. 1c) and that it possesses a membrane-spanning domain. In accordance with these predictions, a high-molecular-mass cross-reactive protein (apparent M_r 140 000) could be detected following Western immunoblotting analysis of detergent-extracted *S. zooepidemicus* cell envelopes but not in analogous *S. equi* cell envelope extracts (Fig. 6b, compare lanes 1–2 with 3–4).

Growth of wild-type *S. equi* was significantly impaired when cultured under iron-limiting conditions (THYE + DIP) and showed a dose-dependent inhibition of growth in response to increasing concentrations of DIP (Fig. 7). Growth was enhanced in iron-rich (THYE) broth or in THYE+DIP medium supplemented with Hb, BSA::haem or whole blood (Fig. 7). This provides evidence for the first time, to our knowledge, that *S. equi* can utilize haem and Hb as iron sources.

S. equi shphtsA::ΩKm2 showed a slight but consistent decrease in generation time (131 ± 21 min) when cultured in THYE+DIP compared with wild-type *S. equi* (125 ± 23 min). However, this difference is not statistically significant ($P=0.136$). Neither was there any significant difference between generation times of mutant and wild-type strains when grown in medium supplemented with Hb, BSA::haem, whole blood or the iron-dependent antibiotic streptonigrin (data not shown; Yeowell & White, 1982).

Fig. 6. *In vitro* expression of SeShr. (a) Purified His₁₀-tagged SeShr (lane 1). Culture supernatants of *S. equi* wild-type (WT, lanes 2, 5, 8 and 11), *S. equi shphtsA::ΩKm* (lanes 3, 6, 9 and 12) and *S. equi shr::ΩKm* (lanes 4, 7, 10 and 13) grown overnight in THYE+ferric citrate (lanes 2–4), THYE (lanes 5–7), THYE+400 µM DIP (lanes 8–10) and THYE+NTA (lanes 11–13) were incubated with Hb-agarose. The position of His₁₀-SeShr and the positions of SeShr and an SeShr degradation product [SeShr(d)] occasionally seen in *S. equi* grown in THYE, DIP and NTA are indicated. Recombinant SeShr proteins also exhibited some evidence of proteolytic degradation (see Figs 2 and 6b). (b) Triton X-100-extracted cell envelopes prepared from *S. zooepidemicus* (lanes 1 and 2) and *S. equi* (lanes 3 and 4) grown in either THYE (lanes 1 and 3) or THYE+NTA (lanes 2 and 4). Cell envelopes (lanes 1–4) and His-tagged SeShr (lane 6) were incubated with Hb agarose. Lane 5 contains purified His₁₀-tagged SeShr only. Following incubation, Hb agarose beads were boiled in Laemmli sample buffer and analysed by SDS-PAGE and Western immunoblotting using anti-SeShr antibodies. The positions of His₁₀-SeShr and SzShr from *S. zooepidemicus* are indicated.

DISCUSSION

S. equi, *S. pyogenes* and *S. zooepidemicus* each possess similar haem-uptake systems (Hts system) containing three ligand-binding proteins. Analyses of the *S. equi* homologues (SeShr, SeShp and SeHtsA) in this report have extended our overall understanding of this family of proteins. We show for the first time, to our knowledge, by using *in vitro* expression of SeShr, SeShp and SeHtsA, their iron-regulated expression, providing support for a role in haem/iron transport. The *S. equi* uptake system differs from the *S. zooepidemicus* and *S. pyogenes* systems by possessing a truncated Shr protein which is not envelope-associated, but which can be detected in culture supernatants. This correlates well with sequence data which indicate the presence of an N-terminal signal sequence directing export and the absence of any obvious

Fig. 7. Growth of wild-type *S. equi* under iron-rich and iron-limiting conditions. Wild-type *S. equi* was grown in THYE and subcultured into THYE, THYE supplemented with 500 µM DIP (THYE+DIP), THYE+DIP supplemented with 30 µM Hb (THYE+DIP+Hb) and THYE+DIP supplemented with 10 µM BSA:haem (THYE+DIP+BSA-Hm). THYE+DIP supplemented with 0.5% sheep blood showed a similar result to THYE+DIP+Hb (data not shown). Growth curves [time (h) versus OD₆₀₀] of *S. equi* grown in THYE and THYE supplemented with 100, 200, 400 and 600 µM DIP are inset. Error bars, SD.

membrane-/cell-wall-anchoring domain/motif. On this basis, it seems reasonable to conclude that SeShr is secreted.

S. equi possesses a number of factors which would result in erythrocyte lysis and release of Hb and haem (Harrington *et al.*, 2002; Holden *et al.*, 2009; Timoney, 2004). We showed that *S. equi* can utilize haem and Hb as iron sources. The lack of significant difference between the growth of wild-type *S. equi* and mutants of the Hts system may be due to compensation by another iron-uptake systems(s). Indeed, the *S. equi* genome possesses homologues of the *S. pyogenes* MtsABC and SuiADEF systems and also expresses a potential siderophore termed equibactin (Hanks *et al.*, 2005; Heather *et al.*, 2008; Holden *et al.*, 2009; Janulczyk *et al.*, 2003).

The Hts haem-uptake system shows remarkable similarity to the Isd system of *Staph. aureus* with Shr, Shp and HtsA probably serving roles in haem uptake similar to those of the IsdB/IsdH, IsdA/IsdC and IsdE proteins, respectively (Muryoi *et al.*, 2008; Zhu *et al.*, 2008b). The first important steps in haem acquisition are its direct capture from Hb or Hb–Hp and subsequent transfer to other proteins in the uptake pathway. Full-length Shr is proposed to share that role with the IsdH and IsdB proteins. Although there is no direct evidence as yet that Shr acquires haem from Hb, most available data support this prediction, since Shr binds Hb and Hb–Hp complexes, is predicted to possess a haem-binding pocket similar to that of the Isd proteins and was recently shown to transfer haem to Shp (Bates *et al.*, 2003; Zhu *et al.*, 2008a). However, there are clear differences between the two groups of proteins. The mode of Hb

binding is distinct between the Isd and Shr proteins since Shr proteins do not possess the YYHFF motif required for Hb binding of IsdH and IsdB (Pilpa *et al.*, 2009). This report shows that residues within and upstream of the NEAT1 domain are required for Hb binding of SeShr and contrasts with the situation for Isd proteins where purified NEAT domains possess ligand-binding functions. Furthermore, haem acquisition by IsdH and IsdB exploits the fact that Hb and haem bind to distinct NEAT domains on the same protein (Pilpa *et al.*, 2009). However, for the Shr proteins, our ligand-binding experiments and structural modelling suggests that there is some overlap in Hb- and haem-binding regions.

In the *S. equi* system, a role for SeShr in haem uptake remains unclear. The protein exhibits significant binding to both Hb and Hb–Hp complexes with greater binding to the latter ligand. However, lack of evidence that SeShr binds haem and facilitates haem transfer from Hb or Hb–Hp to SeShp indicates that SeShr is unlikely to have the same role as Shr/SzShr in haem uptake. SeShp and SeHtsA cannot acquire haem from Hb–Hp complexes. However, the ability to bind and acquire haem from Hb–Hp complexes is physiologically relevant, since MetHb released from lysed erythrocytes is quickly and irreversibly complexed with Hp. Hb–Hp complexes and free Hb are finally cleared from circulation following internalization by CD163 on the surface of macrophages/monocytes (Fabriek *et al.*, 2005). Some pathogens, notably *Neisseria meningitidis* and *Haemophilus influenzae*, can scavenge haem as efficiently from Hb–Hp as from Hb alone (Wilks & Burkhard, 2007). A role in haem acquisition cannot be discounted for SeShr, and one potential role for the protein may be to compete with CD163 for Hb–Hp binding, thereby thwarting the clearance of Hb–Hp and permitting increased circulating Hb–Hp from which haem may eventually diffuse.

Like IsdC, Shp is proposed to be the central conduit in the haem uptake pathway by facilitating haem transfer from Shr to HtsA (Liu & Lei, 2005; Muryoi *et al.*, 2008; Nygaard *et al.*, 2006a, b). Initial direct haem acquisition by Shr would be the most efficient means of acquiring haem from Hb. However, some haem acquisition proteins, notably IsdA and IsdC, can indirectly acquire haem following its diffusion from Hb (Zhu *et al.*, 2008b). We have shown that this is the case for SeShp and SeHtsA. Under conditions of pathogen-induced haemolysis, when concentrations of Hb and haem would be higher than normal, it could be envisaged that SeShp and SeHtsA proteins may acquire enough haem needed to support growth in the absence of a fully functional Shr protein. A model for haem acquisition by *S. equi* and *S. pyogenes*/*S. zooepidemicus* is presented in Fig. 8.

Like IsdA, full-length Shr is a broad spectrum iron-regulated adhesin and binds Fn and laminin and promotes bacterial cell adhesion (Fisher *et al.*, 2008). We have shown that SeShr does not bind Fn. This strongly suggests that the Fn-binding region is located in the C-terminal two-thirds

Fig. 8. Model for haem uptake in *S. equi*. Following erythrocyte destruction, possibly by *S. equi* haemolysin, higher levels of Hb and haem than normal are released. SeShp binds to free haem and/or captures haem from MetHb. Holo-SeShp interacts with and transfers haem to SeHtsA. SeHtsA can also bind haem or capture haem from Hb. Haem is then transported into the cytoplasm using SeHtsBC of the HtsABC transporter. Once released from erythrocytes, free Hb is rapidly complexed with Hp. SeShp or SeHtsA cannot efficiently sequester haem from Hb-Hp. SeShr, which is secreted into the extracellular medium, can bind both Hb and Hb-Hp complexes. However, a role for SeShr in haem transport is unclear at present. In contrast with SeShr, cell-anchored homologues in *S. pyogenes* and *S. zooepidemicus* (Shr and SzShr, respectively) can bind haem, Hb and Hb-Hp. Holo-Shr/SzShr transfers bound haem to Shp/SzShp, the proposed central conduit in the transport pathway.

of Shr/SzShr and probably in the LRR or NEAT2 domains. *S. equi* binds less efficiently to Fn than *S. zooepidemicus*; a factor linked to the increased virulence of *S. equi* and almost certainly as a result of secretion of SeShr and another truncated Fn-binding protein, FNE (Holden *et al.*, 2009; Lindmark *et al.*, 2001). In contrast with SeShr, FNE retains the ability to bind Fn and has been suggested to compete with the Fn-binding mechanisms of competing pathogens (Holden *et al.*, 2009).

The Hts system in *S. equi* highlights common themes observed throughout the genomes of *S. equi* and *S. zooepidemicus* in showing evidence of considerable genetic exchange between these two organisms and *S. pyogenes*, and the presence of a large number of partially deleted/pseudo genes on the *S. equi* genome. These factors, together with the acquisition of genetic mobile elements, are thought to have facilitated the evolution of *S. equi* from *S. zooepidemicus* (Holden *et al.*, 2009). A key event in the evolution of *S. equi* has been proposed to be the acquisition of an integrative conjugative element encoding the potential siderophore termed equibactin (Heather *et al.*, 2008; Holden *et al.*, 2009). Analysis of *Staph. aureus* mutants showed that haem acquisition satisfies iron requirements early in the infectious process and that siderophores play a vital role in iron acquisition, later in infection in niches that are low/devoid of haemoproteins (Dale *et al.*, 2004; Skaar *et al.*, 2004). Whether a similar scenario can be envisaged for *S. equi* remains to be determined. Certainly, the defined mutants in SeShr, SeShp and SeHtsA developed in this study will be invaluable in future studies to unravel the importance of haem/iron acquisition in the pathogenesis and abscess formation of

S. equi and to provide insight into its evolution from the less virulent *S. zooepidemicus*.

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