The streptococcal iron uptake (Siu) transporter is required for iron uptake and virulence in a zebrafish infection model

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A limited understanding of iron uptake mechanisms is available for *Streptococcus pyogenes*, a haemolytic human pathogen capable of using a variety of haemoproteins in addition to ferric and ferrous iron. This study characterizes a transporter named *siu* (for streptococcal iron uptake), which consists of an ATP-binding protein (SiuA), a substrate-binding protein (SiuD), and two membrane permease subunits (SiuBG). An *siuG* mutant was constructed and characterized. The mutant demonstrated growth reduction in comparison to the parent strain when grown in complex medium containing iron in the form of blood, haemoglobin or serum. Only a small reduction in the growth of the *siuG* mutant was observed in medium containing ferric iron. However, in iron uptake assays the *siuG* mutant showed a decrease of approximately 30% in Fe³⁺ incorporation. Addition of 6 µM haem to the medium inhibited Fe³⁺ uptake by the wild-type by 76%, while addition of protoporphyrin IX did not, suggesting that utilization of haem as an iron source is responsible for the inhibition of Fe³⁺ uptake. Inactivation of *siuG* moderately reduced the ability of haem to inhibit Fe³⁺ incorporation by the cells. Inactivation of *siaB* (encoding a membrane permease of a second iron transporter) had a similar outcome, and inactivation of both transporters had a cumulative effect. These observations implicate both the *siu* and *sia* transporters in haem utilization by *Strep. pyogenes*. Studies in a zebrafish infection model revealed that the *siuG* mutant was attenuated in both intramuscular and intraperitoneal routes of infection. Together these observations show that the *siu* system is an iron acquisition pathway in *Strep. pyogenes* that is important both *in vitro* and *in vivo*.

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INTRODUCTION

Pathogenic bacteria use a variety of high-affinity iron-scavenging systems to compete for iron while colonizing the human body, as the vast majority of the iron in mammals is tightly bound to host proteins. Most of the intracellular iron is stored in ferritin or found in the form of haem complexes such as haemoglobin and myoglobin. In the body fluids haemoglobin is bound to haptoglobin and haem is carried by haemopexin or serum albumin (Genco & Dixon, 2001; Wandersman & Stojiljkovic, 2000), while ferric iron is sequestered by transferrin and lactoferrin (Wooldridge & Williams, 1993). The redundancy in iron uptake pathways frequently displayed by individual pathogens underscores their importance to the infection process. Accordingly, mutants in one or more of these pathways are often attenuated for virulence (Brown *et al.*, 2001a; Henderson & Payne,

1994; Ratledge, 2004; Stojiljkovic *et al.*, 1995; Torres *et al.*, 2001). Despite its apparent role in bacterial virulence, iron acquisition is only partially understood in Gram-positive pathogens.

The acquisition of iron from host proteins has been studied in a handful of Gram-positive microbes. Some species employ surface receptors for host proteins like transferrin or lactoferrin (Hartford *et al.*, 1993; Modun *et al.*, 1998) or siderophores to obtain ferric iron (Coulanges *et al.*, 1998; Courcol *et al.*, 1997; De Voss *et al.*, 1999; Russell *et al.*, 1984; Sebalsky & Heinrichs, 2001). The use of haem and host haemoproteins has been demonstrated in *Corynebacterium diphtheriae* (Schmitt, 1997, 1999), *Staphylococcus aureus* (Mazmanian *et al.*, 2003), and several streptococci (Bates *et al.*, 2003; Brown *et al.*, 2001a; Eichenbaum *et al.*, 1996; Francis *et al.*, 1985; Podbielski *et al.*, 1999). Haem uptake in Gram-positive organisms seems to be mediated by dedicated surface receptors for haem or haemoproteins, while the production of haemophores (secreted haem-binding

Abbreviations: ddH₂O, double-distilled water; i.m., intramuscular(ly); i.p., intraperitoneal(ly).

proteins found in several Gram-negative bacteria) has not been reported (Wandersman & Delepelaire, 2004).

The principal machinery involved in the uptake of free or complex iron in Gram-positive bacteria is ABC transporters, which consist of a substrate-binding lipoprotein, one or two membrane permease subunits, and a hydrophilic ATPase (Brown & Holden, 2002; Gilson *et al.*, 1988; Higgins, 1992; Wandersman & Stojiljkovic, 2000). Haem and siderophore transporters share significant homology and belong to a defined cluster of ABC transporters. In addition, Gram-positive pathogens carry ABC metal transporters, which are part of a separate cluster of transporters and have affinity for multiple metals (Brown & Holden, 2002; Claverys, 2001). In some of these multi-metal transporters, the metal binding receptors function as bacterial adhesins as well (Dintilhac *et al.*, 1997; Elsner *et al.*, 2002; Oligino & Fives-Taylor, 1993; Spellerberg *et al.*, 1999).

Streptococcus pyogenes is a haemolytic pathogen capable of producing a diverse array of skin and mucous membrane infections as well as aggressive deep tissue diseases and streptococcal toxic shock syndrome. Untreated streptococcal infections can lead to the serious complications of rheumatic fever and acute glomerulonephritis (Bisno *et al.*, 2003; Cunningham, 2000). Under laboratory conditions *Strep. pyogenes* can use haem and a variety of haemoproteins such as haemoglobin-haptoglobin, haemoglobin, myoglobin, haem-albumin and catalase as a source of iron, but it cannot use transferrin or lactoferrin (Eichenbaum *et al.*, 1996; Francis *et al.*, 1985; Podbielski *et al.*, 1999). *Strep. pyogenes* possesses a multi-metal transporter encoded by *mts* (Janulczyk *et al.*, 1999) and two transporters from the iron-complex family: *sia* (streptococcal iron acquisition) (Bates *et al.*, 2003) or *hts* (Lei *et al.*, 2003) and a transporter which we name here *siu* (streptococcal iron uptake). The *siaABC* genes were suggested to function as a haem transporter, and SiaA (or HtsA), the binding protein homologue, was shown to bind haemoglobin and haem (Bates *et al.*, 2003; Lei *et al.*, 2003). On the other hand, the *mts* transporter is involved in uptake of manganese and ferric iron (Janulczyk *et al.*, 2003), where MtsA binds iron, zinc and manganese *in vitro* (Janulczyk *et al.*, 1999). The ligand and the function of the *siu* transporter have not yet been defined. In this study, we investigated the role of the *siu* transporter in iron acquisition and disease production.

METHODS

Bacterial strains and growth assay conditions. *Escherichia coli* DH5 α was used for gene cloning. *E. coli* cells were grown aerobically in Luria-Bertani broth. *Strep. pyogenes* NZ131 (M49 type; Nordstrand *et al.*, 1998) and the mutant strains were grown statically at 37 °C in Todd-Hewitt (Difco or Oxoid) broth with 0.2% (w/v) yeast extract (THY), in Todd-Hewitt broth buffered with 10 mM Tris and adjusted to pH 6.9 prior to autoclaving (ZTH), or in ZTH medium containing 12 mM nitrilotriacetic acid (NTA). NTA is a metal chelator with high specificity for iron; its first stability constants (log K_1) for Fe³⁺ and Fe²⁺ are 15.87 and 8.83 respectively. Since NTA has affinity for zinc, manganese, calcium and magnesium

(log K_1 of 10.45, 7.44, 6.41 and 5.4 respectively), 0.66 mM of these cations was added to NTA-containing media (Eichenbaum *et al.*, 1996). ZTH was inoculated with a THY overnight culture at a 1/300 dilution. Iron was added to ZTH-NTA in the form of 3 mM ferric chloride (FeCl₃), 0.13% (v/v) sheep's blood (Colorado Serum), 0.13% (v/v) horse serum (Sigma) or 12 μ M human haemoglobin (Sigma). Human haemoglobin was prepared as a 10 mg ml⁻¹ stock solution in phosphate-buffered saline (PBS; pH 7.4).

Todd-Hewitt broth (no yeast extract, TH) was also treated for 3 or 20 h with 5% (w/v) Chelex-100 (Bio-Rad). The pH of the resin-treated medium was adjusted to 7.65, autoclaved, and 0.5 mM CaCl₂ and 0.9 mM MgSO₄ were added before inoculation. Inductivity-coupled plasma mass spectrometry (ICP-MS) analysis (at the Laboratory for Environmental Analysis, University of Georgia at Athens) demonstrated that TH contains about 17.5 $\pm$ 6.5 μ M iron, 0.53 $\pm$ 0.2 μ M manganese and 15.5 $\pm$ 0.2 μ M zinc, depending on the batch and manufacturer. Chelex-100 treatment for 3 h resulted in 2.7 μ M iron, less than 0.18 μ M manganese and about 0.3 μ M zinc in the medium. Treatment with Chelex-100 for 20 h did not significantly change its iron content in comparison to 3 h of treatment.

Strep. pyogenes was also grown in a chemically defined medium (CDM) (Podbielski *et al.*, 1999; van de Rijn & Kessler, 1980). CDM was also treated with 3% (w/v) Chelex-100 for 6 h and filter-sterilized (CxCDM). ICP-MS analysis showed that CxCDM contains 1.6 μ M iron and 0.43 μ M zinc; the manganese concentration is below the detection level. For cell growth, CxCDM was supplemented with 33 μ M MgCl₂ and 68 μ M CaCl₂. All experiments done with *Strep. pyogenes* cells growing in CDM or CxCDM were inoculated using mid-exponential-phase cells, which were prepared as follows: cells cultured in ZTH medium were harvested at the exponential phase (OD₆₀₀ 0.6), washed twice with PBS, and stored in small frozen aliquots in 16% (v/v) glycerol. All glassware used for streptococcal growth was soaked for 30 min in a chromic/sulfuric acid solution (Fisher Scientific) and rinsed with double-distilled water (ddH₂O). When necessary, the antibiotics spectinomycin and erythromycin were used for *E. coli* at 100 μ g ml⁻¹ and 500 μ g ml⁻¹, respectively. For *Strep. pyogenes*, spectinomycin and erythromycin were used at 100 μ g ml⁻¹ and 1 μ g ml⁻¹, respectively. Optical density was measured with a Beckman DU640 spectrophotometer (600 nm) or with a Scienceware 800-3 Klett colorimeter (640–700 transmission filter).

Construction of strains ZE4913, ZE4914 and ZE4915. The primers used in this study are listed in Table 1. Mutants with insertional inactivation of the *siuG* and *siaB* genes were constructed in *Strep. pyogenes* NZ131 (M49 type) using primers designed according to the *Strep. pyogenes* SF370 genome database (Ferretti *et al.*, 2001) (NCBI and TIGR complete genome databases). All of the constructed chromosomal mutations were verified by PCR analysis. The *siuG* mutant (ZE4915) was constructed by amplifying a 2.9 kb fragment from NZ131 chromosomal DNA using primers fluX-S and fluX-A. The PCR fragment, which included the 3'-end of *siuB*, the entire *siuG* gene, and a region downstream of *siuG*, was digested with *Aat*II and *Sal*I and ligated into pBR322, generating the plasmid pSaAa. The *ermAM* gene (erythromycin resistance) from pFW15 (Podbielski *et al.*, 1996) was amplified using primers erm-S and erm-A and cloned into the *Eco*RI site of the *siuG* gene in pSaAa, generating pSaAaerm. A fragment containing the *siuG::ermAM* allele and flanking region was released by *Aat*II/*Sal*I digestion and electroporated into *Strep. pyogenes* NZ131. Allelic replacement clones were selected on THY agar plates containing erythromycin. A *siuG siaB* double mutant (ZE4914) was constructed by introducing a disrupted *siaB* copy in the background of ZE4915. For this purpose, a 2 kb fragment including the 3'-end of *siaA*, the entire *siaB* gene and the 5'-end of *siaC* was amplified from NZ131 chromosomal DNA using primers *stoj5* and *stoj6*. The *siaB* PCR product was

Table 1. Oligonucleotide primer sets

Locus	Primer	Primer sequence
Mutant construction		
<i>ermAM</i>	erm-S	5'-GGGAATTCATGAACAAAAATATAAAATA-3'
	erm-A	5'-GGGAATTCATAAAGGAGGGCAATTTAT3'
<i>aad9</i>	spc-S	5'-GGTGATCAATCGATTTTCGTTTCGTGAAT-3'
	spc-A	5'-GGTGATCAGTATACGTTCCCAAATAAA-3'
<i>siaB</i>	stoj5	5'-GGTTGCTGTGGTTGATATCTGTGAC-3'
	stoj6	5'-GTCATGTAATACTAACACAATCGTTA-3'
<i>siuG</i>	fhuX-A	5'-GCTTAAGCCTGACGTCATTGTTGTTGG-3'
	fhuX-S	5'-GACCAACATCACGAGAGTCGACTTGACCTG-3'
PCR analysis		
<i>sia</i>	204A-F	5'-GCGTTCAGGAGGTCTAGCTC-3'
	204C-R	5'-CTGATGCTACTGCCATAGCAG-3'
<i>siu</i>	fhu3-S	5'-GCCATGGCTGCAGAAGG-3'
	fhu-A	5'-CCCTGCAGCCCATCTTAGCAAAGAC-3'
RT-PCR analysis		
<i>recA</i>	sraL	5'-GCGTTCAGGAGGTCTAGCTC-3'
	sraR	5'-CTGATGCTACTGCCATAGCAG-3'
<i>siuG</i>	fhuG-S	5'-CCCTGCAGCAGTTTCTGGCGCTAG-3'
	fhuG-A	5'-CCCTGCAGTACAACATACCAGCTGC-3'

cloned into the *XmnI* site of pACYC184, producing plasmid pStoj3. The *aad9* gene (spectinomycin resistance) was amplified from pUCSpec (Husmann *et al.*, 1997) using spc-S and spc-A primers and cloned into the *BclI* site of *siaB*, generating plasmid p5spc1. The fragment containing the *siaB::aad9* allele and flanking chromosomal regions was released by *XmnI/StuI* digestion and electroporated into the ZE4915 strain. Allelic replacement mutants were selected on THY agar containing erythromycin and spectinomycin. The construction of the *siaB* mutant (ZE4913) was the same as the construction of ZE4914, except that the *siaB::aad9* allele was introduced into the wild-type NZ131 strain.

RNA analysis of *Strep. pyogenes* NZ131 grown in chemically defined medium. *Strep. pyogenes* cells were used to inoculate 50 ml CxCDM and CxCDM supplemented with 20 μ M FeCl₃ or with 33 μ M MnSO₄ in side-arm (Klett) flasks at 37 °C. Cells were harvested at late-exponential phase (60 Klett units) and RNA was isolated using RiboPure-Bacteria (Ambion), following the manufacturer's recommendations. Reverse transcriptase (RT) reactions were done using Superscript III reverse transcriptase, following the manufacturer's protocol (Invitrogen). Approximately 50 ng of the cDNA was used as the template for each PCR reaction (25 cycles with Taq polymerase, Roche). Gene-specific primer sets listed in Table 1 were used in the RT and PCR reactions at a concentration of 4 μ M and 30 μ M, respectively.

Ferric iron uptake assays. Iron uptake assays were essentially performed as previously described, with small modifications (Brown *et al.*, 2001a; Janulczyk *et al.*, 2003). CxCDM was inoculated with *Strep. pyogenes* cells (1/250) and the cultures were grown to mid-exponential phase (35 Klett units) at 37 °C. ⁵⁵FeCl₃ (0.2 μ Ci μ l⁻¹, 7.4 kBq μ l⁻¹; 0.02 μ M) was added to 1 ml cultures and incubated at 37 °C. Culture samples (200 μ l) were drawn every 30 min and washed twice with 500 μ l CxCDM containing 10 mM NTA. The radioactivity associated with the cell pellet and the supernatant was measured as counts per minute (c.p.m.) for 5 min against a ³H standard using a Beckman LS6500 scintillation counter. The culture's OD₆₀₀ was measured at the same time using a Beckman DU640 spectrophotometer. ⁵⁵Fe incorporation for each time point

was standardized for the cell quantity by dividing the c.p.m. by the culture OD₆₀₀. Competition assays with iron and manganese were performed as above except that increasing concentrations of non-radioactive FeCl₃ or MnSO₄ were provided in addition to ⁵⁵FeCl₃. Culture samples were drawn after about 60 min, washed twice, and their radioactivity was measured. For the inhibition of ferric iron uptake, increasing concentrations of haem or protoporphyrin IX were added with the ⁵⁵FeCl₃. Samples were taken after 60 min (OD₆₀₀ about 1), washed, and the radioactivity measured. ⁵⁵Fe incorporation was defined as the fraction of c.p.m. of the pellet divided by the sum of the c.p.m. in pellet and the supernatant. Haem was prepared as a 10 mg ml⁻¹ stock solution of haemin chloride (Sigma) in 0.1 M NaOH pH 10. Protoporphyrin IX was prepared as a 10 mg ml⁻¹ stock solution dissolved in 1:1 dimethyl formamide/methanol. As a control, a 10 mg ml⁻¹ haem solution was treated with 5% (w/v) Chelex-100 for 1 h prior to adding it to the cells to remove any unbound iron. When necessary, dilutions were prepared in ddH₂O.

Zebrafish care and virulence assays. Care and feeding of zebrafish (*Danio rerio*) followed published methods (Neely *et al.*, 2002; Westerfield, 1995). Streptococci were cultured overnight in THY plus 20% (w/v) peptone (THYP) at 37 °C, diluted 1:100 the next day in THYP, and incubated at 37 °C. The cells were harvested at OD₆₀₀ 0.3, washed once with THYP, and diluted to the appropriate concentration in fresh THYP. Injection of zebrafish followed a previously described method (Neely *et al.*, 2002). Briefly, streptococcal cells (10 μ l of 10⁵ ml⁻¹) were aseptically injected into groups of four to six anaesthetized male breeder zebrafish (Scientific Hatcheries). Following intraperitoneal (i.p.) or intramuscular (i.m.) injection, the fish were allowed to recover in 225 ml sterilized ddH₂O supplemented with aquarium salts (Instant Ocean; Aquarium Systems) at a concentration of 60 mg l⁻¹ in a 29 °C incubator. A control animal group was injected with sterile medium. Infected fish were monitored for 48 h and death recorded in intervals of 12 h. For *Strep. pyogenes*, the 50% lethal dose (LD₅₀) for infection of zebrafish was determined by the method of Neely *et al.* (2002), where zebrafish were challenged over a range of 10¹–10⁶ c.f.u. of each streptococcal strain.

Zebrafish tissue analysis. Selected whole zebrafish were fixed following euthanasia at 40 h after infection and 5 µm thick longitudinal sections of the dorsal muscle were prepared for staining as described previously (Neely *et al.*, 2002). Fixed samples were stained with haematoxylin and eosin and examined with an Olympus BX60 microscope equipped with a digital camera and a motorized stage.

Statistics and data analysis. Statistical significance was determined by using the two-sample Student *t*-test. The standard error of the mean (SEM) was calculated by dividing the standard deviation by the square root of *n*.

RESULTS AND DISCUSSION

The streptococcal iron uptake (*siu*) transporter

Sequence analysis of the M1 SF370 *Strep. pyogenes* genome identified two ABC transporters that belong to the family of iron-complex transporters. One of these streptococcal systems, *sia* (streptococcal iron acquisition), was previously demonstrated to be involved in haemoglobin binding and acquisition of iron (Bates *et al.*, 2003; Lei *et al.*, 2003). Here we characterize the second iron-complex transporter, which is currently annotated as *fhuADB.1G* (*Spy0383-0386*) due to the homology to the corresponding genes of the *Bacillus subtilis* ferrichrome uptake system. We renamed this transporter *siu* (streptococcal iron uptake), since we found it to be involved in iron uptake. The *siu* transporter is conserved in all of the published *Strep. pyogenes* genomes (five complete and two unfinished genomes). It consists of four genes encoding an ATP-binding protein (*siuA*), a substrate-binding protein with a lipoprotein signal (*siuD*), and two subunits of a membrane permease (*siuB* and *siuG*). The *siuADBG* genes constitute an operon that is induced by depletion of iron and other metals (Lei *et al.*, 2003; Smoot *et al.*, 2001). Sequence alignments show that proteins in the *Siu* system have homology with siderophore uptake systems such as the *Staph. aureus* SirABC (Heinrichs *et al.*, 1999) and SstABCD (Morrissey *et al.*, 2000) and with transporters involved in haem and haemoprotein utilization such as the staphylococcal HtsABC (Skaar *et al.*, 2004) and IsdDEF (Mazmanian *et al.*, 2003), the pneumococcal PiaABCD and PiuBCDA (Brown *et al.*, 2001a), and the streptococcal SiaABC (Bates *et al.*, 2003).

The *siu* transporter is involved in iron acquisition

The iron needs of *Strep. pyogenes* NZ131 were investigated in various media treated with different resins. NZ131 grew in TH medium treated with the chelating resin Chelex-100 or in CxCDM (see Methods), demonstrating that it can proliferate in media containing only 1.6 µM iron and trace amounts of manganese and zinc. Similar observations were made in THY medium that was treated with Chelex-100 (Janulczyk *et al.*, 2003; Ricci *et al.*, 2002). RNA analysis showed that growth in CxCDM allows significant expression of both the *siu* and the *sia* transporters (data not shown), which are both negatively regulated by iron (Bates *et al.*, 2003; Lei *et al.*, 2003; Smoot *et al.*, 2001). The addition of

20 µM iron (but not of 20 µM manganese) repressed *siu* expression, confirming that while the iron concentration found in this medium is sufficient to support growth, it is low enough to produce an iron-stress signal. Growth of NZ131 was significantly impaired in a buffered Todd-Hewitt broth containing 12 mM NTA (ZTH-NTA) that was supplemented with a mix of the bivalent metals calcium, magnesium, manganese and zinc as described by Bates *et al.* (2003) (black bar, NTA in Fig. 1). Similarly, 20 mM NTA was previously used to restrict the growth of a second M49 strain, CS101, in THY (Podbielski *et al.*, 1999).

Growth of NZ131 in the NTA-containing medium was significantly enhanced by the addition of different iron sources. The addition of 3 mM ferric chloride, 0.13 % whole blood or 12 µM haemoglobin to ZTH-NTA medium enhanced cell growth up to 86 %, 76 % and 66 %, respectively, of the growth seen in ZTH. On the other hand, the addition of 0.13 % serum resulted in only a small increase of cell growth (32 % of the growth seen in ZTH), indicating that the iron available for the bacterium in serum is low and growth limiting (Fig. 1). These observations suggest that restricted iron availability plays a major role in the restriction of *Strep. pyogenes* growth in the ZTH-NTA medium. It is possible that this medium limits the availability of other metals as well, since ferric chloride and haemoglobin could not completely restore growth. A mutation in *siuG*, one of the membrane permease genes, was constructed in *Strep. pyogenes* NZ131 by the insertional inactivation method using an *ermAM* cassette (strain ZE4915). The construction of the mutant was verified by PCR analysis. In agreement with the presence of the *siuG::ermAM* allele, the PCR product obtained from the *siuG* mutant was 1.2 kb larger

Fig. 1. Growth analysis of the wild-type and the *siuG* mutant in iron-depleted media supplemented with various iron sources. *Strep. pyogenes* wild-type (NZ131, black bars) and *siuG* (ZE4915, grey bars) cells were grown in iron-depleted medium, ZTH-NTA (ZTH with 12 mM NTA, 0.66 mM MgCl₂, MnCl₂, CaCl₂ and ZnCl₂), or in iron-depleted medium supplemented with 3 mM ferric chloride, 0.13 % sheep's blood, 12 µM human haemoglobin or 0.13 % horse serum. The culture's OD₆₀₀ was determined following overnight incubation. Results are shown as percentage growth with respect to that obtained in complete medium (ZTH). Data represent the mean of at least three experiments. Error bars represent SEM; significant *P* values (<0.05) are indicated by *.

than that obtained from the wild-type strain (data not shown). Growth of the *siuG* mutant (grey bars in Fig. 1) was compared to that of the parent strain NZ131. The addition of ferric chloride to the iron-depleted medium enhanced the growth of the *siuG* mutant to a level slightly lower than that of the wild-type (70 % vs 86 % of that observed in ZTH). On the other hand, growth of the *siuG* mutant was significantly reduced in ZTH-NTA medium containing whole blood or haemoglobin, to only 34 % and 21 %, respectively, of that observed in ZTH. The most significant effect of *siuG* inactivation was on the ability of the bacteria to grow in ZTH-NTA medium supplemented with serum, where only 3 % of the level of growth observed in ZTH was obtained. Since *Strep. pyogenes* cannot use transferrin as an iron source, the *Siu* transporter may contribute to the use of haem bound to serum albumin or haemopexin. While the growth phenotype of the *siuG* mutant is partial, it establishes the role of the *Siu* transporter in iron acquisition. Redundancy in iron uptake pathways in *Strep. pyogenes* is probably a major factor in the partial growth phenotype. However, it is also possible that the *siuG* mutation allows residual transporter activity (with the remaining membrane permease subunit, *siuB*), and therefore may hinder the full appreciation of this transporter's role in iron acquisition.

Inactivation of the *siu* transporter results in decreased Fe^{3+} utilization

The presence of NTA in the ZTH-NTA medium complicates the study of the cell's use of ferric iron. Therefore, we investigated Fe^{3+} utilization by the wild-type and the *siuG* mutant using a $^{55}\text{Fe}^{3+}$ uptake assay in a low-iron medium that does not contain a chelator. $^{55}\text{FeCl}_3$ was added to cells growing in CxCDM at the early exponential phase and incorporation by the cells was monitored every 30 min. Iron accumulation by both the wild-type and the *siuG* mutant cells increased over time (Fig. 2), with maximum

Fig. 2. Incorporation of radioactive ferric iron by the wild-type and the *siuG* mutant. $^{55}\text{FeCl}_3$ was added to *Strep. pyogenes* wild-type (NZ131, $\blacktriangle$) and *siuG* (ZE4915, $\bullet$) cultures at the early exponential phase grown in CxCDM. Cells were harvested every 30 min, washed, and the radioactivity associated with the cell pellet was measured. Results are expressed as c.p.m. divided by the OD_{600} of the culture. Data points represent the mean of three experiments done in duplicate; error bars represent SEM.

incorporation by wild-type cells observed after about 30 min incubation. The addition of 2 μM non-radioactive iron ($^{56}\text{Fe}^{3+}$) or manganese (Mn^{2+}) inhibited $^{55}\text{Fe}^{3+}$ incorporation into NZ131 cells by 30 % and 75 %, respectively. Inhibition reached 62 % and 80 % with 6 μM iron or manganese (data not shown). Inhibition of Fe^{3+} uptake by manganese suggests that at least some of the Fe^{3+} uptake in NZ131 is mediated by a multi-metal transporter such as the *mts* transporter (RT-PCR confirmed the presence of *mts* transcript in the NZ131 strain; data not shown). Similarly, manganese could compete with Fe^{3+} uptake in the AP1 (M1 type) strain, in which inactivation of *mts* reduced accumulation of both $^{54}\text{Mn}^{2+}$ and $^{55}\text{Fe}^{3+}$ (Janulczyk *et al.*, 2003).

Comparison between the wild-type and the *siuG* mutant revealed that the rate of Fe^{3+} uptake was reduced in the mutant, in which maximum incorporation was observed only after 60 min incubation and reached about 70 % of that observed in the wild-type. Therefore, the *siu* transporter is involved in Fe^{3+} uptake, although to a lesser extent than the *mts* transporter, inactivation of which resulted in a 90 % decrease of Fe^{3+} accumulation (Janulczyk *et al.*, 2003). Similarly to *siu*, contribution both to Fe^{3+} uptake and to the cell's ability to grow in iron-restricted medium containing haemoglobin was also demonstrated by the homologous *piu* and *pia* transporters in *Streptococcus pneumoniae* (Brown *et al.*, 2001a).

Haem inhibits Fe^{3+} accumulation by *Strep. pyogenes*

We studied the effect of haem present in the growth medium on Fe^{3+} accumulation by *Strep. pyogenes* NZ131. Haem at a concentration as low as 0.75 μM inhibits the accumulation of $^{55}\text{Fe}^{3+}$ by 55 % as compared to the uptake in the absence of haem, and the addition of 6 μM haem results in about 76 % inhibition (Fig. 3a, black bars). Treatment of haem with Chelex-100 to remove free iron possibly present in the solution did not change the percentage inhibition of $^{55}\text{Fe}^{3+}$ uptake by haem (Fig. 3a, white bar). This is consistent with the proposal that it is the haem, and not free iron, that inhibits $^{55}\text{Fe}^{3+}$ uptake. On the other hand, the addition of protoporphyrin IX, the core structure of haem, did not significantly interfere with $^{55}\text{Fe}^{3+}$ incorporation, even at a concentration as high as 6 μM (Fig. 3a, grey bars). Since iron is important for the ability of haem to hinder ferric transport, we suggest that this inhibition is not the outcome of non-specific interference of the haem moiety, but that the use of haem as an iron source leads to repression of the Fe^{3+} uptake pathways. Haem may also compete with ferric iron for some of the transporters that contribute to Fe^{3+} uptake.

Since we found that haem utilization reduced the accumulation of $^{55}\text{Fe}^{3+}$ by the wild-type cells, we asked whether inactivation of *siuG* would interfere with this phenomenon. The ability of haem to hinder $^{55}\text{Fe}^{3+}$ uptake in the *siuG* mutant was tested using a range of haem concentrations. The addition of 2 and 4 μM haem resulted in about 54 %

Fig. 3. Inhibition of ^{55}Fe incorporation by haem. $^{55}\text{FeCl}_3$ was added to *Strep. pyogenes* cultures grown in CxCDM at the early exponential phase. (a) ^{55}Fe accumulation by *Strep. pyogenes* wild-type (NZ131) in the presence of 6 μM Chelex-treated haem (white bar), or increasing concentrations of haem (black bars), or protoporphyrin IX (grey bars). (b) ^{55}Fe accumulation by the wild-type (NZ131, black bars), the *siuG* mutant (ZE4915, white bars), the *siaB* mutant (ZE4913, hatched bars) and the *siuG siaB* mutant (ZE4914, grey bars) in the presence of increasing concentrations of haem. ^{55}Fe uptake was calculated as incorporation in the presence of haem or protoporphyrin IX as a percentage of the incorporation without the inhibitors. Fe^{3+} accumulation for each strain was calculated as incorporation in the presence of haem as a percentage of the incorporation in the same strain in the absence of haem. Data represent the mean of at least four experiments; error bars represent SEM.

and 76 % inhibition of $^{55}\text{Fe}^{3+}$ uptake in the wild-type strain (Fig. 3b, black bars). However, the ability of haem to inhibit uptake was lessened in the *siuG* mutant; at 2 and 4 μM haem concentration Fe^{3+} uptake by the *siuG* strain was reduced only by 36 % and 60 % ($P < 0.05$, $n = 4$) (Fig. 3b, white bars). Therefore, it seems that the *siu* is required for effective inhibition of $^{55}\text{Fe}^{3+}$ transport by haem.

When a higher concentration of haem was used, $^{55}\text{Fe}^{3+}$ accumulation by the *siu* mutant was inhibited to a level similar to that observed in the wild-type cells. This suggests that an additional transporter(s) is involved in haem utilization and/or is affected by haem. To test this hypothesis, a mutant in *siaB* (the *sia* membrane permease component, strain ZE4913) and a *siuG siaB* double mutant (strain ZE4914) were constructed by the insertional inactivation method using an *aad9* cassette. The presence of the *siaB::aad9* allele in both mutant strains was confirmed by PCR; as expected the *sia* fragment amplified from the *siaB* and the *siuG siaB* strains was 1.25 kb larger than that

produced from the wild-type strain. To test if the *sia* transporter contributes to the haem effect seen in the *siuG* mutant, we repeated the assay with the *siaB* and the *siuG siaB* strains. Similar to the *siuG* mutant, 2 μM haem did not efficiently inhibit $^{55}\text{Fe}^{3+}$ uptake in the *siaB* mutant (Fig. 3b, hatched bars, $P < 0.025$, $n = 6$), while 4 μM haem led to a decrease in Fe^{3+} uptake similar to that seen in the wild-type strain. Inactivation of both *siaB* and *siuG* had a cumulative effect at 4 and 6 μM haem. In the presence of 6 μM haem, only 52 % inhibition was observed in the double mutant (Fig. 3b, grey bar), while about 76–80 % inhibition was observed in the wild-type, *siuG* or *siaB* strains. While this reduction is not striking, it is statistically significant ($P < 0.005$, $n = 4$) when compared to the wild-type. Based on these observations we suggest that haem utilization is partially impaired if the *siu* transporter is disrupted and that inactivation of the *sia* transporter reduces haem usage by the cell even further. Additionally, the ability of haem to reduce $^{55}\text{Fe}^{3+}$ uptake by 52 % in the double mutant may result from the residual activity of the *siu* system and the presence of other haem utilization pathways. Redundancy in haem utilization pathways has been demonstrated in several Gram-positive bacteria, such as *isdDEF* and *htsABC* in *Staph. aureus* (Mazmanian *et al.*, 2003; Skaar *et al.*, 2004), *piaABCD* and *piuBCDA* in *Strep. pneumoniae* (Brown *et al.*, 2001a), *hmuTUV* and an uncharacterized transporter in *C. diphtheriae* (Drazek *et al.*, 2000; Schmitt & Drazek, 2001).

siuG is required for virulence of *Strep. pyogenes* in zebrafish

Competitive index studies showed that *piaA* in *S. pneumoniae* was important in both a pulmonary and a systemic murine model for disease (Brown *et al.*, 2001a), and mice immunized with recombinant PiuA and PiaA were protected against systemic pneumococcal challenge (Brown *et al.*, 2001b). Likewise, the *Strep. pyogenes mtsA* and the *Staph. aureus hts* mutants were attenuated in animal infection models (Janulczyk *et al.*, 2003; Skaar *et al.*, 2004). Using a zebrafish animal model we investigated the role of *siuG* in disease progression by *Strep. pyogenes*. The zebrafish immune system has many similarities to the mammalian system (Postlethwait *et al.*, 1998; Trede *et al.*, 2001) and numerous studies have characterized its cardiovascular components (MacRae & Fishman, 2002). Recent studies established that the zebrafish is a suitable model to investigate streptococcal infections. I.p. and i.m. injection of *Strep. pyogenes* HSC5 (M5 type) produced lethal infections in the fish, along with hypopigmented lesions and tissue necrosis (Miller & Neely, 2004; Neely *et al.*, 2002). We used this model to investigate the role of iron acquisition in disease production and progression by *Strep. pyogenes* NZ131.

When zebrafish were injected i.m. with a range of 10^1 – 10^6 c.f.u. of the wild-type NZ131, the dose response was similar to that reported for HSC5 (LD_{50} 10^4 cells ml^{-1}) (Neely *et al.*, 2002). I.m. injection of NZ131 also produced a hypopigmented lesion with extensive muscular necrosis. A control animal group, mock injected with sterile medium,

showed no signs of distress. Forty hours after injection, infected zebrafish were fixed and longitudinal sections were prepared. Staining of the tissue revealed streptococcal cells arranged in clusters at the site of infection, as well as the appearance of some host immune cells (data not shown). When the fish were injected i.p. with a range of 10^1 – 10^6 c.f.u. of the wild-type NZ131, the LD₅₀ was higher ($>10^5$ cells ml⁻¹) than when they were injected i.m. This observation is different from the observations made with the HCS5 strain, where the LD₅₀ in the i.p. route was lower than that in the i.m. infection (Neely *et al.*, 2002).

We investigated the role of the *siu* transporter in virulence by comparing the mutant strain to the parent strain when injected separately by both the i.m. and i.p. routes of infection. Groups of four to six zebrafish were challenged with 10^5 cells ml⁻¹ of *Strep. pyogenes* wild-type and the *siuG* mutant and monitored for 2 days. I.m. injection with NZ131 resulted in only 14 % survival of the fish by 48 h (Fig. 4). In the *siuG* mutant the ability to cause death of the fish was significantly reduced (88 % survival, $P<0.0115$, $n=3$). I.p. injection with 10^5 cells ml⁻¹ of *Strep. pyogenes* wild-type resulted in about 50 % increase in animal survival as compared to the i.m. injection. Still, the *siuG* was less virulent as compared to the wild-type (data not shown). These results suggest that acquisition of iron is important for *Strep. pyogenes* pathogenesis in the zebrafish model and that *siuG* function has an important role *in vivo* in the establishment of infection. Iron metabolism and erythroid development in zebrafish is analogous to that of higher vertebrates; zebrafish produce haem and haemoglobin, carry out haemoglobin switching during development (Brownlie *et al.*, 2003), use transferrin receptors (Wingert *et al.*, 2004) and divalent metal transporter 1 (DMT1, Donovan *et al.*, 2002) to transport iron into and within the cell's compartments, and employ ferroprotein 1 (Fpr1) as an intestinal

Fig. 4. Zebrafish survival curve following *Strep. pyogenes* infection. Groups of four to six zebrafish were challenged with 10^5 c.f.u. of *Strep. pyogenes* wild-type (NZ131, ▲) and the *siuG* mutant (ZE4915, ●) by i.m. injection and monitored for 2 days. Data were pooled from three to five independent experiments and presented as total percentage fish survival as a function of time.

and macrophage iron exporter (Donovan *et al.*, 2000). Therefore, it is likely that the iron acquisition mechanisms used by *Strep. pyogenes* during infection of zebrafish are relevant for iron acquisition during human infection.

In conclusion, *siuADBG* is the third ABC-type iron transporter in *Strep. pyogenes* shown to be involved in iron uptake. The growth assays and the reduced ability of haem to decrease ferric transport by the *siuG* mutant implicated the *siu* transporter in iron acquisition from haemoglobin, haem, blood and serum. However, additional studies are required to determine the ligand of the *siu* transporter and how it contributes to the use of haemoglobin and haem.

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REFERENCES

- Bates, C. S., Montanez, G. E., Woods, C. R., Vincent, R. M. & Eichenbaum, Z. (2003). Identification and characterization of a *Streptococcus pyogenes* operon involved in binding of hemoproteins and acquisition of iron. *Infect Immun* **71**, 1042–1055.
- Bisno, A. L., Brito, M. O. & Collins, C. M. (2003). Molecular basis of group A streptococcal virulence. *Lancet Infect Dis* **3**, 191–200.
- Brown, J. S. & Holden, D. W. (2002). Iron acquisition by Gram-positive bacterial pathogens. *Microbes Infect* **4**, 1149–1156.
- Brown, J. S., Gilliland, S. M. & Holden, D. W. (2001a). A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol* **40**, 572–585.
- Brown, J. S., Ogunniyi, A. D., Woodrow, M. C., Holden, D. W. & Paton, J. C. (2001b). Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. *Infect Immun* **69**, 6702–6706.
- Brownlie, A., Hersey, C., Oates, A. C. & 10 other authors (2003). Characterization of embryonic globin genes of the zebrafish. *Dev Biol* **255**, 48–61.
- Claverys, J. P. (2001). A new family of high-affinity ABC manganese and zinc permeases. *Res Microbiol* **152**, 231–243.
- Coulanges, V., Andre, P. & Vidon, D. J. (1998). Effect of siderophores, catecholamines, and catechol compounds on *Listeria* spp. Growth in iron-complexed medium. *Biochem Biophys Res Commun* **249**, 526–530.
- Courcol, R. J., Trivier, D., Bissinger, M. C., Martin, G. R. & Brown, M. R. (1997). Siderophore production by *Staphylococcus aureus* and identification of iron-regulated proteins. *Infect Immun* **65**, 1944–1948.
- Cunningham, M. W. (2000). Pathogenesis of group A streptococcal infections. *Clin Microbiol Rev* **13**, 470–511.
- De Voss, J. J., Rutter, K., Schroeder, B. G. & Barry, C. E., 3rd (1999). Iron acquisition and metabolism by mycobacteria. *J Bacteriol* **181**, 4443–4451.

- Dintilhac, A., Alloing, G., Granadel, C. & Claverys, J. P. (1997). Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. *Mol Microbiol* **25**, 727–739.
- Donovan, A., Brownlie, A., Zhou, Y. & 17 other authors (2000). Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* **403**, 776–781.
- Donovan, A., Brownlie, A., Dorschner, M. O. & 7 other authors (2002). The zebrafish mutant gene chardonnay (*cdy*) encodes divalent metal transporter 1 (DMT1). *Blood* **100**, 4655–4659.
- Drazek, E. S., Hammack, C. A. & Schmitt, M. P. (2000). *Corynebacterium diphtheriae* genes required for acquisition of iron from haemin and haemoglobin are homologous to ABC haemin transporters. *Mol Microbiol* **36**, 68–84.
- Eichenbaum, Z., Muller, E., Morse, S. A. & Scott, J. R. (1996). Acquisition of iron from host proteins by the group A streptococcus. *Infect Immun* **64**, 5428–5429.
- Elsner, A., Kreikemeyer, B., Braun-Kiewnick, A., Spellerberg, B., Buttar, B. A. & Podbielski, A. (2002). Involvement of Lsp, a member of the LraI-lipoprotein family in *Streptococcus pyogenes*, in eukaryotic cell adhesion and internalization. *Infect Immun* **70**, 4859–4869.
- Ferretti, J. J., McShan, W. M., Ajdic, D. & 20 other authors (2001). Complete genome sequence of an M1 strain of *Streptococcus pyogenes*. *Proc Natl Acad Sci U S A* **98**, 4658–4663.
- Francis, R. T., Jr, Booth, J. W. & Becker, R. R. (1985). Uptake of iron from hemoglobin and the haptoglobin-hemoglobin complex by hemolytic bacteria. *Int J Biochem* **17**, 767–773.
- Genco, C. A. & Dixon, D. W. (2001). Emerging strategies in microbial haem capture. *Mol Microbiol* **39**, 1–11.
- Gilson, E., Alloing, G., Schmidt, T., Claverys, J. P., Dudler, R. & Hofnung, M. (1988). Evidence for high affinity binding-protein dependent transport systems in gram-positive bacteria and in *Mycoplasma*. *EMBO J* **7**, 3971–3974.
- Hartford, T., O'Brien, S., Andrew, P. W., Jones, D. & Roberts, I. S. (1993). Utilization of transferrin-bound iron by *Listeria monocytogenes*. *FEMS Microbiol Lett* **108**, 311–318.
- Heinrichs, J. H., Gatlin, L. E., Kunsch, C., Choi, G. H. & Hanson, M. S. (1999). Identification and characterization of SirA, an iron-regulated protein from *Staphylococcus aureus*. *J Bacteriol* **181**, 1436–1443.
- Henderson, D. P. & Payne, S. M. (1994). *Vibrio cholerae* iron transport systems: roles of heme and siderophore iron transport in virulence and identification of a gene associated with multiple iron transport systems. *Infect Immun* **62**, 5120–5125.
- Higgins, C. F. (1992). ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8**, 67–113.
- Husmann, L. K., Yung, D. L., Hollingshead, S. K. & Scott, J. R. (1997). Role of putative virulence factors of *Streptococcus pyogenes* in mouse models of long-term throat colonization and pneumonia. *Infect Immun* **65**, 1422–1430.
- Janulczyk, R., Pallon, J. & Bjorck, L. (1999). Identification and characterization of a *Streptococcus pyogenes* ABC transporter with multiple specificity for metal cations. *Mol Microbiol* **34**, 596–606.
- Janulczyk, R., Ricci, S. & Bjorck, L. (2003). MtsABC is important for manganese and iron transport, oxidative stress resistance, and virulence of *Streptococcus pyogenes*. *Infect Immun* **71**, 2656–2664.
- Lei, B., Liu, M., Voyich, J. M., Prater, C. I., Kala, S. V., DeLeo, F. R. & Musser, J. M. (2003). Identification and characterization of HtsA, a second heme-binding protein made by *Streptococcus pyogenes*. *Infect Immun* **71**, 5962–5969.
- MacRae, C. A. & Fishman, M. C. (2002). Zebrafish: the complete cardiovascular compendium. *Cold Spring Harb Symp Quant Biol* **67**, 301–307.
- Mazmanian, S. K., Skaar, E. P., Gaspar, A. H., Humayun, M., Gornicki, P., Jelenska, J., Joachmiak, A., Missiakas, D. M. & Schneewind, O. (2003). Passage of heme-iron across the envelope of *Staphylococcus aureus*. *Science* **299**, 906–909.
- Miller, J. D. & Neely, M. N. (2004). Zebrafish as a model host for streptococcal pathogenesis. *Acta Trop* **91**, 53–68.
- Modun, B., Evans, R. W., Joannou, C. L. & Williams, P. (1998). Receptor-mediated recognition and uptake of iron from human transferrin by *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Infect Immun* **66**, 3591–3596.
- Morrissey, J. A., Cockayne, A., Hill, P. J. & Williams, P. (2000). Molecular cloning and analysis of a putative siderophore ABC transporter from *Staphylococcus aureus*. *Infect Immun* **68**, 6281–6288.
- Neely, M. N., Pfeifer, J. D. & Caparon, M. (2002). Streptococcus-zebrafish model of bacterial pathogenesis. *Infect Immun* **70**, 3904–3914.
- Nordstrand, A., Norgren, M., Ferretti, J. J. & Holm, S. E. (1998). Streptokinase as a mediator of acute post-streptococcal glomerulonephritis in an experimental mouse model. *Infect Immun* **66**, 315–321.
- Oligino, L. & Fives-Taylor, P. (1993). Overexpression and purification of a fimbria-associated adhesin of *Streptococcus parasanguis*. *Infect Immun* **61**, 1016–1022.
- Podbielski, A., Spellerberg, B., Woischnik, M., Pohl, B. & Luticken, R. (1996). Novel series of plasmid vectors for gene inactivation and expression analysis in group A streptococci (GAS). *Gene* **177**, 137–147.
- Podbielski, A., Woischnik, M., Kreikemeyer, B., Bettenbrock, K. & Buttar, B. A. (1999). Cysteine protease SpeB expression in group A streptococci is influenced by the nutritional environment but SpeB does not contribute to obtaining essential nutrients. *Med Microbiol Immunol* **188**, 99–109.
- Postlethwait, J. H., Yan, Y. L., Gates, M. A. & 26 other authors (1998). Vertebrate genome evolution and the zebrafish gene map. *Nat Genet* **18**, 345–349.
- Ratledge, C. (2004). Iron, mycobacteria and tuberculosis. *Tuberculosis* **84**, 110–130.
- Ricci, S., Janulczyk, R. & Bjorck, L. (2002). The regulator PerR is involved in oxidative stress response and iron homeostasis and is necessary for full virulence of *Streptococcus pyogenes*. *Infect Immun* **70**, 4968–4976.
- Russell, L. M., Cryz, S. J., Jr & Holmes, R. K. (1984). Genetic and biochemical evidence for a siderophore-dependent iron transport system in *Corynebacterium diphtheriae*. *Infect Immun* **45**, 143–149.
- Schmitt, M. P. (1997). Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. *J Bacteriol* **179**, 838–845.
- Schmitt, M. P. (1999). Identification of a two-component signal transduction system from *Corynebacterium diphtheriae* that activates gene expression in response to the presence of heme and hemoglobin. *J Bacteriol* **181**, 5330–5340.
- Schmitt, M. P. & Drazek, E. S. (2001). Construction and consequences of directed mutations affecting the hemin receptor in pathogenic *Corynebacterium* species. *J Bacteriol* **183**, 1476–1481.
- Sebulsky, M. T. & Heinrichs, D. E. (2001). Identification and characterization of *fluD1* and *fluD2*, two genes involved in iron-hydroxamate uptake in *Staphylococcus aureus*. *J Bacteriol* **183**, 4994–5000.

- Skaar, E. P., Humayun, M., Bae, T., DeBord, K. L. & Schneewind, O. (2004). Iron-source preference of *Staphylococcus aureus* infections. *Science* **305**, 1626–1628.
- Smoot, L. M., Smoot, J. C., Graham, M. R., Somerville, G. A., Sturdevant, D. E., Migliaccio, C. A., Sylva, G. L. & Musser, J. M. (2001). Global differential gene expression in response to growth temperature alteration in group A *Streptococcus*. *Proc Natl Acad Sci U S A* **98**, 10416–10421.
- Spellerberg, B., Rozdzinski, E., Martin, S., Weber-Heynemann, J., Schnitzler, N., Luttkien, R. & Podbielski, A. (1999). Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of *Streptococcus agalactiae* to human laminin. *Infect Immun* **67**, 871–878.
- Stojiljkovic, I., Hwa, V., de Saint Martin, L., O'Gaora, P., Nassif, X., Heffron, F. & So, M. (1995). The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. *Mol Microbiol* **15**, 531–541.
- Torres, A. G., Redford, P., Welch, R. A. & Payne, S. M. (2001). TonB-dependent systems of uropathogenic *Escherichia coli*: aerobactin and heme transport and TonB are required for virulence in the mouse. *Infect Immun* **69**, 6179–6185.
- Trede, N. S., Zapata, A. & Zon, L. I. (2001). Fishing for lymphoid genes. *Trends Immunol* **22**, 302–307.
- van de Rijn, I. & Kessler, R. E. (1980). Growth characteristics of group A streptococci in a new chemically defined medium. *Infect Immun* **27**, 444–448.
- Wandersman, C. & Delepelaire, P. (2004). Bacterial iron sources: from siderophores to hemophores. *Annu Rev Microbiol* **58**, 611–647.
- Wandersman, C. & Stojiljkovic, I. (2000). Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. *Curr Opin Microbiol* **3**, 215–220.
- Westerfield, M. (1995). *The Zebrafish Book: Guide for the Laboratory use of Zebrafish (Danio rerio)*. Eugene: University of Oregon Press.
- Wingert, R. A., Brownlie, A., Galloway, J. L. & 9 other authors (2004). The chianti zebrafish mutant provides a model for erythroid-specific disruption of transferrin receptor 1. *Development* **131**, 6225–6235.
- Wooldridge, K. G. & Williams, P. H. (1993). Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiol Rev* **12**, 325–348.