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Crystallization and preliminary X-ray diffraction analysis of the haem-binding protein HemS from *Yersinia enterocolitica*

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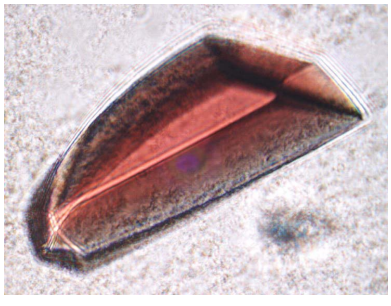

Bacteria have evolved strategies to acquire iron from their environment. Pathogenic microbes rely on specialized proteins to 'steal' haem from their host and use it as an iron source. HemS is the ultimate recipient of a molecular-relay system for haem uptake in Gram-negative species, functioning as the cytosolic carrier of haem. Soluble expression and high-quality diffraction crystals were obtained for HemS from *Yersinia enterocolitica*. Crystals belong to the orthorhombic space group *I*222, with unit-cell parameters a = 74.86, b = 77.45, c = 114.09 Å, and diffract X-rays to 2.6 Å spacing in-house. Determination of the structure of the haem–HemS complex will reveal the molecular basis of haem binding.

1. Introduction

Iron is an essential nutrient for the majority of living organisms, given its key role in biological processes such as oxygen transport and cellular respiration. Despite being one of the most abundant chemical elements, iron is scarcely available under physiological conditions because of its relatively low solubility. Microorganisms have therefore evolved a range of specialized proteins to circumvent iron-dependency, often involving, in pathogenic bacteria, the uptake of haem as a source of iron from the host's haem proteins.

The haem-uptake system shared by many Gram-negative species includes four structurally distinct but functionally interlinked components (Stojiljkovic & Perkins-Balding, 2002). In *Yersinia enterocolitica*, the outer membrane receptor HemR sequesters haem from the host's haem proteins or directly binds free haem; the ligand is then passed on by means of the periplasmic carrier HemT to the heterodimeric integral inner membrane permease HemUV/HemP, which internalizes the haem for final delivery to the cytosolic recipient HemS (Stojiljkovic & Hantke, 1994; Faraldo-Gómez & Samson, 2003). In this ABC transporter system, translocation of haem across the outer membrane into the periplasmic space by HemR is driven by the proton-motive force, mediated through conformational changes in the energy-transducing TonB system (Andrews *et al.*, 2003). However, the exact mechanism of haem transport and the molecular basis and specificities of ligand binding and release in the HemR, HemT, HemUV/HemP and HemS proteins are not known. The basic four-component organization is common to pathogens such as *Escherichia*, *Yersinia*, *Vibrio*, *Shigella* and *Pseudomonas*, although the names given to equivalent proteins in distinct species are different: *e.g.* the HemS protein of *Y. enterocolitica* is called ShuS in *S. dysenteriae*, ChuS in *E. coli*, HmuS in *Y. pestis etc*.

The main investigations carried out on the HemRSTUV uptake system include genetic and biochemical experiments, predominantly using the enterobacteria *Yersinia pestis* (Thompson *et al.*, 1999) and *Y. enterocolitica* (Stojiljkovic & Hantke, 1992, 1994). It has been established that the genes of the components of the uptake system are all encoded within a single operon (Stojiljkovic & Hantke, 1994) and that expression from this operon is regulated by iron (Thompson *et al.*, 1999). Further work focused on the HemR outer membrane receptor and involved sequence comparisons coupled with mutagenesis experiments on HemR from *Y. enterocolitica*. These studies showed that two of the four conserved histidine residues are involved in haem binding (Bracken *et al.*, 1999).

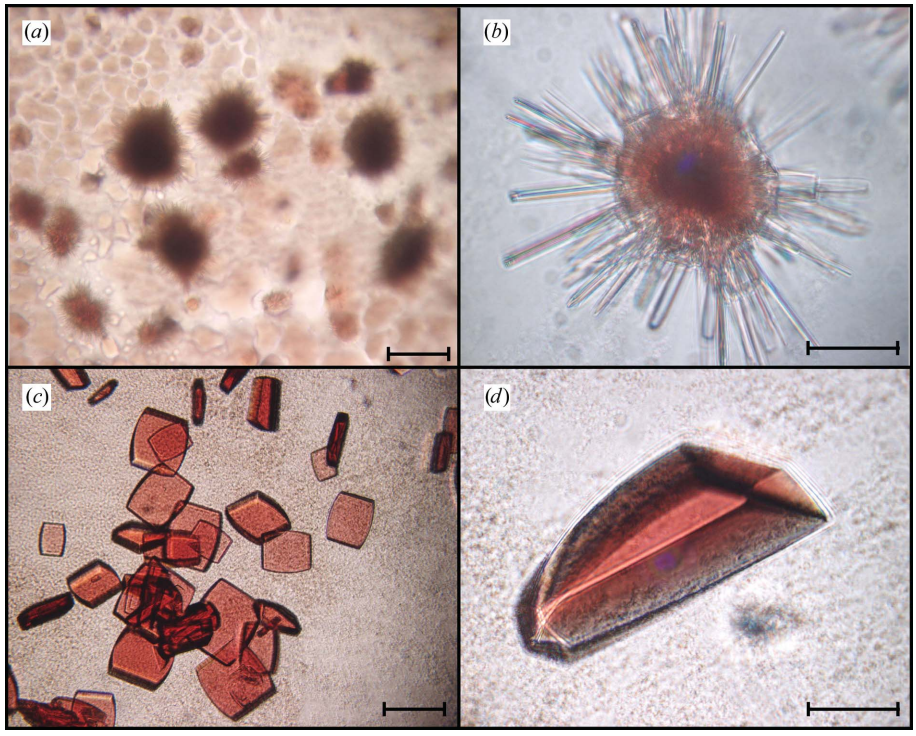

Figure 2
Crystallization of HemS from *Y. enterocolitica*. (*a*) Needle clusters observed in the 'hit' condition identified from the high-throughput screening of 864 conditions in sitting-drop vapour-diffusion experiments: 0.1 *M* MES, 2.0 *M* ammonium sulfate, 5% PEG 400 pH 6.5. (*b*) Improved conditions in hanging drops: 0.1 *M* MES pH 6.5, 1.8 *M* ammonium sulfate and 4% PEG 400. (*c*) and (*d*) Crystals obtained through further optimization in hanging drops in 0.1 *M* Tris–HCl pH 8.5, 1.8 *M* ammonium sulfate, 2% PEG 400; drop size was 2 + 2 µl and protein concentration 30 mg ml^{-1} (bar indicates 100 µm).

The best characterized molecular component of the system is the cytosolic recipient HemS. Originally thought to be a bacterial haem oxygenase, HemS from *Y. enterocolitica* was shown to be a 40 kDa protein likely to be responsible for protecting the cellular environment from the toxic effects of free haem (Stojiljkovic & Hantke, 1994). HemS was also found to be an essential protein for *Yersinia* and has therefore been considered as a possible target for the development of new antibacterial drug agents. Homologues of HemS are characterized by a sequence identity greater than 30% and are unique to the phylum of the proteobacteria. A comprehensive biochemical study was carried out on a HemS homologue, the ShuS protein from *S. dysenteriae* (Wilks, 2001), which shares 65% identical residues with the protein from *Y. enterocolitica*. ShuS was shown to bind one haem per molecule with an affinity in the micromolar range.

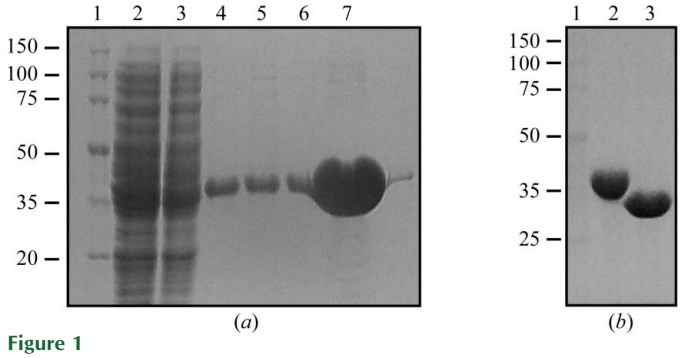

Figure 1
SDS–PAGE showing the two major steps in the purification of HemS from *Y. enterocolitica*. (*a*) His-tag purification. Lane 1, molecular-weight markers (kDa); lane 2, flowthrough; lanes 3, 4, 5 and 6, washing with 5, 10, 15 and 20 m*M* imidazole, respectively; lane 7, elution of bound HemS with 150 m*M* imidazole. (*b*) Purified HemS protein samples before (lane 2) and after (lane 3) cleavage with thrombin.

Gel-filtration and electron-microscopy work revealed that ShuS forms oligomeric particles, apparently spherical in shape and larger than those of ferritin. Finally, it was observed that ShuS is able to bind DNA, perhaps for protection against haem-induced oxidative damage, and acts as haem storage during active transport (Wilks, 2001).

So far, neither HemS nor any of the components of the HemRSTUV uptake system have been structurally studied. The only known structure of a bacterial haem binder/carrier is that of the secreted siderophore HasA from *Serratia marcescens*, which has been elucidated in complex with its haem ligand (Arnoux *et al.*, 1999, 2000), although this protein appears to be part of a distinct haem-uptake system.

Analysis of the structure of HemS in complex with haem will allow us to decipher the molecular basis of ligand binding. Here, we describe the cloning, purification and crystallization of the *Y. enterocolitica* HemS protein.

2. Materials and methods

2.1. Cloning and expression

The *hemS* gene of *Y. enterocolitica* (accession Nos. NCBI gi2507043 or Swiss-Prot P31517) was PCR amplified from genomic DNA and cloned into the expression vector pGAT2 (Peränen *et al.*, 1996). The GST tag had been previously removed from this vector, thus leaving only an N-terminal His tag which can be cleaved by thrombin. For protein expression, transformed *E. coli* BL21(DE3) cells (Novagen) were grown in 2×YT media supplemented with 50 µg ml^{-1} carbenicillin (Sigma) at 310 K and shaken at 220 rev min^{-1} in baffled flasks. Once an OD_{600} of 0.6–1.0 was reached, the incubation temperature was decreased to 303 K and protein

expression was induced by adding IPTG to a final concentration of 0.4 m*M*. Cultures were further grown overnight at 303 K and shaken at 160 rev min^{-1}. Cells were harvested by centrifugation at 4000 rev min^{-1}, resuspended in lysis buffer (50 m*M* HEPES pH 8.0, 150 m*M* NaCl) with a cocktail of protease inhibitors (Roche) and finally lysed using a French Press.

2.2. Purification

The cells are characterized by a distinctive dark colour, indicating that the HemS protein had incorporated haem during expression. The dark-red supernatant obtained after two centrifugation steps (15 000*g* and 30 000*g* for 30 min at 277 K) was passed through a 0.45 µm filter and incubated at 277 K for 1 h with 6 ml Ni–NTA Superflow (Qiagen) slurry per litre of culture. The nickel resin had been pre-equilibrated with lysis buffer and imidazole was added to a final concentration of 5 m*M* to minimize non-specific binding. The resin was applied onto a column and washed by gravity flow with a series of four wash-buffer solutions containing 5, 10, 15 and 25 m*M* imidazole, respectively. The protein was finally eluted with elution buffer containing 150 m*M* imidazole (Fig. 1*a*). The buffer was changed to 50 m*M* HEPES pH 8.0, 150 m*M* NaCl using centrifugal membrane devices (Vivaspin). The protein concentration was determined by spectroscopic measurement of the absorbance at 280 nm and an estimated millimolar extinction coefficient of 1.13 ml cm^{-1} mg^{-1} was used.

The N-terminal His tag was removed by proteolytic cleavage with thrombin (5 U thrombin per milligram of HemS overnight at room temperature). To remove uncleaved protein and the cleaved His tags, the solution was incubated with the Ni–NTA resin for 1 h at 277 K, the resin was packed on a column and the flowthrough containing untagged HemS was collected. Thrombin was finally removed by incubation with 2 µl *p*-aminobenzamidine-agarose beads (Sigma) per unit of thrombin. The thrombin-loaded beads were separated by centrifugation and the supernatant containing HemS was recovered (Fig. 1*b*).

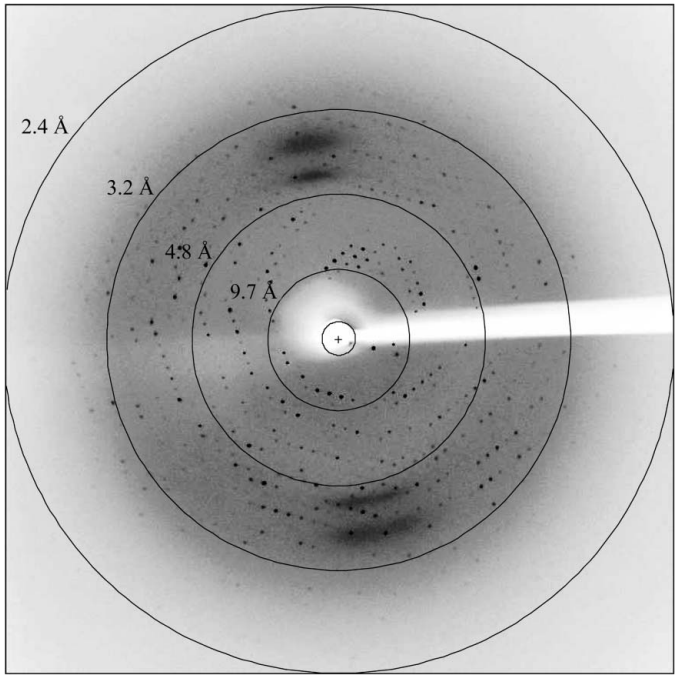

Figure 3
Image showing a typical diffraction pattern from HemS crystals obtained using the laboratory radiation source. The figure was produced with *MOSFLM* (Leslie, 1992).

The HemS sample was saturated with its ligand by incubation with a sixfold molar excess of haemin for 1 h at room temperature. The stock haemin solution was prepared by dissolving haemin chloride (Sigma) in 0.1 *M* NaOH and the concentration of the haemin solution was determined spectroscopically (ε_{385} = 58.44 mM^{-1} cm^{-1} in 0.1 *M* NaOH) as previously described (Choi *et al.*, 1999). A final gel-filtration purification step (with the resin GLC 300; Isco Inc.) was carried out to remove excess haemin. The protein was then concentrated to a concentration of 20 mg ml^{-1} in 50 m*M* HEPES pH 8.0, 150 m*M* NaCl using centrifugal membrane devices (Vivaspin).

2.3. Crystallization

Initial high-throughput crystallization screening was carried out using the sitting-drop vapour-diffusion method in 96-well Intelliplates (Hampton Research). The experiments were set up with the Hydra II micro-dispensing system (Robertson Scientific), testing conditions from five crystallization screens from Nextal Biotechnology (Classics, PEGs, AmSO4, Cations, MPDs) and a number of screens from Hampton Research (Crystal Screen, Crystal Screen 2, Index, SaltRx and Grid Screens: Ammonium Sulfate, Sodium Malonate, PEG/LiCl, PEG 6000). The effect of volume was checked by using two different drop sizes (2 + 2 µl and 0.7 + 0.7 µl). The protein concentration used in these initial screens was 15 mg ml^{-1}. Of the 864 conditions screened, only one gave a positive result, producing clusters of red needles (Fig. 2*a*): 0.1 *M* MES pH 6.5, 5% PEG 400, 2.0 *M* ammonium sulfate (from the screen AmSO4, Nextal Biotechnology). A simple manual screen around the hit condition yielded larger better clusters of crystals (Fig. 2*b*), although the crystal size and shape were not ideal for X-ray work.

A protocol was devised to carry out a rational grid screen with robotics to simultaneously test the effect on the crystal formation of both a range of buffers and ammonium sulfate concentrations (0.8–2.8 *M*). The buffers used in this screen included MES pH 6.5, sodium cacodylate pH 6.5, Bis-Tris pH 6.5, HEPES pH 7.5, Tris–HCl pH 8.5, bicine pH 9.0 and CAPS pH 10.0, all at a concentration of 0.1 *M*. Experiments with Tris and HEPES showed a marked improvement in crystal formation relative to the MES buffer used in the hit condition.

Other screening included testing against sodium malonate (1.2–2.4 *M*), which has been shown to be a successful precipitant in protein crystallization trials (McPherson, 2001). Additives such as imidazole or glycerol in different concentrations were also tested, as well as the effect of varying the concentration of sodium chloride, PEG 400 and protein. Finally, crystal growth was also carried out at the decreased temperature of 277 K, but this did not give improvements.

After optimization of these conditions, the best results were obtained by the hanging-drop method with drops consisting of 2 µl HemS at 30 mg ml^{-1} mixed with 2 µl 0.1 *M* Tris–HCl pH 8.5, 1.8 *M* ammonium sulfate and 2% PEG 400, incubated at 293 K. Bright red crystals typically grew to 0.3 × 0.2 × 0.05 mm (Figs. 2*c* and 2*d*) over 48–72 h. The crystals did not grow any larger after this time. Variation of drop size or higher protein concentration did not improve crystal size.

2.4. Diffraction analysis

Crystals were transferred into an artificial cryoprotected mother liquor consisting of 0.1 *M* Tris–HCl pH 8.5, 1.8 *M* ammonium sulfate, 150 m*M* sodium chloride, 2% PEG 400, 1.2 *M* sodium malonate prior to flash-freezing. Optimization of the cryoprotectant solution was a key issue, since mixtures with higher concentrations of either PEG

Table 1
Statistics for the processing and merging of the diffraction data.

Values in parentheses are for the highest resolution shell.

Space group	*I*222
Unit-cell parameters (Å)	a = 74.86, b = 77.45, c = 114.09
Resolution range (Å)	20.0–2.6 (2.74–2.6)
No. of observations	36422 (5168)
No. of unique reflections	10493 (1477)
Completeness (%)	99.0 (98.5)
$R_{\rm merge}$† (%)	6.8 (31.2)
Mean $I/\sigma(I)$	12.8 (4.0)
Redundancy	3.5 (3.5)
Solvent content (%)	42
Molecules per AU	1

† $R_{\rm merge} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i |I_{h,i}|$, where the outer summation is over all unique reflections with multiple observations and the inner summation over all observations of each reflection.

400, ammonium sulfate or sodium malonate resulted in phase separation, serious crystal damage and/or unsatisfactory freezing.

Initial diffraction experiments were carried out in-house using a Rigaku MicroMax 007 rotating-anode generator with a rotating copper anode equipped with an R-AXIS IV^{++} image-plate detector and an X-stream 2000 cryocooling vapour jet. Well ordered diffraction patterns were observed, with data extending to 2.6 Å spacing depending on crystal size and quality (Fig. 3). A data set was collected consisting of 180 frames with 0.5° oscillation and 30 min exposure and a crystal-to-detector distance of 200 mm. Data were processed using *MOSFLM* v.6.2.4 (Leslie, 1992) and *SCALA* (Evans, 1997). The lattice symmetry belongs to the orthorhombic space group *I*222 and the statistics for the crystal unit-cell parameters and data processing are shown in Table 1.

3. Conclusion

The 39.2 kDa HemS protein from *Y. enterocolitica* was cloned from genomic DNA, expressed using a T7-based vector and purified by metal-affinity chromatography. The His tag was removed using thrombin. This bacterial haem-binding protein purifies with a red colour when recombinantly expressed in *E. coli*. Diffraction-quality crystals were obtained and cryoprotecting conditions were optimized, enabling the measurement of good-quality data to 2.6 Å spacing. Since we have crystallized HemS in complex with haem, structure determination by Fe-MAD (Nioche *et al.*, 2004; Vallone *et al.*, 2004) may be possible, although this might prove challenging given the size of the structure to be phased from a single Fe atom.

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References

Andrews, S. C., Robinson, A. K. & Rodriguez-Quinones, F. (2003). *FEMS Microbiol. Rev.* **27**, 215–237.
Arnoux, P., Haser, R., Izadi-Pruneyre, N., Lecroisey, A. & Czjzek, M. (2000). *Proteins*, **41**, 202–210.
Arnoux, P., Haser, R., Izadi, N., Lecroisey, A., Delepierre, M., Wandersman, C. & Czjzek, M. (1999). *Nature Struct. Biol.* **6**, 516–520.
Bracken, C. S., Baer, M. T., Abdur-Rashid, A., Helms, W. & Stojiljkovic, I. (1999). *J. Bacteriol.* **181**, 6063–6072.
Choi, C. Y., Cerda, J. F., Chu, H. A., Babcock, G. T. & Marletta, M. A. (1999). *Biochemistry*, **38**, 16916–16924.
Evans, P. (1997). *Jnt CCP4/ESF–EACMB Newsl. Protein Crystallogr.* **33**, 22–24.
Faraldo-Gómez, J. D. & Sansom, M. S. (2003). *Nature Rev. Mol. Cell Biol.* **4**, 105–116.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACMB Newsl. Protein Crystallogr.* **26**.
McPherson, A. (2001). *Protein Sci.* **10**, 418–422.
Nioche, P., Berka, V., Vipond, J., Minton, N., Tsai, A. L. & Raman, C. S. (2004). *Science*, **306**, 1550–1553.
Peränen, J., Rikkonen, M., Hyvonen, M. & Kaariainen, L. (1996). *Anal. Biochem.* **236**, 371–373.
Stojiljkovic, I. & Hantke, K. (1992). *EMBO J.* **11**, 4359–4367.
Stojiljkovic, I. & Hantke, K. (1994). *Mol. Microbiol.* **13**, 719–732.
Stojiljkovic, I. & Perkins-Balding, D. (2002). *DNA Cell Biol.* **21**, 281–295.
Thompson, J. M., Jones, H. A. & Perry, R. D. (1999). *Infect. Immun.* **67**, 3879–3892.
Vallone, B., Nienhaus, K., Brunori, M. & Nienhaus, G. U. (2004). *Proteins*, **56**, 85–92.
Wilks, A. (2001). *Arch. Biochem. Biophys.* **387**, 137–142.