The Hem and Has haem uptake systems in *Serratia marcescens*

Najla Benevides-Matos and Francis Biville

Correspondence
Francis Biville
fbiville@pasteur.fr

Unité des Membranes Bactériennes, Institut Pasteur (CNRS URA 2172), 25 Rue du Dr Roux, Paris CEDEX 15, France

Serratia marcescens, like several other Gram-negative bacteria, possesses two functional haem uptake systems. The first, referred to as the Hem system, can transport haem present at a concentration equal to or above 10^{-6} M. It requires an active outer-membrane receptor which uses proton-motive force energy transmitted by the inner-membrane TonB protein. The other system, Has, takes up haem at lower concentrations and utilizes a small secreted haem-binding protein (haemophore) and its cognate TonB-dependent outer-membrane receptor HasR. Various combinations of mutations were used to examine haem uptake activity by the two systems in *S. marcescens*. The Hem uptake system enables *S. marcescens* to take up haem at a concentration of 10^{-6} M in the presence of various levels of iron depletion. The Has system, which enables such uptake even in the presence of lower haem concentrations, requires higher iron depletion conditions for function. Has haem uptake requires the presence of HasB, a TonB paralogue encoded by the *has* operon. These two systems enable *S. marcescens* to take up haem under various conditions from different sources, reflecting its capacity to confront conditions encountered in natural biotopes.

Received 7 September 2009

Revised 16 March 2010

Accepted 16 March 2010

INTRODUCTION

Iron is an essential element for most living organisms. For microbes developing in living hosts, and particularly in vertebrates, haem is the most abundant source of iron. Because of its high reactivity, it is usually not free; rather, it is bound both intracellularly to haemoglobin and myoglobin, and extracellularly to haemopexin and albumin (Wandersman & Stojiljkovic, 2000). Bacteria have cell-surface receptors for haem, haemoprotein, or both. In addition, several bacteria secrete haemophores, which are small proteins that scavenge free or protein-bound haem due to their high affinity for haem, and return it to surface receptors (Letoffe *et al.*, 1994), (Ghigo *et al.*, 1997). In Gram-negative bacteria, haem sequentially crosses the two membranes. Both steps are energy-dependent processes. Transport through the outer membrane involves specific surface-exposed receptors powered by the proton-motive force supplied by an inner-membrane machine, the TonB–ExbB–ExbD multiprotein complex (Fischer *et al.*, 1989). Transport through the inner membrane involves periplasmic-binding-protein-dependent ABC transporters energized by ATP hydrolysis (Davidson *et al.*, 2008).

Serratia marcescens, an enterobacterium found in water, on plants and in humans, can use both oxidized and reduced iron, and is able to obtain iron from haem or haemoproteins (Angerer *et al.*, 1992; Wandersman & Delepelaire, 2004).

The genome of *S. marcescens* strain DB11 encodes three potential haem uptake systems (http://www.sanger.ac.uk/Projects/S_marcescens/). The first to be characterized in *S. marcescens* strain SM365, the Has system, enables uptake of haem at 2.5×10^{-8} M due to the synthesis and secretion of a small polypeptide exhibiting high affinity for haem (Letoffe *et al.*, 1994). The second functional haem uptake system, termed Hem based on its sequence similarities to the Hem system from *Yersinia enterocolitica*, functions without haemophores and requires a higher haem concentration for its uptake activity in *S. marcescens* strain SM365 (Benevides-Matos *et al.*, 2008). A third putative haem transporter shares strong identity with putative haem receptors from *Erwinia carotovora* (71 % identical), *Photobacterium luminescens* (72 % identical) and *Bordetella bronchiseptica* (68 % identical). In *S. marcescens* strain SM365, the search for structural genes encoding the third putative haem transporter using primers enabling amplification of these genes in strain DB11 was unsuccessful (N. Benevides-Matos, unpublished results). Inactivation of the Has and Hem systems leads to loss of haem uptake activity in *S. marcescens* strain SM365 (Benevides-Matos *et al.*, 2008). Thus, in *S. marcescens* SM365, two haem uptake systems are functional (Benevides-Matos *et al.*, 2008). Other Gram-negative bacteria, such as *Helicobacter pylori* (Worst *et al.*, 1999), *Neisseria meningitidis* (Perkins-Balding *et al.*, 2003), *Pseudomonas aeruginosa* (Ochsner *et al.*, 2000), *Vibrio cholerae* (Wyckoff *et al.*, 2004), *Yersinia pestis* (Rossi *et al.*, 2001) and *Haemophilus influenzae* (Morton *et al.*, 2004), have multiple haem uptake

Abbreviations: ALA, Δ -aminolaevulinic acid; Dip, 2,2'-dipyridyl.

systems. For some of these bacteria, the apparent redundancy of haem uptake systems has been explained by the fact that the haem sources recognized by the different systems are distinct. In *N. meningitidis*, haem from haemoglobin is transported by HmbR, whereas HpuA/HpuB can obtain haem from the haemoglobin-haptoglobin complex (Perkins-Balding *et al.*, 2004). In *H. influenzae*, uptake of haemoglobin-haptoglobin is mediated by phase-variable Hgb-binding proteins (Cope *et al.*, 2000). In the case of haem-haemopexin, haem transport requires haemophore HxuA and a receptor, HxC, encoded by the *hxCBA* operon (Morton *et al.*, 2007). Hup has been hypothesized to be a high-affinity haemoglobin acquisition system (Morton *et al.*, 2004). For other bacteria such as *Helicobacter pylori* (Worst *et al.*, 1999), specificity of haem uptake systems has not yet been thoroughly investigated.

Previous studies have shown that the Has system in *S. marcescens*, when expressed in *Escherichia coli*, works with a wide variety of haem sources (Ghigo *et al.*, 1997; Letoffe *et al.*, 2004). Expression in *E. coli* of outer-membrane transporters HasR and HemR allows TonB-dependent haem uptake (Ghigo *et al.*, 1997; Benevides-Matos *et al.*, 2008). In the case of the Has system, TonB can be replaced by HasB6, a HasB allele mutated in its transmembrane domain (Paquelin *et al.*, 2001; Benevides-Matos *et al.*, 2008). HasB6 does not complement other TonB functions such as iron-siderophore uptake, $\phi 80$ infection or killing by colicin B (Paquelin *et al.*, 2001). Subsequent experiments monitored with HemR cloned in *E. coli* revealed that this TonB-dependent transport receptor (TBDT) does not function with HasB6, thus demonstrating that HasB displays specific pairing with HasR (Benevides-Matos *et al.*, 2008).

The *in vivo* haem transport efficiencies of HasR and HemR expressed in *E. coli* cannot be distinguished (Benevides-Matos *et al.*, 2008). In contrast, in the presence of its cognate haemophore HasA, the haem uptake activity of HasR expressed in *E. coli* is 25-fold more efficient (Ghigo *et al.*, 1997). This raises the question whether Hem and Has system activities can be distinguished in *S. marcescens*. This question is addressed in the present report.

METHODS

Bacterial strains. Bacterial strains used in this study are listed in Table 1.

Media and growth conditions. Haemin, bovine haemoglobin, 2,2'-dipyridyl (Dip) and Δ -aminolaevulinic acid (ALA) were obtained from Sigma. Haem was dissolved immediately before use in 0.02 M NaOH. Haemoglobin was dissolved in 100 mM NaCl. The haemoglobin concentration was calculated on the basis of the haem monomer. Haemin and haemoglobin solutions were filter-sterilized with 0.45 μ m pore size Millipore filters for bacterial growth experiments. *S. marcescens* strains were grown on LB medium (Miller, 1972) aerobically at 30 °C. *E. coli* strains were grown on LB medium (Miller, 1972) aerobically at 37 °C. When required, ALA was used at a concentration of 50 μ g ml⁻¹. Iron-depleted medium was obtained with the addition of Dip at a 150 μ M or 300 μ M final concentration (LBD). Antibiotics were added to the following final concentrations (μ g ml⁻¹): ampicillin, 1000; chloramphenicol, 200; kanamycin, 100; streptomycin, 100. Solid media contained 1.5 % Difco agar. Soft LB agar medium contained 0.7 % Difco agar.

Haem utilization assays. To test the ability of SMH3 haem auxotroph of *S. marcescens* and isogenic mutants *hasR::Km*, *hemR::Km*, *hasR::Km hemR::Km*, *hasA::Km*, *hasA::Km hemR::Km*, *hasB::Km*, and *hasB::Km hemR::Km* to utilize haem, approximately 100 cells were spread onto LB ALA and LB agar supplemented with 150 μ M or 300 μ M Dip to create iron depletion

Table 1. Strains and plasmids

Strain or plasmid	Genotype	Source or reference
<i>S. marcescens</i> strains		
SM365	wt	Laboratory collection
SMH3	Str ^R , haem auxotroph (block in ALA synthesis)	Rossi <i>et al.</i> (2003)
SMH3 <i>hasR</i>	<i>hasA::aphA</i> (Str ^R Km ^R , Hem ⁻)	Rossi <i>et al.</i> (2003)
SMH3 <i>hasA</i>	<i>hasA::aphA</i> (Str ^R Km ^R , Hem ⁻)	Rossi <i>et al.</i> (2003)
SMH3 <i>hasB</i>	<i>hasB::aphA</i> (Str ^R Km ^R , Hem ⁻)	Rossi <i>et al.</i> (2003)
SMH3 <i>hemR</i>	<i>hemR::cat</i> (Str ^R Cm ^R , Hem ⁻)	Benevides-Matos <i>et al.</i> (2008)
SMH3 <i>hasR hemR</i>	<i>hasR::aphA hemR::cat</i> (Str ^R Km ^R Cm ^R , Hem ⁻)	Benevides-Matos <i>et al.</i> (2008)
SMH3 <i>hasA</i> (pKOBEGA)	λ red functions (Str ^R Km ^R Amp ^R , Hem ⁻)	This study
SMH3 <i>hasB</i> (pKOBEGA)	λ red functions (Str ^R Km ^R , Amp ^R , Hem ⁻)	This study
SMH3 <i>hasA hemR</i>	<i>hasR::aphA hemR::cat</i> (Str ^R Km ^R Cm ^R , Hem ⁻)	This study
SMH3 <i>hasB hemR</i>	<i>hasR::aphA hemR::cat</i> (Str ^R Km ^R Cm ^R , Hem ⁻)	This study
SMH3 <i>hasR-lacZ</i>	Integrated pGPhasR-lacZ (Amp ^R Str ^R , Hem ⁻)	Rossi <i>et al.</i> (2003)
SMH3 <i>hasA hasR-lacZ</i>	<i>hasA::aphA</i> , integrated pGPhasR-lacZ (Str ^R Km ^R Amp ^R , Hem ⁻)	Rossi <i>et al.</i> (2003)
SMH3 <i>hasB hasR-lacZ</i>	<i>hasB::aphA</i> , integrated pGPhasR-lacZ (Str ^R Km ^R Amp ^R , Hem ⁻)	Rossi <i>et al.</i> (2003)
Plasmids		
pKOBEGA	λ red functions, 30 °C Ts (Amp ^R derivative of pKOBEG)	Chaveroche <i>et al.</i> (2000)
pGPhasR-lacZ	<i>hasR-lacZ</i> (Amp ^R)	Rossi <i>et al.</i> (2003)

and haemoglobin at a concentration ranging from 10^{-5} M to 10^{-7} M. Plates were incubated at 30 °C and colony sizes were measured at 48 h using a dissecting microscope fitted with an optical reticule. The diameters of at least 10 isolated colonies were measured and the means, standard deviations and analysis of variance (Statview software) were determined using Microsoft Excel 98.

Growth assays on agar plates supplemented with purified HasA. A 100 µl sample of an overnight culture of the tested strain was mixed with 4 ml soft agar and poured onto LB plates supplemented with Dip (150 or 300 µM), and haemoglobin (10^{-5} , 10^{-6} , 10^{-7} M). Wells (5 mm diameter) were cut in the agar and filled with 100 µl 10^{-7} M sterile apo-HasA prepared from a C600 (pSYC34) culture supernatant as described previously (Cwerman *et al.*, 2006). Growth around the wells was recorded after overnight incubation at 30 °C.

Growth assays on agar plates supplemented with purified haem-loaded haemopexin. A 100 µl sample of an overnight culture of the tested strain was mixed with 4 ml soft agar and poured onto LB plates containing 300 µM Dip (LBD). Wells were cut in the agar and filled with 100 µl sterile haem-loaded haemopexin (2.3×10^{-6} M), or haem (10^{-6} M).

Genetic techniques. Cells were transformed by electroporation as described previously (Dower *et al.*, 1988).

DNA manipulations. Chromosomal DNA was isolated using the Wizard Genomic DNA purification kit (Promega, catalogue number A1120). Large-scale plasmid DNA preparations were performed using the Plasmid Maxi kit (Qiagen) as recommended by the manufacturer. Small-scale plasmid DNA preparations were made using the QIAprep Spin Miniprep kit (Qiagen). Restrictions, modifications and ligations were carried out according to the manufacturers' recommendations. DNA fragments of interest were separated by gel electrophoresis and isolated using the QIAquick gel extraction kit (Qiagen). DNA fragments were amplified in a Hybaid PCR thermocycler using TaKaRa LA *Taq* (TaKaRa).

Deletion of *hemR* in *S. marcescens* by red linear DNA gene inactivation. The 1.9 kb DNA fragment, in which *hemR* was substituted for the *cat* gene, was amplified from SMH3 *hemR::Cmp* strains using the following primers: left 500 bp *hemR* homology arm, *hemR*.500-5: 5'-ACCGCATCGAAAGCTTGCTGAA-ATAAC-3'; right 500 bp *hemR* homology arm, *hemR*.500-3: 5'-ACCAGCGCCATCCAGGCCGGCAG-3'

Briefly, plasmid pKOBEGA (an Amp^R derivative of pKOBEG; see Table 1) was introduced into the target strain and electrocompetent cells were prepared at 30 °C after induction with 0.2 % arabinose of the λ red system carried by pKOBEGA.

The PCR product (10 µg) was introduced into SMH3 *hasA::Km*/pKOBEGA and SMH3 *hasB::Km*/pKOBEGA, respectively, using electroporation, and chloramphenicol-resistant deletion mutants produced by allelic exchange were selected at 37 °C (to eliminate the thermosensitive plasmid pKOBEGA). Correct localization of the chromosomal insertion was checked by PCR amplification using *cat* primers *cat*-5 (5'-AATTTCTGCCATTTCATCCGC-3') and *cat*-3 (5'-TTGATCGGCCGTAAGAGGT-3'), in combination with primers *hemR*.500-5 and *hemR*.500-3, respectively.

Preparation of apo-HasA protein and loading with haem. Apo-HasA protein was prepared as previously described (Cwerman *et al.*, 2006). Haem was initially dissolved in 0.02 M NaOH to a concentration of 10^{-3} M. Solutions were prepared immediately before use. A 50 µl volume of 1.75×10^{-4} M apo-HasA was mixed with 17.5 µl 4×10^{-4} M haem in TN buffer (50 mM Tris/HCl pH 7.5, 100 mM NaCl). The mixture was incubated for 15 min at

room temperature. Haem binding was monitored by UV-visible absorption spectra on a Kontron spectrophotometer. Holo-HasA haem loading was determined on a Soret band (407 nm) (Izadi *et al.*, 1997). Since less than 10 % of free haem was still present in the reaction mixture, purification of the haem-loaded Has was not required.

Protein analysis by electrophoresis and HasR immunodetection. Strains SMH3 *hemR* and SMH3 *hemR hasB* were grown in LB-ALA and LB-ALA Dip (300 µM) media either in the absence or in the presence of holo-HasA (10^{-7} M) at 30 °C. Cells were harvested at OD₆₀₀ 3.0 and 100 µl 100 % trichloroacetic acid was added to 1 ml cell suspension. The mixture was then centrifuged for 10 min at 5000 g at 4 °C. Cell pellets were collected, washed with 1 ml 80 % acetone, dried and suspended in sample buffer for SDS page gels. A 0.2 OD₆₀₀ equivalent was loaded onto 12.5 % SDS-PAGE gels in each lane. Immunodetection with anti-HasR antibodies was performed as described previously (Letoffe *et al.*, 1999).

β -Galactosidase assay. β -Galactosidase was assayed by the method described by Pardee *et al.* (1959).

RESULTS

The Has haem uptake system requires severe iron depletion in order to function

Haem uptake activity of *S. marcescens* strain SMH3, grown in the presence of various concentrations of the iron chelator Dip, was examined. In *S. marcescens* haem auxotroph strain SMH3, and its derivatives used in this study, transported haem was used as a haem source to support growth. The results clearly indicated that strain SMH3 took up haem only in the presence of severe iron limitation due to Dip added at a final concentration of 150 µM and above (data not shown). The effect of HasR and/or HemR disruption on haem uptake ability was thus investigated in strain SMH3 by monitoring growth in the presence of 150–300 µM Dip. Disruption of both the Has and Hem systems completely abolished haem uptake by *S. marcescens* without affecting growth in the presence of the haem biosynthesis precursor ALA, which passes through the outer membrane through porins and is then transported through the inner membrane by a dipeptide permease (Carter *et al.*, 2002) (Table 2). Haem uptake by both the Has and Hem systems was absent for bacteria grown in iron-replete conditions (Table 2). When strain SMH3 was cultured in the presence of 150 µM Dip, it was unable to grow at haemoglobin concentrations lower than 10^{-6} M. When the haemoglobin concentration decreased to 10^{-7} M, addition of Dip at 300 µM was required to obtain growth. Similar results were observed with strain SMH3 *hemR*, in which haem is transported by the Has system. In strain SMH3 *hasR*, in which haem is transported by the Hem system, 10^{-6} M haem was required for growth irrespective of the concentration of Dip added above 150 µM. This implies that the Has system has a higher affinity for available haem, but requires more severe iron depletion for its activity. The question arose whether the level of iron depletion provoked by the addition of Dip at a 300 µM final concentration was required for expression of

Table 2. Haem uptake in *S. marcescens* strain SMH3 and its derivatives impaired in haem uptake systems

The SMH3 haem auxotroph of *S. marcescens* and isogenic mutants *hasR::Km*, *hemR::Km*, *hasR::Km hemR::Km*, *hasA::Km*, *hasA::Km hemR::Km*, *hasB::Km*, and *hasB::Km hemR::Km* were plated on LB ALA and LB agar supplemented with 150 μ M or 300 μ M Dip to create iron depletion, and haemoglobin (Hb) at a concentration ranging from 10^{-5} M to 10^{-7} M. A 6 h culture of the tested strain, performed in LB ALA medium, was first diluted to obtain about 10^3 c.f.u. ml $^{-1}$. One hundred microlitres of the cell suspension was plated on each test plate. Plates were incubated for 48 h at 30 °C. Sizes of 10 isolated colonies were then measured. Data are the mean diameter (mm) of 10 colonies from one representative experiment. Standard deviations were calculated using Statview software. All experiments were repeated three times. –, No growth.

Strain	LB	LB ALA	LB Hb 10^{-5}			LB Hb 10^{-6}		LB Hb 10^{-7}	
			No Dip	Dip 150	Dip 300	Dip 150	Dip 300	Dip 150	Dip 300
SMH3	–	3.7 $\pm$ 0.2	–	2.6 $\pm$ 0.0	1.9 $\pm$ 0.0	1.7 $\pm$ 0.0	1.4 $\pm$ 0.0	–	0.3 $\pm$ 0.0
SMH3 <i>hemR</i>	–	3.3 $\pm$ 0.0	–	1.0 $\pm$ 0.0	1.6 $\pm$ 0.0	0.5 $\pm$ 0.0	1.2 $\pm$ 0.0	–	0.3 $\pm$ 0.0
SMH3 <i>hasR</i>	–	3.2 $\pm$ 0.0	–	2.3 $\pm$ 0.0	2.1 $\pm$ 0.0	0.7 $\pm$ 0.0	0.8 $\pm$ 0.0	–	–
SMH3 <i>hasR hemR</i>	–	3.2 $\pm$ 0.0	–	–	–	–	–	–	–
SMH3 <i>hasA</i>	–	3.4 $\pm$ 0.0	–	2.7 $\pm$ 0.0	2.2 $\pm$ 0.0	0.4 $\pm$ 0.0	1.1 $\pm$ 0.0	–	–
SMH3 <i>hasA hemR</i>	–	3.2 $\pm$ 0.0	–	–	0.8 $\pm$ 0.0	–	–	–	–
SMH3 <i>hasB</i>	–	3.3 $\pm$ 0.0	–	2.5 $\pm$ 0.0	1.6 $\pm$ 0.0	0.3 $\pm$ 0.0	1.0 $\pm$ 0.0	–	–
SMH3 <i>hasB hemR</i>	–	3.2 $\pm$ 0.0	–	–	–	–	–	–	–

the *has* operon. Expression of the *has* operon was shown to be repressed by iron and induced by a TonB-dependent signalling cascade started by binding of the holo-haemophore to its cognate receptor HasR. Thus, the effect of addition of various concentrations of Dip upon *has* operon expression was investigated in strain SMH3 *hasR-lacZ*, in which expression of *lacZ* is under the control of the *has* operon promoter. Strain SMH3 *hasR-lacZ* was grown in the presence of ALA and various concentrations of Dip. In the presence of ALA, *S. marcescens* strain SMH3 *hasR-lacZ* excretes haem, which can be bound by HasA and thus form the holo-haemophore. The holo-haemophore can then bind its cognate receptor, HasR, and in the presence of iron depletion initiate the *has* signalling cascade, thus inducing expression of the *has* operon. Similar levels of expression of the *has* operon were observed for strain SMH3 *hasR-lacZ* grown in the presence of ALA and added haem (data not shown).

When added at concentrations below 150 μ M, Dip had no effect on the expression level of the *has* operon (data not shown). Added at a 150 μ M final concentration, Dip only slowly and slightly induced expression of *hasR-lacZ* fusion (Fig. 1). Addition of a 300 μ M Dip final concentration provoked rapid and sharp induction of *hasR-lacZ* fusion expression (Fig. 1). Iron depletion provoked by addition of Dip at a final concentration of 300 μ M was thus required to enable full expression and activity of the Has system in the presence of haem. The same level of induction was also observed at higher Dip concentrations.

Haem uptake activity of the Has system of *S. marcescens* in the absence of the HasA haemophore

We sought to elucidate how the Has and Hem systems coordinate their activity in *S. marcescens*. To determine

this, the haem uptake capacity of the Hem and Has systems in the absence of haemophore HasA was compared. For this purpose, *S. marcescens* strain SMH3 and its *hasA* and *hasA hemR* derivatives were tested for growth in the presence of exogenous haem at various concentrations in the presence of 150 μ M or 300 μ M Dip. When disrupted for both *hemR* and *hasA*, *S. marcescens* was unable to take up haem provided at 10^{-6} M whatever the concentration of added Dip (Table 2).

In the *hasA hemR* mutant, very weak growth was observed only in the presence of 10^{-5} M haem when Dip was added at a 300 μ M final concentration. The addition of exogenous haemophore restored full haem uptake activity of the Has system (Table 3). This clearly demonstrated that

Fig. 1. Effect of Dip addition on expression level of *S. marcescens* *has* operon. An overnight culture of strain SMH3 *hasR-lacZ* was diluted, in LB ALA, to OD₆₀₀ 0.05 and grown at 30 °C. After 2 h, the culture was dispensed into flasks and grown in the presence of various concentrations of Dip (●, no addition; ■, 150 μ M Dip; ▲, 300 μ M Dip). β -Galactosidase activity was measured every 15 min for 2 h. Cultures, sample processing and β -galactosidase assays were performed as described in Methods. β -Galactosidase activities reported are means of at least three experiments; error bars represent one standard deviation.

Table 3. Haemophore-dependent haem uptake assay of *S. marcescens* strains SMH3, SMH3 *hasA*, SMH3 *hasA hemR*, SMH3 *hasB* and SMH3 *hasB hemR*

A 100 µl sample of an overnight culture of the tested strain was mixed with 4 ml soft agar and poured onto LB plates supplemented with Dip (150 or 300 µM), and with various concentrations of haemoglobin (Hb, 10^{-7} – 10^{-5} M). Wells (5 mm in diameter) were cut in the agar and filled with 100 µl sterile apo HasA (10^{-7} M). Growth around the wells was recorded after overnight incubation at 30 °C. + + *, Thick growth on the whole plate; + +, thick growth radius of 10 mm around the well; +, weak growth radius of 3 mm around the well. All experiments were repeated three times. C, Control; H, 10^{-7} M haemophore added.

		SMH3 <i>hasA</i>	SMH3 <i>hasA hemR</i>	SMH3 <i>hasB</i>	SMH3 <i>hasB hemR</i>
LB	C	–	–	–	–
Hb 10^{-5} Dip ₁₅₀	C	+ + *	–	+ + *	–
	H	+ + *	–	+ + *	–
Hb 10^{-5} Dip ₃₀₀	C	+ + *	–	+ + *	–
	H	+ + *	+ +	+ + *	+ +
Hb 10^{-6} Dip ₁₅₀	C	+ + *	–	+ + *	–
	H	+ + *	–	+ + *	–
Hb 10^{-6} Dip ₃₀₀	C	+ + *	–	+ + *	–
	H	+ + *	+ +	+ + *	+ +
Hb 10^{-7} Dip ₁₅₀	C	–	–	–	–
	H	–	–	–	–
Hb 10^{-7} Dip ₃₀₀	C	–	–	–	–
	H	+ +	+ +	+	+

the haem uptake activity of the Has system in *S. marcescens* required the presence of the HasA haemophore when haem was present at concentrations lower than 10^{-5} M.

Haem uptake activity of the Has system of *S. marcescens* requires a functional HasB

The TonB paralogue HasB in its mutated HasB6 form is a substitute only for TonB with HasR expressed in *E. coli*. The question arises as to the role of HasB in *S. marcescens* with respect to Has-dependent haem uptake. The putative role of HasB was thus examined in strain SMH3 *hemR*, devoid of a functional Hem uptake system. We sought to determine whether the haem uptake capacity of the Has system in the absence of HasB was affected. In a *hasB hemR* mutant, haem transport was abolished in the presence of both 150 µM and 300 µM Dip whatever the concentration of haemoglobin (Table 2). This demonstrated that HasB was required for haem transport by the Has system regardless of the concentration of haemoglobin. Previous experiments had failed to demonstrate such a role for HasB, mainly due to the fact that they were performed with a strain expressing HemR (Rossi *et al.*, 2003).

Effect of *hasA* and *hasB* disruption on *has* operon expression

The absence of haem transport activity in a *hasB hemR* mutant may have been related to the loss of Has system activity and/or a decrease in *has* operon expression. This decrease in *has* operon expression leads to a decrease in the amount of HasA required for activity of the Has system and induction of the *has* operon. To investigate these two

hypotheses, the effect of HasA addition on haem transport ability in strains SMH3 *hasB* and SMH3 *hasB hemR* mutants was tested. As shown in Table 3, addition of HasA restored haem uptake activity of the Has system only in the presence of 300 µM Dip. Restoration of uptake activity of the Has system in the *hasB hemR* mutant by addition of HasA suggested that exogenously added HasA initiated the *has* signalling cascade, thus enabling expression of *has*-operon-encoded components of the Has system, and consequently haem uptake activity of the Has system (Rossi *et al.*, 2003). To test this hypothesis, the effect of *hasB* disruption upon the amount of HasR was examined using Western blot analysis. Strains SMH3 *hemR* and SMH3 *hasB hemR* were grown in LB-ALA medium either without or with 300 µM Dip to create iron depletion, and in the absence or presence of added HasA. The results presented in Fig. 2 show that, in strain SMH3 *hemR*, the level of HasR increased similarly for bacteria grown under iron-depleted conditions both in the absence and in the presence of exogenous HasA. In contrast, in the SMH3 *hasB hemR* strain, the level of HasR was not increased by the addition of Dip alone but required the addition of both Dip and haemophore HasA (Fig. 2). Thus, phenotypes related to disruption of HasB could be the consequence of a decrease in *has* operon expression. The effect of *hasB* disruption on the *has* operon expression level was examined using strain SMH3 *hasR-lacZ*, containing a fusion between the promoter of the *has* operon and *lacZ*. Strain SMH3 *hasR-lacZ* and its *hasB* derivative were grown in LB-ALA medium in the absence or in the presence of 300 µM Dip, with or without an added haemophore. As seen in Table 4, the basal expression level of *hasR-lacZ* fusion was similar in the two strains tested. In the presence

Fig. 2. Immunodetection of HasR in strains SMH3 *hemR* and SMH3 *hemR hasB*. Strains were grown in LB ALA, or LB ALA supplemented with Dip (300 μ M) in the absence or presence of holo-HasA (10^{-7} M). Cells were harvested during the stationary phase (OD₆₀₀ 3). Sample treatment, electrophoresis and detection with anti-HasR antibodies (final dilution 1 : 5000) were performed as described in Methods. M.M., size standard.

of 300 μ M Dip, the expression level of *hasR-lacZ* fusion increased sharply. For strains SMH3 *hasR-lacZ hasA*, and SMH3 *hasR-lacZ hasB* grown in LB-ALA, addition of Dip up to 300 μ M did not increase the *hasR-lacZ* fusion expression level. In the presence of 300 μ M Dip and added haemophore, the expression level of *hasR-lacZ* fusion increased strongly in strain SMH3 *hasR-lacZ hasA*, but only slightly in strain SMH3 *hasR lacZ-hasB*. The latter results are consistent with those obtained by Western blot analysis. They demonstrate that the effect of *hasB* disruption on haem uptake activity was related to weak expression of the *has* operon, which cannot provide enough haemophore to initiate its signalling cascade and consequently to increase Has system expression and activity. Moreover, the kinetics of induction of *hasR-lacZ* fusion provoked by Dip addition (300 μ M) in strains SMH3 *hasR-lacZ* and SMH3 *hasR-lacZ hasB* also clearly demonstrated the importance of HasB for sharp induction of the *has* operon in the presence of haem and iron depletion (Fig. 3).

Has-dependent use of haem-loaded haemopexin as a haem source by *S. marcescens* strains SMH3

Haemopexin, by binding haem, acts as an extracellular antioxidant preventing the toxic effects of haem during tissue damage or intravascular haemolysis. Haem-haemopexin is also a source of haem for *H. influenzae* which has the operon *hxcCBA*, in which HxcC is an outer-membrane receptor of haem (Morton *et al.*, 2004). When expressed in *E. coli*, the complete Has system enables haem uptake from haem-loaded haemopexin, (*S. Ltoff*, Institut Pasteur, personal communication). *S. marcescens* strain SMH3 and its *hasR* and *hemR* derivatives were investigated for their ability to use haem-loaded haemopexin as haem source. Strains SMH3, SMH3 *hasR* and SMH3 *hemR* were tested for growth, as described in Methods, in the presence of haem-loaded haemopexin (2.3×10^{-6} M), or haem (10^{-6} M). The results show that inactivation of *hasR* completely abolished haem uptake activity when haem-loaded haemopexin was used as a haem source (Fig. 4). The use of haem-loaded haemopexin as a haem source was not affected by *hemR* disruption (Fig. 4). This result demonstrates that, in *S. marcescens*, only the *has* system allowed haem uptake from haem-loaded haemopexin.

DISCUSSION

The results presented in this study clearly suggest that the Has and Hem systems do not function in a redundant manner in *S. marcescens*. Indeed, while neither haem transport system works in iron-rich medium, they respond differently to iron chelation. Hem system uptake activity is constant and independent of the amount of Dip added above 150 μ M. In contrast, the complete Has system requires a higher amount of Dip and has higher affinity for haem.

Haem uptake by *S. marcescens* can thus be summarized as follows. In the presence of iron depletion due to the addition of 150 μ M Dip, haem is transported only by the Hem system.

Table 4. Role of HasB in induction of the *has* operon by haem-loaded haemophore

Overnight cultures of strains SMH3 *hasR-lacZ*, SMH3 *hasA hasR-lacZ*, *hasB* and SMH3 *hasB hasR-lacZ* were diluted in LB ALA medium to OD₆₀₀ 0.05 and grown at 30 $^{\circ}$ C. After 2 h, each culture was dispensed into flasks in which Dip (300 μ M) and HasA haemophore (10^{-7} M) were added when required and grown at 30 $^{\circ}$ C. After 2 h, β -galactosidase activity was assayed. Cultures, sample processing and β -galactosidase assays were performed as described in Methods. β -Galactosidase activities reported are means of at least three experiments; the error range represents one standard deviation.

Strain	β -Galactosidase specific activity [nmol substrate converted min ⁻¹ (mg dry weight bacteria) ⁻¹]		
	LB ALA	LB ALA Dip 300 μ M	LB ALA Dip 300 μ M + HasA 10^{-7} M
SMH3 <i>hasR-lacZ</i>	30 $\pm$ 2	2200 $\pm$ 110	2500 $\pm$ 125
SMH3 <i>hasA hasR-lacZ</i>	35 $\pm$ 2	150 $\pm$ 6	2000 $\pm$ 15
SMH3 <i>hasB hasR-lacZ</i>	35 $\pm$ 2	120 $\pm$ 6	400 $\pm$ 20

Fig. 3. Effect of *hasB* disruption on induction of the *has* operon. Overnight cultures of strains SMH3 *hasR-lacZ* and SMH3 *hasB hasR-lacZ* were diluted, in LB ALA, to OD₆₀₀ 0.05, and grown at 30 °C. After 2 h, the culture was dispensed into flasks and grown in LB ALA medium in the presence of 300 μM Dip. ■, SMH3 *hasR-lacZ*; ▲, SMH3 *hasB hasR-lacZ*. β-Galactosidase activity was measured every 30 min for 2 h. Cultures, sample processing and β-galactosidase assays were performed as described in Methods. β-Galactosidase activities reported are means of at least three experiments; error bars represent one standard deviation.

Functional redundancy of Hem and Has systems is prevented, since the iron depletion level due to addition of 150 μM Dip does not permit induction of *has* operon expression. Under these growth conditions, the basal level of HasA does not interfere with haem uptake activity of the Hem system. This conclusion is in agreement with the repression of the *has* operon expression observed in the presence of 200 μM Dip and haemoglobin added at

10⁻⁶ M (Rossi *et al.*, 2003). This repression was suggested to be related to the transport activity of the Hem system, thus providing enough iron to promote Fur-dependent repression of the *has* operon without inhibiting its own activity. Repression of the Has system under conditions permitting activity of the Hem system might be related to the fact that Fur-dependent regulation of the Has system involves two Fur boxes, respectively located upstream from the *has* operon and upstream from *hasI*, the gene encoding the sigma factor required for induction of the *has* operon (Rossi *et al.*, 2003). In the presence of higher concentrations of Dip causing stronger iron depletion and low haem concentrations not compatible with the activity of the Hem system, the *has* signalling cascade can be induced by a haem-loaded haemophore, resulting in sharp induction of the *has* operon and uptake of haem by the Has system. In spite of expression of both systems, functional redundancy is prevented, since the Hem system is not able to take up haem at this low level.

Analysis of the *hasA hemR* mutant reveals that haem uptake activity of the Has system in the absence of its haemophore is very low, even in the presence of high haem and Dip concentrations. Full haem uptake activity is restored in the *hasA hemR* mutant by the addition of an exogenous haemophore. In the case of *hasB hemR* mutants, which produce HasA, haem uptake activity of the Has system is nearly abolished whatever the amount of Dip added, even in the presence of high haem concentrations. For this mutant, addition of the haemophore restores weak haem uptake only in the presence of a high Dip concentration. In the case of the *hasB* mutant, the level of HasA synthesized by the strain is very low (data not shown) and does not permit induction of the *has* operon and consequent uptake of haem by the Has system. Only the addition of HasA in the medium can enable induction of the *has* operon to a level sufficient for weak haem uptake. Western blot experiments demonstrate that the effect of *hasB* disruption on haem uptake by the Has system in *S. marcescens* is related to a strong decrease in the HasR level. Quantitative data obtained with assays of β-galactosidase activity in strains SHM3 *hasR-lacZ* and SMH3 *hasR-lacZ hasB* confirm this conclusion. These data indicate the importance of HasB for induction of the *has* operon and clearly demonstrate that TonB cannot substitute for HasB in induction, nor for efficient haem uptake by the Has system. The structural gene for HasB is contained in the *has* operon, which is only expressed in the presence of severe iron depletion. Thus, two components (HasR and HasB) needed for the first step in Has signalling cascade initialization require strong iron depletion for expression. The requirement of HasB for initialization of the Has signalling cascade is a complementary lock for induction and haemophore-dependent activity when iron depletion is not sufficient. In *S. marcescens*, only HasB can induce conformational changes in HasR required for initiation of the signalling cascade responsible for induction of the *has* operon, thus distinguishing between the

Fig. 4. Haemopexin and haem utilization by *S. marcescens* strains SMH3, SMH3 *hasR* and SMH3 *hemR*. A 100 μl sample of an overnight culture of the tested strain was mixed with 4 ml soft agar and poured onto LB plates containing 300 μM Dip (LBD). Wells (5 mm diameter) were cut in the agar and filled with 100 μl sterile haemopexin (2.3×10⁻⁶ M) or/and haem (10⁻⁶ M). Growth around the wells was recorded after overnight culture at 30 °C. All experiments were repeated three times. The plates were photographed after 36 h incubation at 30 °C.

requirement for haem uptake and signal transduction. This may be related to the strong affinity of the C-terminus of HasB for HasR, which is more than 50 times higher than the affinity of the C-terminus of TonB_{E.c} for HasR (Lefevre *et al.*, 2008). This higher affinity may be related to the fact that, along with the TonB box, other regions of HasR are involved in the interaction with HasB (Lefevre *et al.*, 2008). These regions could be specifically involved in the signal transduction process.

Finally, the haem sources used by the Hem and Has systems are not strictly identical. Investigation of haem uptake by *S. marcescens* strain SMH3 and its *hasR* and *hemR* mutants using haem-loaded haemopexin as a haem source clearly demonstrated that only the *has* system can take up haem from this haemoprotein. *S. marcescens* can colonize various biotopes, such as water, earth, plants, insect gut and rodents and may be responsible for urinary infections and septicaemia in humans. The levels of haem and iron encountered inside vertebrates are very low. Only 0.1 % of total iron is extracellular and bound to proteins such as transferrin and lactoferrin. In addition, most extra-erythrocytic haem is bound to proteins such as haemopexin and haemoglobin-haptoglobin. Haemophore-dependent Has system activity could therefore be required when bacteria invade vertebrates. In other biotopes, when only iron is limiting, *S. marcescens* can take up haem using the Hem system.

ACKNOWLEDGEMENTS

Financial support was provided by the Institut Pasteur, the Centre National de la Recherche Scientifique (URA2172) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. We thank Ann Smith and Clémence Fournier for the generous gift of haem-loaded haemopexin. We thank Cécile Wandersman for conceptual discussions and comments when writing the manuscript.

REFERENCES

- Angerer, A., Klupp, B. & Braun, V. (1992). Iron transport systems of *Serratia marcescens*. *J Bacteriol* **174**, 1378–1387.
- Benevides-Matos, N., Wandersman, C. & Biville, F. (2008). HasB, the *Serratia marcescens* TonB paralog, is specific to HasR. *J Bacteriol* **190**, 21–27.
- Carter, R. A., Yeoman, K. H., Klein, A., Hosie, A. H., Sawers, G., Poole, P. S. & Johnston, A. W. (2002). *dpp* genes of *Rhizobium leguminosarum* specify uptake of δ -aminolevulinic acid. *Mol Plant Microbe Interact* **15**, 69–74.
- Chaverroche, M. K., Ghigo, J. M. & d'Enfert, C. (2000). A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res* **28**, E97.
- Cope, L. D., Hrkál, Z. & Hansen, E. J. (2000). Detection of phase variation in expression of proteins involved in haemoglobin and haemoglobin-haptoglobin binding by nontypeable *Haemophilus influenzae*. *Infect Immun* **68**, 4092–4101.
- Cwerman, H., Wandersman, C. & Biville, F. (2006). Heme and a five-amino-acid hemophore region form the bipartite stimulus triggering the *has* signaling cascade. *J Bacteriol* **188**, 3357–3364.
- Davidson, A. L., Dassa, E., Orelle, C. & Chen, J. (2008). Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev* **72**, 317–364.
- Dower, W. J., Miller, J. F. & Radgale, C. W. (1988). High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res* **16**, 6127–6145.
- Fischer, E., Gunter, K. & Braun, V. (1989). Involvement of ExbB and TonB in transport across the outer membrane of *Escherichia coli*: phenotypic complementation of *exb* mutants by overexpressed *tonB* and physical stabilization of TonB by ExbB. *J Bacteriol* **171**, 5127–5134.
- Ghigo, J. M., Letoffe, S. & Wandersman, C. (1997). A new type of hemophore-dependent heme acquisition system of *Serratia marcescens* reconstituted in *Escherichia coli*. *J Bacteriol* **179**, 3572–3579.
- Izadi, N., Henry, Y., Haladjian, J., Goldberg, M. E., Wandersman, C., Delepierre, M. & Lecroisey, A. (1997). Purification and characterization of an extracellular haem-binding protein, HasA, involved in haem iron acquisition. *Biochemistry* **36**, 7050–7057.
- Lefevre, J., Delepelaire, P., Delepierre, M. & Izadi-Pruneyre, N. (2008). Modulation by substrates of the interaction between the HasR outer membrane receptor and its specific TonB-like protein, HasB. *J Mol Biol* **378**, 840–851.
- Letoffe, S., Ghigo, J. M. & Wandersman, C. (1994). Iron acquisition from haem and haemoglobin by a *Serratia marcescens* extracellular protein. *Proc Natl Acad Sci U S A* **91**, 9876–9880.
- Letoffe, S., Nato, F., Goldberg, M. E. & Wandersman, C. (1999). Interactions of HasA, a bacterial haemophore, with haemoglobin and with its outer membrane receptor HasR. *Mol Microbiol* **33**, 546–555.
- Letoffe, S., Delepelaire, P. & Wandersman, C. (2004). Free and hemophore-bound heme acquisitions through the outer membrane receptor HasR have different requirements for the TonB–ExbB–ExbD complex. *J Bacteriol* **186**, 4067–4074.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Morton, D. J., Smith, A., Ren, Z., Madore, L. L., VanWagoner, T. M., Seale, T. W., Whitby, P. W. & Stull, T. L. (2004). Identification of a haem-utilization protein (Hup) in *Haemophilus influenzae*. *Microbiology* **150**, 3923–3933.
- Morton, D. J., Seale, T. W., Madore, L. L., VanWagoner, T. M., Whitby, P. W. & Stull, T. L. (2007). The haem–haemopexin utilization gene cluster (*hxCBA*) as a virulence factor of *Haemophilus influenzae*. *Microbiology* **153**, 215–224.
- Ochsner, U. A., Johnson, Z. & Vasil, M. L. (2000). Genetics and regulation of two distinct haem-uptake systems, *phu* and *has*, in *Pseudomonas aeruginosa*. *Microbiology* **146**, 185–198.
- Paquelin, A., Ghigo, J. M., Bertin, S. & Wandersman, C. (2001). Characterization of HasB, a *Serratia marcescens* TonB-like protein specifically involved in the haemophore-dependent haem acquisition system. *Mol Microbiol* **42**, 995–1005.
- Pardee, A. B., Jacob, F. & Monod, J. (1959). The genetic control and cytoplasmic expression of inducibility in the synthesis of β -galactosidase of *Escherichia coli*. *J Mol Biol* **1**, 165–178.
- Perkins-Balding, D., Baer, M. T. & Stojiljkovic, I. (2003). Identification of functionally important regions of a haemoglobin receptor from *Neisseria meningitidis*. *Microbiology* **149**, 3423–3435.
- Perkins-Balding, D., Ratliff-Griffin, M. & Stojiljkovic, I. (2004). Iron transport systems in *Neisseria meningitidis*. *Microbiol Mol Biol Rev* **68**, 154–171.

Rossi, M. S., Fetherston, J. D., Letoffe, S., Carniel, E., Perry, R. D. & Ghigo, J. M. (2001). Identification and characterization of the hemophore-dependent heme acquisition system of *Yersinia pestis*. *Infect Immun* **69**, 6707–6717.

Rossi, M. S., Paquelin, A., Ghigo, J. M. & Wandersman, C. (2003). Haemophore-mediated signal transduction across the bacterial cell envelope in *Serratia marcescens*: the inducer and the transported substrate are different molecules. *Mol Microbiol* **48**, 1467–1480.

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.

Worst, D. J., Maaskant, J., Vandenbroucke-Grauls, C. M. & Kusters, J. G. (1999). Multiple haem-utilization loci in *Helicobacter pylori*. *Microbiology* **145**, 681–688.

Wyckoff, E. E., Schmitt, M., Wilks, A. & Payne, S. M. (2004). HutZ is required for efficient haem utilization in *Vibrio cholerae*. *J Bacteriol* **186**, 4142–4151.

Edited by: J. Cavet