Genetics and regulation of two distinct haem-uptake systems, *phu* and *has*, in *Pseudomonas aeruginosa*

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A gene cluster similar to haem iron uptake loci of bacterial pathogens was identified in *Pseudomonas aeruginosa*. This *phu* locus ('*Pseudomonas* haem uptake') consisted of the *phuR* receptor gene and the *phuSTUVW* operon encoding a typical ABC transporter. Expression of *phuR* and *phuSTUVW* from mapped transcriptional-start sites occurred under iron-restricted growth conditions and was directly controlled by the Fur protein. Binding of Fur was demonstrated by DNase footprinting of two adjacent 'Fur boxes' that overlapped both the *phuR* and *phuSTUVW* promoters. Two tandem repeats of 154 bp were identified downstream of the *phuSTUVW* operon, each of which contained a strong Fur-dependent promoter driving expression of iron-regulated RNAs antisense to *phuSTUVW*. Mutant strains with deletions in *phuR* and *phuSTUV* showed greatly reduced growth with either haem or haemoglobin as the only iron source: the defects were complemented by plasmids harbouring the *phuR* or the *phuSTUV* genes, respectively. Deletions of *phuW* or of the tandem repeats had only minor effects on haem utilization. The remaining haem and haemoglobin uptake still observed in the Δ*phuR* or Δ*phuSTUV* deletion mutants was due to a second haem-acquisition system, *has*, which was also under the direct control of Fur. This second haem-receptor gene, *hasR*, was identified upstream of and in an operon with *hasA*, encoding a haem-binding extracellular protein. A Δ*hasR* mutant also exhibited decreased utilization of haem and haemoglobin, and a Δ*phuR* Δ*hasR* double mutant was virtually unable to take up either compound. Both the PhuR and HasR proteins were detected in the outer-membrane fraction of *P. aeruginosa* grown in low-iron media. Taken together, the evidence suggests that the *phu* and *has* loci encode two distinct systems required for the acquisition of haem and haemoglobin in *P. aeruginosa*.

Keywords: *Pseudomonas*, iron transport, haem receptor, ferric uptake regulator

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen and has the potential to severely infect injured or immunocompromised hosts such as patients with cystic fibrosis, emphysema, cancer or serious burns (Woods & Vasil, 1994). A large variety of potential virulence factors play a role in colonization, survival, tissue invasion and in the ultimate damage of the host (Nicas & Iglewski, 1985). *P. aeruginosa* prefers an aerobic metabolism, requiring respiratory enzymes that need iron for their function. Extremely low concentrations of free iron are encountered during growth in a human host because virtually all host iron is bound to haemoglobin, transferrin and lactoferrin (Crosa, 1997; Payne, 1993; Litwin & Calderwood, 1993). Iron restriction plays a central role in the stress response in *P. aeruginosa*, which has thus evolved numerous iron-acquisition systems. These include the release of siderophores, production of extracellular proteases, secretion of cytotoxic exotoxin

A, and, as described in this report, the capacity to utilize haem and haemoglobin from exogenous sources. Typically, the factors relevant for these iron-uptake mechanisms are tightly regulated and only expressed during iron-limited growth. The 'ferric uptake regulator' protein (Fur) plays the central role in this iron-starvation response. Fur is a dimeric DNA-binding repressor which employs Fe(II) as a co-repressor and binds to a so-called 'Fur box' in the promoter regions of iron-regulated genes (Ochsner et al., 1995; Prince et al., 1993). Upon iron limitation, the Fur–Fe(II) complex dissociates and is displaced from the DNA, thereby allowing transcription of iron-regulated genes. A large number of Fur-regulated genes have been identified in P. aeruginosa and their role in iron acquisition has been characterized (Ochsner & Vasil, 1996). In this report, we present the isolation and characterization of the phu ('Pseudomonas haem uptake') gene cluster encoding an outer-membrane receptor and a specific ABC (ATP-binding cassette) transporter required for haem and haemoglobin uptake. The ability to use free haem or haem complexed to haemoglobin is characteristic for a growing number of pathogens, including Vibrio cholerae (Henderson & Payne, 1994), Shigella dysenteriae (Mills & Payne, 1997), enterohaemorrhagic Escherichia coli O157:H7 (Torres & Payne, 1997), Yersinia enterocolitica (Stojiljkovic & Hantke, 1992), Yersinia pestis (Hornung et al., 1996), Neisseria spp. (Stojiljkovic et al., 1996), Haemophilus influenzae (Cope et al., 1995; Maciver et al., 1996; Hanson et al., 1992) and Serratia marcescens (Ghigo et al., 1997).

These known haem-acquisition systems can be separated into three categories, based on the factors they involve. The largest category, into which the P. aeruginosa phu system falls, comprises uptake systems similar to those for the uptake of ferrisiderophores, colicins and vitamin B12 (Braun & Hantke, 1991). They involve a specific TonB-dependent receptor at the cell surface and a periplasmic binding-protein-dependent transport machinery (PBT) required for the passage through the cytoplasmic membrane. The PBT system belongs to the larger family of so-called ABC transporters and typically consists of a periplasmic substrate-binding protein, one or two hydrophobic integral membrane-spanning proteins, and one or two hydrophilic proteins with ATPase activity (Ames et al., 1990; Higgins, 1990; Linton & Higgins, 1998). The best studied haem-uptake systems in this category include the hemR–hemSTUV system of Y. enterocolitica (Stojiljkovic & Hantke, 1992) and the hmuRSTUV system of Y. pestis (Hornung et al., 1996).

The haem-uptake systems in the second category consist of an outer-membrane receptor, an extracellular haem-binding protein and a type I secretion apparatus, referred to as an ABC export system. The ABC protein-mediated exporters are an inner-membrane ATPase, an inner-membrane fusion protein, and an outer-membrane component (Pugsley, 1993; Linton & Higgins, 1998). Such a haem-acquisition system has been demonstrated for Ser. marcescens (Binet & Wandersman, 1996; Ghigo

et al., 1997) and was also postulated for P. aeruginosa after the isolation and characterization of an extra-cellular haem-binding protein, HasA, from this organism (Létoffé et al., 1998). HasA is an iron-regulated protein required for the utilization of haemoglobin in P. aeruginosa. In this paper, we provide further evidence for such a system in P. aeruginosa through the genetic and biochemical characterization of the corresponding outer-membrane receptor, HasR.

The third category of haem-uptake systems involves a haem-binding outer-membrane lipoprotein. The best studied species containing such a lipoprotein are H. influenzae type b (Hanson et al., 1992) and H. influenzae Rd (Reidl & Mekalanos, 1996).

Haem from intracellular host haemoglobin becomes available as an iron source after lysis of erythrocytes, and it is thus not surprising that several haem-utilizing pathogens have been shown to produce efficient haemolysins. Well-studied examples include V. cholerae El-Tor, which produces a cytotoxic haemolysin (HlyA) under iron deprivation (Stoebner & Payne, 1988; Menzl et al., 1996). Similarly, iron starvation triggers the secretion of ShlA haemolysin in Ser. marcescens (Poole & Braun, 1988). Besides lysing erythrocytes, purified ShlA haemolysin was also shown to cause lysis of human epithelial cells at nanomolar concentrations (Hertle et al., 1999). P. aeruginosa secretes several factors that have the potential to facilitate the acquisition of haem from exogenous sources. A heat-labile haemolysin, phospholipase C, was purified from P. aeruginosa culture supernatants (Berka & Vasil, 1982) and the corresponding gene, plcH, was subsequently cloned (Pritchard & Vasil, 1986). In addition, P. aeruginosa secretes a heat-stable glycolipid haemolysin composed of rhamnose and β-hydroxydecanoate; the genes required for the production of these rhamnolipids are regulated by quorum sensing and iron starvation (Ochsner et al., 1994; Ochsner & Reiser, 1995). Furthermore, iron depletion activates the production of exotoxin A, which has a broad cytotoxic activity toward eukaryotic cells (Vasil et al., 1977). Lysis of host cells makes wide sources of haem and free iron accessible for P. aeruginosa, especially in concert with extracellular proteases that degrade the host haem-containing proteins and siderophores that immediately scavenge free iron (Wolz et al., 1994).

METHODS

Strains, media and growth conditions. The strains and plasmids used in this study are shown in Table 1. Luria broth was used for strain maintenance. The low-iron medium was tryptic soy broth treated with Chelex 100 Resin (Bio-Rad), dialysed, and supplemented with 1% (w/v) glycerol and 50 mM glutamate; 50 µg $FeCl_3$ ml^{-1} was also added for high-iron medium (Prince et al., 1993). For growth-stimulation experiments, P. aeruginosa wild-type and mutant strains were grown for 8 h at 37 °C in Chelex-treated M9 medium containing 0·2% glucose and 0·1 mM dipyridyl (Sigma) as a non-metabolizable iron chelator. The cultures were diluted to 10^4–10^5 cells ml^{-1}, and 100 µl of the diluted cells were mixed with 3 ml Chelex-treated M9 medium, 0·2 % glucose, 0·8 %

Table 1. Strains and plasmids

Gm^R^, gentamicin resistance; Tc^R^, tetracycline resistance; Amp^R^, ampicillin resistance; Km^R^, kanamycin resistance; Cm^R^, chloramphenicol resistance.

Strain/plasmid	Relevant characteristics	Source/reference
P. aeruginosa strains		
PAO1 (ATCC 15692)	Prototroph	Holloway *et al.* (1979)
Δ*phuR*	PAO1 with 0·4 kb of *phuR* replaced by Gm^R^ cassette	This study
Δ*phuSTUV*	PAO1 with 2·4 kb of the *phuSTUVW* operon replaced by Gm^R^ cassette	This study
Δ*phuW*	PAO1 with a 0·2 kb of *phuW* replaced by Gm^R^ cassette	This study
Δ*phu*-R1R2	PAO1 with 0·3 kb of R1/R2 replaced by Gm^R^ cassette	This study
Δ*hasR*	PAO1 with 1·5 kb of *hasR* replaced by Tc^R^ cassette	This study
Δ*phuR* Δ*hasR*	PAO1 with 0·4 kb of *phuR* replaced by Gm^R^ cassette and 1·5 kb of *hasR* replaced by Tc^R^ cassette	This study
E. coli strains		
DH5α	*hsdR recA lacZYA* φ80 *lacZ*ΔM15	BRL
HB101	*hsdS recA proA lacY*	Boyer & Roulland-Dussoix (1969)
BL21(DE3)	High-stringency T7 expression host, *hsdS* DE3	Novagen
Plasmids		
pBluescript SK/KS	Amp^R^, lacZ′, T7; cloning vector	Stratagene
pCRII-2.1	Amp^R^ Km^R^; PCR cloning vector	Invitrogen
pETW999	pET23a containing *phuW* under P_{T7} control	This work
pHAS1	Translational *hasR*∷*lacZ* fusion	This work
pHAS2	Translational *hasA*∷*lacZ* fusion	This work
pHAS3	Translational *hasR–hasA*∷*lacZ* fusion	This work
pHAS3051	pUCP19 containing *hasR* as a 3·05 kb PCR fragment under P_{lac} control	This work
pPHU1	pUCP19 containing *phuR* as a 6 kb *Eco*RV fragment under P_{lac} control	This work
pPHU2	pUCP19 containing *phuSTUV* as a 5·5 kb *Sph*I fragment under P_{lac} control	This work
pPHU3	pUCP19 containing *phuW* as a 1·3 kb *Stu*I fragment under P_{lac} control	This work
pPHU359−	pBluescript SK containing a 359 bp *Xho*I–*Stu*I *phuV* fragment in the *Xho*I/*Eco*RV sites	This work
pPHU359+	pBluescript KS containing a 359 bp *Xho*I–*Stu*I *phuV* fragment in the *Xho*I/*Eco*RV sites	This work
pPHU-R1	pCRII-2.1 containing a 346 bp R1 fragment	This work
pPHU699-R	pBluescript SK+ containing a 699 bp *Hinc*II–*Eco*RV fragment of *phuR*/*phuS* in anti-*phuR* orientation relative to the T7 promoter	This work
pPHU699-S	pBluescript SK+ containing a 699 bp *Hinc*II–*Eco*RV fragment of *phuR*/*phuS* in anti-*phuS* orientation relative to the T7 promoter	This work
pPZ20	Amp^R^, ′*lacZ*-based promoter probe vector	Schweizer (1991a)
pRK2013	Km^R^ conjugation helper plasmid	Figurski & Helinski (1979)
pSUP203	Amp^R^ Tc^R^ Cm^R^ mob, suicide vector	Simon *et al.* (1983)
pUCP19	Amp^R^ P_{lacZ}, broad-host-range cloning and expression vector	Schweizer (1991b)

low-melting-point agarose (Sigma) at 37 °C and poured onto Chelex-treated M9 medium, 0·2% glucose, 1·5% agarose plates containing 100 μg EDDHA [ethylenediamine di(*o*-hydroxyphenylacetic acid)] ml^−1^ (Sigma) as an iron chelator.

Sterile filter disks were placed on top of the plates and spotted with 15 μl 5 mg ml^−1^ solutions of bovine haemin chloride in 10 mM NaOH, bovine haemoglobin in PBS, bovine lactoferrin in PBS or holo-transferrin in PBS; all these compounds were

from Sigma. Antibiotics were used at the following concentrations: for *E. coli*, 100 µg ampicillin ml^{-1}, 15 µg gentamicin ml^{-1}, 100 µg kanamycin ml^{-1} and 15 µg tetracycline ml^{-1} were used; for *P. aeruginosa*, 750 µg carbenicillin ml^{-1}, 75 µg gentamicin ml^{-1} and 150 µg tetracycline ml^{-1} were used.

General genetic methods. PCR was performed using *Taq* polymerase (BRL) and appropriate custom-made primers (BRL) in a Perkin Elmer Cetus thermal cycler, with 30 cycles of denaturing (1 min, 94 °C), annealing (1 min, 54 °C) and extending (1 min, 72 °C). The PCR products were purified in preparative agarose gels and cloned into pCRII-2.1 (Invitrogen). DNA sequencing of the *phu* genes and of all cloned PCR products was performed with Sequenase 2.0 (United States Biochemical) and M13 primers or custom-made 18-mer oligonucleotides. Published procedures were followed for Southern blot analysis (Sambrook *et al.*, 1989) and colony hybridization (Forster *et al.*, 1990).

RNase protection analyses were performed using the Riboprobe system (Promega). Radiolabelled riboprobes from suitable cloned DNA fragments were generated by run-off transcription from the T7 promoter of linearized plasmids using T7 RNA polymerase and [α-^{32}P]CTP as described in detail elsewhere (Barton *et al.*, 1996). A 699 bp *Eco*RV–*Hin*cII fragment containing the *phuR*–*phuS* divergent promoters with the first 195 bp of the *phuR* coding region and the first 314 bp of the *phuS* coding region was cloned in either orientation into the *Eco*RV site of pBluescript SK+, resulting in plasmids pPHU699-R and pPHU699-S, which served to generate riboprobes antisense to *phuR* and to *phuS*, respectively. Plasmid pPHU-R1 contained a 346 bp PCR fragment obtained with primers 5′-GATCGAAGGCATGGCCCG-3′ and 5′-GACAA-GCCGGTTCTCATTC-3′ and was used to generate a riboprobe to detect T1 and T2 transcripts. pPHU359− and pPHU359+ were constructed by cloning an internal *Xho*I–*Stu*I *phuV* fragment into pBluescript SK (*Xho*I–*Eco*RV) and into pBluescript KS (*Xho*I–*Eco*RV), and served to generate riboprobes for the detection of sense and antisense *phuV* transcripts.

Isolation of the *phu* and *has* loci. The repeats R1 and R2 that were originally isolated in a cycle-selection procedure for Fur targets (Ochsner & Vasil, 1996) were used as probes to obtain overlapping larger DNA clones by Southern blot analysis followed by colony hybridization. First, a 0·85 kb *Dde*I fragment containing the 3′ end of repeat R2 was cloned and used as a probe to isolate a 1·3 kb *Stu*I fragment containing the *phuW* gene, which was cloned behind the *lac* promoter in pUCP19, resulting in pPHU3. Using *phuW* as a probe, a 5·5 kb *Sph*I fragment harbouring the *phuV*, *phuU*, *phuT* and *phuS* genes, and the 5′ portion of *phuR* was isolated and cloned into pUCP19, yielding pPHU2. Subsequently, a 6 kb *Eco*RV fragment containing the entire *phuR* gene was cloned into pUCP19, yielding pPHU1. The DNA sequence of a total 7·7 kb portion of the *phu* locus was determined. The DNA sequence of the *hasR* gene was pulled out from the unfinished *P. aeruginosa* genome project (Pathogenesis Corporation) using a TBLASTN search with the *Ser. marcescens* HasR amino acid sequence. The *P. aeruginosa hasR* homologue was subsequently isolated by PCR using the primers 5′-CCTCGTTG-CAGTCGATCAG-3′ and 5′-aagcttGCTCATGCCAAAACT-CCAA-3′ (lower-case letters indicate a non-complementary *Hin*dIII site). The 3·05 kb PCR product was cloned into pCRII-2.1, partially sequenced and directionally cloned as an *Eco*RI–*Hin*dIII fragment into pUCP19, yielding pHAS3051. Plasmid pETW999 contained a 999 bp PCR fragment of the *phuW* gene and was generated with the primers 5′-catatgC-GCATTCGCCTGTTCGC-3′ (lower-case letters indicate a

non-complementary *Nde*I site) and 5′-AGATTATCTCCT-CATCAGGCT-3′. The *phuW* gene was then directionally cloned as an *Nde*I–*Hin*dIII fragment from pCRII-2.1 into pET23a. In the resulting plasmid, pETW999, *phuW* was under T7 promoter control and also translational initiation signals were plasmid derived.

Generation of mutant strains affected in the *phu* and *has* loci. Specific deletion mutants were obtained by replacing portions of the relevant genes with a gentamicin-resistance (Gm^R) or a tetracycline-resistance (Tc^R) cassette. The mobilizable suicide plasmid pSUP203 (Simon *et al.*, 1983) containing the selection marker with suitable cloned DNA flanking regions served as the donor plasmid and *E. coli* HB101/pRK2013 (Figurski & Helinski, 1979) served as the helper strain in triparental matings using *P. aeruginosa* PAO1 as the recipient, as described in detail elsewhere (Ochsner *et al.*, 1996). The successful gene replacement in the transconjugants was verified by Southern blot analysis. In brief, the deletion mutants created for this study had the following genotypes. In the Δ*phuR* mutant, an internal 0·4 kb *Hin*cII fragment of the 5′ part of the *phuR* gene was replaced with a Gm^R cassette. Similarly, the Δ*phuSTUV* mutant had a replacement of a 2·4 kb *Stu*I fragment covering the 3′ end of *phuS*, the entire coding regions of *phuT* and *phuU* and the 5′ half of *phuV* with a Gm^R cassette. The Δ*phuW* mutant harboured a 0·22 kb *Mlu*I–*Sph*I deletion in the 3′ portion of the *phuW* gene and carried a Gm^R cassette in that location. Mutant Δ*phu*-R1R2 had a 0·3 kb *Nhe*I–*Dde*I fragment replaced with a Gm^R cassette, thereby deleting the entire R1 repeat and the 5′ portion of the R2 repeat, including the promoter located within R2. In the Δ*hasR* mutant, two adjacent internal *Sma*I fragments of 0·45 kb and 1·05 kb of the *hasR* gene were deleted and replaced with a Tc^R cassette. The double mutant Δ*phuR* Δ*hasR* was obtained by deleting the *hasR* gene in the Δ*phuR* background using the same donor plasmid constructs as for the generation of the single mutants.

Translational fusions to the *lacZ* reporter gene. pHAS1, pHAS2 and pHAS3, containing translational fusions to the *lacZ* reporter gene, were constructed as follows. A 0·32 kb PCR product was generated using the primers 5′-CCTCGT-TGCAGTCGATCAG-3′ and 5′-aagcttGCGACCGCCTCG-CACG-3′ (lower-case letters indicate a non-complementary *Hin*dIII site), cloned into pCRII-2.1, sequenced and directionally cloned as an *Eco*RI–*Hin*dIII fragment into pPZ20 (Schweizer, 1991a), resulting in pHAS1 harbouring a *hasR*::*lacZ* fusion. Similarly, a 0·23 kb PCR product was generated with the primers 5′-TGAGCGATGAACTGA-CCCT-3′ and 5′-aagcttGCTCATGCCAAAACTCCAA-3′ (includes *Hin*dIII site), and yielded pHAS2 harbouring a *hasA*::*lacZ* fusion. A 0·29 kb PCR fragment comprising the *hasR* promoter without the *hasR* translational signals was obtained with the primers 5′-CCTCGTTGCAGTCGA-TCAG-3′ and 5′-aagcttCATGACAGCGCCTACACT-3′ (includes *Hin*dIII site) and cloned into the *Eco*RI site of pHAS2. In the resulting plasmid, pHAS3, the *hasR* promoter fragment was upstream of the *hasA* promoter and had the same orientation.

Biochemical procedures. Outer-membrane proteins were isolated by EDTA/lysozyme treatment of osmotically stabilized cells followed by differential centrifugation, as published elsewhere (Ochsner *et al.*, 1999). The proteins in the crude outer-membrane preparation were separated on an 8% SDS-polyacrylamide gel and stained with Coomassie blue. High-molecular-mass proteins and the Benchmark ladder (BRL) were used as size standards. The Bradford assay (Bio-Rad)

was used to measure protein concentrations. β-Galactosidase activities were determined spectrophotometrically using ONPG (Sigma) as the substrate with 1 U corresponding to a substrate conversion of 1 μmol min^{-1}. The β-galactosidase activities given as U mg^{-1} were normalized to the amount of soluble protein.

RESULTS

Isolation of the *P. aeruginosa* haem uptake (*phu*) locus

A large number of *P. aeruginosa* iron-regulated genes (*pigs*) have been previously identified in a cycle-selection procedure based on the binding of the Fur protein to DNA sequences containing a 'Fur box' (Ochsner &

Vasil, 1996). Two isolates, *pig9* and *pig20*, mapped to the same region at 0·9–6·6 min on the *P. aeruginosa* chromosome by PFGE and resulted in identical Southern hybridization patterns (data not shown). Further DNA sequence analysis indicated that *pig9* and *pig20* were located adjacent to each other and contained 154 bp repeat sequences (R1 and R2) with 90% nucleotide sequence identity which were spaced 58 bp apart (Fig. 1a). Both repeat elements contained a strong Fur box matching 15 of the 19 residues of the conserved GATAATGATAATCATTATC Fur-binding consensus sequence (Fig. 1a), which resulted in DNaseI footprints that covered an area of 30 bp each (data not shown). Putative −35 and −10 promoter elements overlapping these Fur boxes correlated well with the mapped RNA
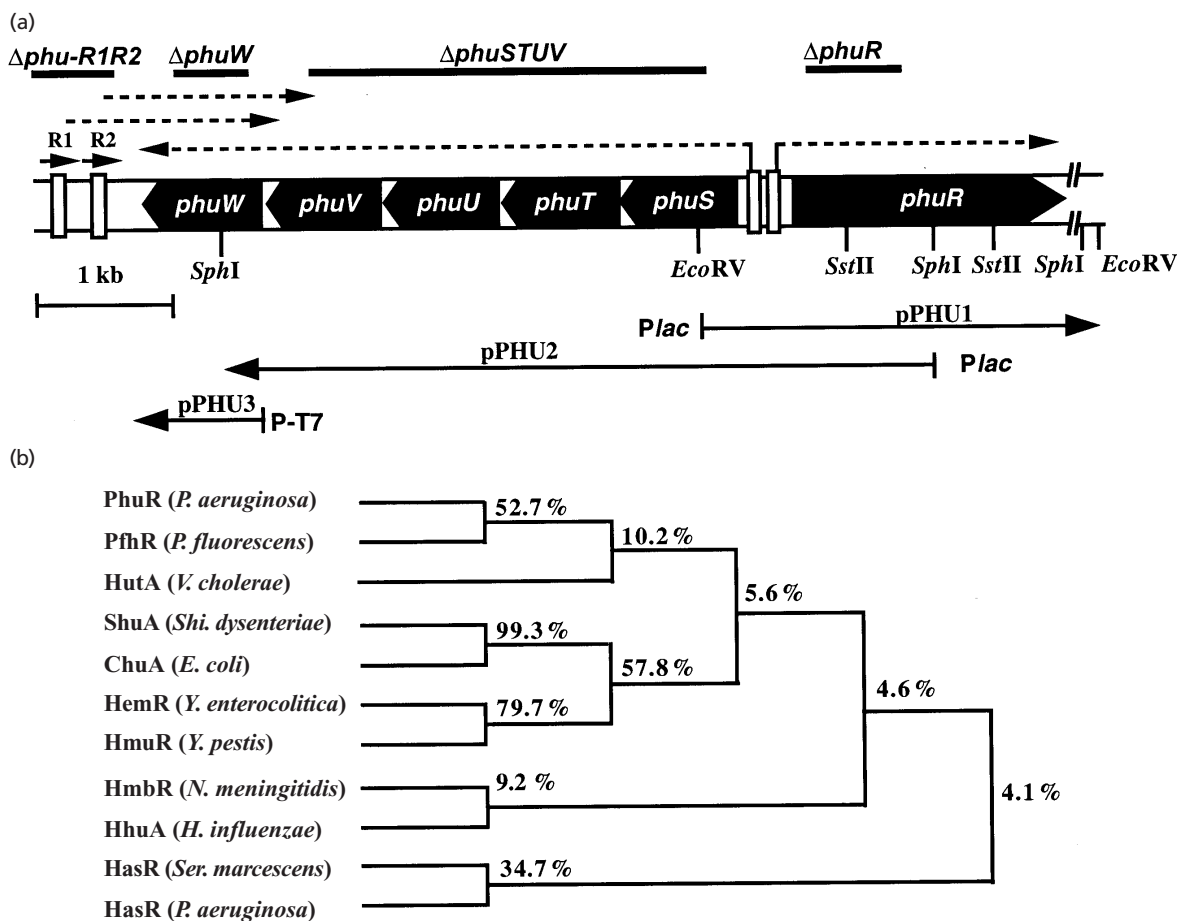
Fig. 1. (a) Map of the *P. aeruginosa* haem uptake locus containing the *phuR* gene and the *phuSTUVW* operon. Relevant restriction sites and the composition of relevant plasmids are shown. Also indicated are transcripts (dashed arrows), tandem repeat elements (R1, R2), and the deleted parts in *phu* mutants (thick black lines). Four Fur-binding elements shown as white boxes were present within the *phu* locus. The Fur box sequences and the number of matching residues to the Fur box consensus (GATAATGATAATCATTATC) were as follows. Upstream of repeat R1, GGAAATGAGAATCATTATT (15 of 19); upstream of repeat R2, GATAATGAGAATAGTTATT (15 of 19); upstream of *phuS*, CAAAACGCATATCTGAATC (11 of 19 on the complementary DNA strand); and upstream of *phuR*, GATAATTATTTGCATTAGC (15 of 19 on the complementary DNA strand). (b) Phylogenetic tree based on bacterial haem and haemoglobin receptors. The percentages indicate the amino acid sequence matching at each branching point and are a measure of evolutionary distance. The protein primary structures were derived from GenBank entries for *P. aeruginosa* PhuR (accession no. AF055999), *Pseudomonas fluorescens* PfhR (accession no. AF127222), *V. cholerae* HutA (L27149), *Shi. dysenteriae* ShuA (U64516), *E. coli* ChuA (U67920), *Y. eneterocolitica* HemR (S49393), *Y. pestis* HmuR (U60647), *N. meningitidis* HmbR (U18558), *H. influenzae* HhuA (43198), *Ser. marcescens* HasR (Y08983) and *P. aeruginosa* HasR (AF127223).

Table 2. Characterization of the predicted proteins encoded in the *P. aeruginosa* haem-uptake (*phu*) locus

Protein	Cellular location*	Molecular mass (Da)	pI	Homologues (% identity)	Putative function
PhuR	Outer membrane	84 600	5·7	*Y. enterocolitica* HemR (24)	Haemin receptor
PhuS	Periplasm	39 000	5·4	*Y. enterocolitica* HemS (39)	Haemin degradation
PhuT	Periplasm	31 100	7·7	*Y. enterocolitica* HemT (31)	Haemin binding
PhuU	Inner membrane	33 600	9·7	*Y. enterocolitica* HemU (48)	Haemin permease
PhuV	Inner membrane	27 500	6·2	*Y. enterocolitica* HemV (45)	ATPase component
PhuW	Inner membrane	32 900	7·3	*C. jejuni orf*W product (27)	Unknown

* While the cellular location was determined experimentally for PhuR, the typical location of bacterial homologues of the ABC transport components is indicated.

start sites of strong iron-regulated transcripts originating within R1 and R2 (Fig. 1a). Possible roles of the R1 and R2 repeats are discussed below. The end of an iron-regulated transcript in the opposite direction was detected immediately downstream of repeat R2 (Fig. 1a). A 5·5 kb *Sph*I DNA fragment containing this region and an overlapping 6 kb *Eco*RV DNA fragment extending even further downstream were cloned into pUCP19, yielding pPHU2 and pPHU1, respectively (Fig. 1a). The DNA sequence of pPHU2 and of parts of pPHU1 was determined and a 7702 bp portion has been deposited in GenBank (accession no. AF055999).

Characterization of the PhuR and HasR outer-membrane receptors and of an ABC transporter encoded by *phuSTUVW*

The *phu* locus consisted of six ORFs, as depicted in Fig. 1(a). The characterization of the encoded proteins, such as biochemical properties, presumed cellular locations and homologies to other bacterial proteins, is summarized in Table 2. The *phuR* gene encoded an 84·6 kDa protein with a 25 aa signal sequence typical for exported proteins; the predicted size of the mature PhuR protein was 82 kDa (Table 2). The amino acid sequence of the PhuR protein had a high degree of overall identity to the family of outer-membrane receptors for haemin and haemoglobin, such as *V. cholerae* HutA (28 %) (Henderson & Payne, 1994), enterohaemorrhagic *E. coli* O157:H7 ChuA (25 %) (Torres & Payne, 1997), *Shi. dysenteriae* ShuA (25 %) (Mills & Payne, 1997; Wyckoff *et al.*, 1998), *Y. enterocolitica* HemR (24 %) (Stojiljkovic & Hantke, 1992) and *Y. pestis* HmuR (24 %) (Hornung *et al.*, 1996). The latter two are encoded in an operon with the small *hemP* and *hmuP* genes, respectively; however, the *phu* locus lacked an ORF encoding a homologue of these hypothetical factors. PfhR was isolated from *P. fluorescens* and had 64 % amino acid sequence identity with *P. aeruginosa* PhuR. These proteins appear to form a subfamily of haem receptors and have less homology to the members of the second subfamily which contains the *Ser. marcescens* HasR receptor (Ghigo *et al.*, 1997). A HasR homologue was isolated from *P. aeruginosa* using the DNA sequence

information of the unfinished *P. aeruginosa* genome project (Pathogenesis Corporation) and exhibited 58 % amino acid sequence identity to *Ser. marcescens* HasR. A phylogenetic tree of selected receptors with the relative evolutionary distances given as percentages is shown in Fig. 1(b).

The PhuR protein contained the amino acid sequence motifs conserved among TonB-dependent receptors, previously designated regions I, II and III (Bitter *et al.*, 1991). Region I is the 'TonB box' with the consensus sequence (D/E)T..V.A(A/S) and is typically located near the N terminus of the protein (Ankenbauer & Quan, 1994; Bitter *et al.*, 1991). A somewhat weak 'TonB box'-like motif, S$\underline{T}$VS$\underline{V}$QTR, was located at amino acid positions 25–32 of the mature PhuR protein. Region II has the consensus sequence (F/I/L/M/V)... (I/L/V).NL.(D/N)(K/R).Y and is typically located in the C-terminal portion of the receptor protein. In PhuR, the motif $\underline{L}$NAG$\underline{L}$Y$\underline{N}$LT$\underline{D}$KK$\underline{Y}$, completely matching this consensus, was detected at positions 686–698 of the mature PhuR sequence. Region III, typically located at a distance of roughly 100 aa from region I, is characterized by the consensus sequence R(V/I)(D/E)(I/V)(I/V/L)-(K/R)G..(G/S/A)...G....G(G/A).(V/I) and the matching pattern $\underline{R}$$\underline{V}$$\underline{E}$$\underline{I}$$\underline{L}$$\underline{R}$$\underline{G}PA\underline{S}A\underline{L}Y\underline{G}$SNAI$\underline{G}$$\underline{G}A\underline{V}$ was found 110 aa C-terminal from region I. The finding of these three characteristic elements and the presence of an N-terminal signal sequence suggested that the PhuR protein was an outer-membrane receptor. Outer-membrane-protein profiles from iron-starved PAO1 wild-type and from Δ*phuR* or Δ*hasR* mutant strains were compared (Fig. 2a). SDS-PAGE analysis revealed the lack of a roughly 80 kDa protein in the Δ*phuR* mutant, which was in good agreement with the predicted size of 82 kDa for the mature PhuR receptor. A prominent protein band at that position was detected upon genetic complementation of the Δ*phuR* mutant with pPHU1, which contains a constitutively expressed functional copy of the *phuR* gene. Similarly, the second *P. aeruginosa* haem receptor, HasR, was detected by SDS-PAGE analysis of outer-membrane-protein preparations. In PAO1 wild-type extracts, a faint protein band was present at roughly 94 kDa, which was the
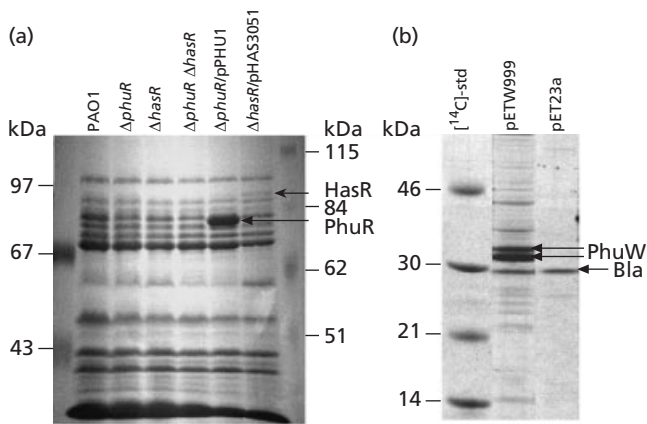
Fig. 2. SDS-PAGE analysis of *P. aeruginosa* proteins involved in haem acquisition. (a) Outer-membrane proteins of PAO1 wild-type, single mutants Δ*phuR*, Δ*hasR*, double mutant Δ*phuR* Δ*hasR* and complemented mutants Δ*phuR*/pPHU1 and Δ*hasR*/pHAS3051. The positions of the putative 82 kDa PhuR and 94 kDa HasR outer-membrane receptors are indicated by arrows. (b) Overexpression and labelling of PhuW using a T7 expression system. Whole-cell extracts of *E. coli* BL21(DE3) containing plasmid pETW999 (pET23a-*phuW*) or the pET23a vector control were separated by SDS-PAGE and auto-radiographed. The positions of the unprocessed and processed forms of the putative 32 kDa PhuW inner-membrane protein as well as of the plasmid-encoded Bla protein are indicated by arrows.

predicted size of the HasR receptor. This band was missing in a Δ*hasR* mutant and was restored in Δ*hasR* by the plasmid pHAS1, which contained a copy of the *hasR* gene (Fig. 2a).

Immediately upstream and divergently transcribed of the *phuR* gene was an operon consisting of five genes: *phuS*, *phuT*, *phuU*, *phuV* and *phuW*. The putative proteins encoded by the first four of these genes were highly similar to the ABC transport components HemS, HemT, HemU and HemV required for haem acquisition in *Y. enterocolitica* (Stojiljkovic & Hantke, 1994); their properties are described in Table 2. PhuT contained a 33 aa signal sequence typical for exported proteins. PhuV contained two motifs common for ABC transporter ATPases, the Walker nucleotide-binding consensus motif GPNGAGKS at positions 34–41 and the motif VLLLDEP at positions 158–165 (Walker *et al.*, 1982). PhuW harboured a possible signal sequence for export and an α-helical hydrophobic stretch in the centre part of the protein sequence at residues 148–163 which may function as a potential membrane-spanning domain. A PhuW homologue of 27 % amino acid sequence identity was identified in an iron-acquisition locus of *Campylobacter jejuni*. (A. van Vliet, unpublished results). The hypothetical *phuW* gene was overexpressed in *P. aeruginosa* using pPHU3, which contained the *phuW* gene under *lac* promoter control, but no protein of the predicted size was detectable in total cell extracts (data not shown). However, T7 expression and specific
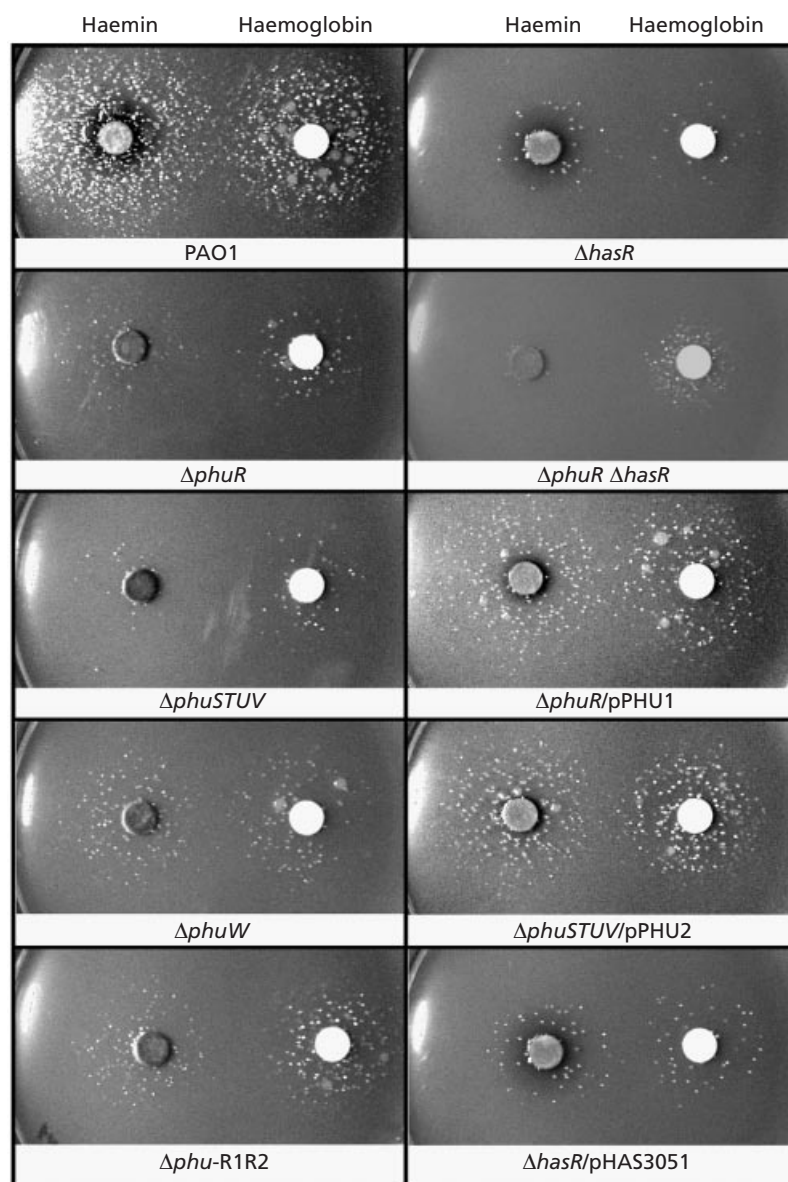
labelling of PhuW in *E. coli* using plasmid pETW999 resulted in the detection of putative PhuW protein. A double band at roughly 34 and 32 kDa was present, which corresponded very well to the size of the unprocessed and processed forms of PhuW, respectively (Fig. 2b).

Utilization of haem and haemoglobin by *P. aeruginosa* wild-type and by mutants affected in the *phu* and *hasR* loci

The proteins encoded by the *phu* genes exhibited a high degree of identity to the factors involved in haem uptake in other micro-organisms. However, their role in iron acquisition from haem-containing compounds remained to be formally demonstrated, since different iron-uptake systems were often found to share a similar genetic organization and had substantial homologies among different systems. In particular, the known outer-membrane receptors for iron chelates such as haem, haemoglobin, transferrin, lactoferrin, ferrisiderophores and ferric citrate appear to be highly conserved among Gram-negative bacteria in spite of their distinct specificities.

The *phu* locus was not essential for *P. aeruginosa* and separate deletions in the *phuR* gene, in the *phuSTUV* operon, in the *phuW* gene and in the repeat elements *phu*-R1R2 were constructed (Fig. 1a). Growth curves and pigmentation of these mutant strains were indistinguishable from PAO1 wild-type cells in low-iron tryptic soy broth or M9 medium. Iron-starved wild-type and mutant cells were placed on iron-restricted M9 agar and scored for growth stimulation around filter disks onto which various iron-containing compounds have been spotted. In this assay, 10^3–10^4 c.f.u. were mixed with top agarose and poured onto the iron-restricted agar plates. This method allowed qualitative growth monitoring by colony counting and discriminating between slow-growing small colonies still embedded in the top agarose and faster-growing colonies that had reached the plate surface. While all strains grew equally well with exogenously supplied transferrin or lactoferrin (data not shown), significant growth stimulation differences were observed when haemin or haemoglobin was used as the sole iron source (Fig. 3). Both a Δ*phuR* and a Δ*phuSTUV* mutant were markedly impaired for utilization of either haem or haemoglobin and formed only very few small colonies around the disks. Growth of these mutants was partially restored by supplying functional copies of the *phuR* gene or the *phuSTUV* operon in *trans* on the plasmids pPHU1 and pPHU2, respectively (Fig. 3). A Δ*phuW* mutant also exhibited reduced growth zones, although the effect was less dramatic. The Δ*phu*-R1R2 mutant strain lacking the tandem repeats downstream of the *phuW* gene was only mildly affected.

The faint yet detectable growth of the Δ*phuR* and the Δ*phuSTUV* mutants with haemin or haemoglobin as the sole iron source suggested the existence of a backup

Haemin   Haemoglobin   Haemin   Haemoglobin

PAO1 | ΔhasR
ΔphuR | ΔphuR ΔhasR
ΔphuSTUV | ΔphuR/pPHU1
ΔphuW | ΔphuSTUV/pPHU2
Δphu-R1R2 | ΔhasR/pHAS3051

Fig. 3. Growth stimulation by haemin or haemoglobin as the sole iron source. PAO1 wild-type, single mutants ΔphuR, ΔphuSTUV, ΔphuW, Δphu-R1R2 and ΔhasR; double mutant ΔphuR ΔhasR; and the complemented mutants ΔphuR/pPHU1, ΔphuSTUV/pPHU2 and ΔhasR/pHAS3051 were pre-grown in M9 medium containing dipyridyl. Roughly 10^3–10^4 such iron-starved cells were then embedded in a top layer of agarose onto M9 plates containing the iron chelator EDDHA. Growth of colonies around sterile filter disks impregnated with haemin or haemoglobin was monitored after 36 h at 37 °C. Note: colonies appeared big when they reached the surface of the plates; the small colonies were located within the top agar.

system for the acquisition of iron from these compounds. In fact, the extracellular haem-binding protein HasA, a so-called haemophore, has been recently described in *P. aeruginosa*, suggesting the presence of a haem-acquisition system analogous to the *Ser. marcescens* system (Létoffé *et al.*, 1998, 1999). Immediately upstream of, and contained in, an operon with the *hasA* gene, we identified the *hasR* gene, which encoded a second putative haem receptor. Downstream of *hasA* was a three-gene operon (data not shown) encoding factors with high homology to the *Ser. marcescens* HasD, HasE and HasF ABC-exporter proteins required for HasA secretion in *S. marcescens* (Binet & Wandersman, 1996; Ghigo *et al.*, 1997). A ΔhasR mutant was affected in the utilization of haemin and haemoglobin as severely as the ΔphuR mutant and a ΔphuR ΔhasR double mutant did not grow significantly under these conditions (Fig. 3). Attempts to complement the ΔhasR mutant

with pHAS3051, containing a functional *hasR* gene, failed, presumably due to polar effects on the downstream genes as discussed below.

Fur-mediated iron regulation of the *phuR* gene and of the *phuSTUVW* operon

Expression of *phuR* and *phuS*, as measured with translational fusions to the *lacZ* reporter gene, occurred preferentially under iron-limiting conditions and was repressed 12-fold and 25-fold, respectively, in high-iron media (data not shown). Two tandem Fur boxes spaced 5 bp apart were detected in the intergenic region between the divergently transcribed *phuR* and *phuS* genes (Fig. 4a). Fur box 1 had the sequence GATAATTATTTG-CATTAGC, which matched the Fur-binding consensus sequence in 15 of 19 residues. Fur box 2 was less obvious and its sequence CAAAACGCATATCTGAATC
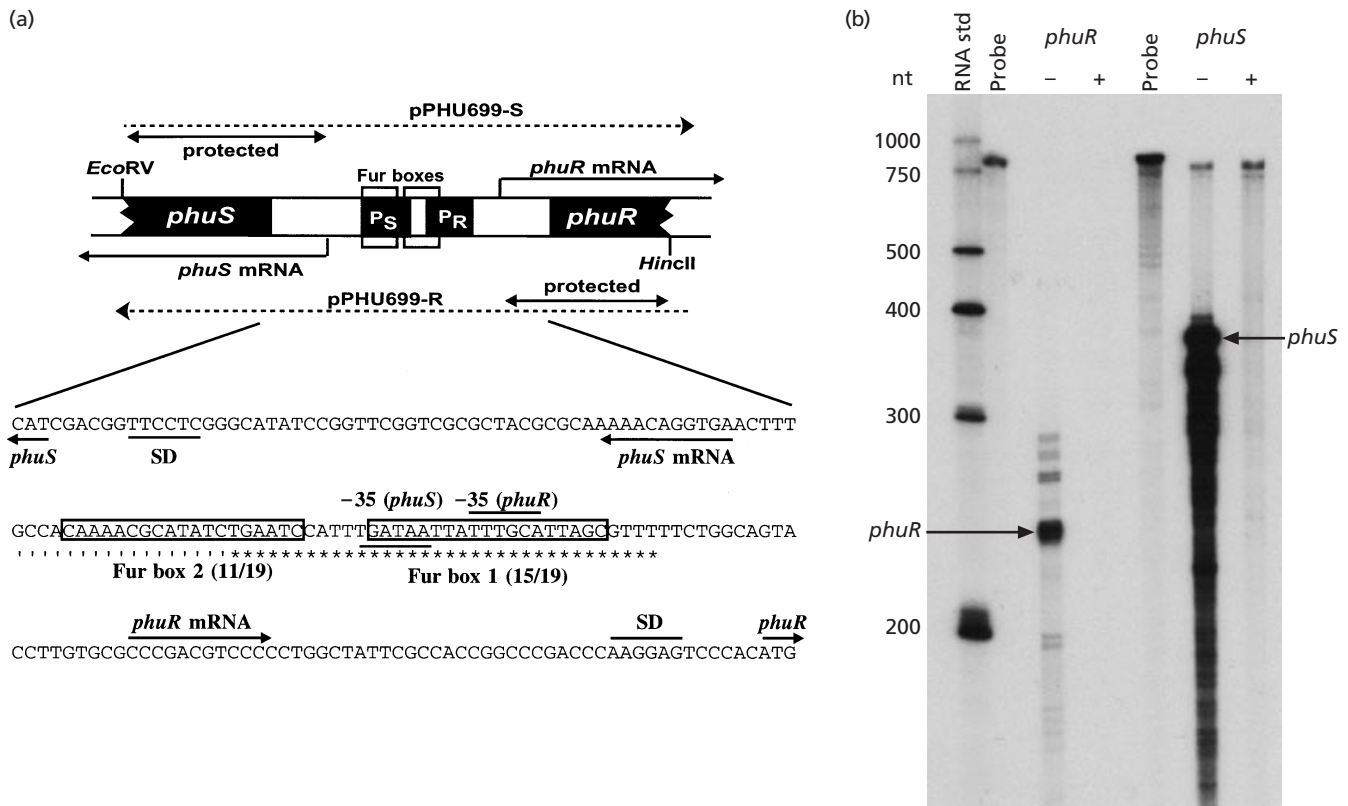
Fig. 4. Coregulation of *phuR* and *phuS* by Fur. (a) Map of a 0·7 kb *Eco*RV–*Hinc*II DNA fragment containing the *phuR*–*phuS* intergenic region. The 5′ portions of the *phuR* and *phuS* genes, their promoters, Fur boxes and transcriptional- and translational-start sites are shown. Riboprobes to detect *phuR* and *phuS* transcripts were generated from plasmids pPHU699-R and pPHU699-S, respectively, as indicated by dashed arrows. Also shown is the DNA sequence between the divergent *phuR* and *phuS* start codons. The location of the two adjacent Fur boxes is indicated by the boxed sequences, and the areas protected by Fur in DNase I footprint analyses are emphasized with asterisks (primary Fur binding site) and primes (secondary Fur binding site). (b) RNase protection of *phuR* and *phuS* transcripts. RNA was isolated from PAO1 grown under low (−) or high (+) iron conditions for 12 h at 37 °C. Normalized samples (20 μg) were hybridized with excess anti-*phuR* riboprobe generated from pPHU699-R or anti-*phuS* riboprobe made from pPHU699-S. The sizes of the protected portions of *phuR* and *phuS* probes were estimated by comparison with the bands of radiolabelled RNA ladder on a 5 % polyacrylamide/8 M urea gel, to allow the rough mapping of the corresponding transcriptional-start sites.

matched the consensus in only 11 of 19 residues. DNase I footprint reactions were carried out to verify the binding of purified Fur to the *phuR*–*phuS* intergenic region (data not shown). A Fur concentration of 30 nM resulted in a primary footprint of 34 bp which covered the stronger Fur box 1. At Fur concentrations of 100 nM or higher, an additional 17 bp were protected; this extended footprint also covered the weaker Fur box 2 (Fig. 4a). The transcriptional-start sites for *phuR* and *phuS* were roughly mapped by RNase-protection analysis. Plasmids pPHU699-R and pPHU699-S containing a 699 bp *Eco*RV–*Hinc*II *phuS*–*phuR* DNA fragment cloned in either direction with respect to the T7 promoter served to generate riboprobes specific for *phuR* and *phuS* (Fig. 4a). Transcripts of both *phuR* and *phuS* were detected in RNA isolated from cells grown to stationary phase in low-iron media, while transcripts were absent in RNA from cells cultivated under high-iron conditions (Fig. 4b). The *phuR*-specific riboprobe

protected a 245 ± 5 nt portion of *phuR* mRNA, and the *phuS*-specific riboprobe protected a 380 ± 10 nt portion of the *phuS* transcript (Fig. 4b). These experimentally determined transcriptional-start sites correlated very well with the hypothetical start sites obtained through a promoter-prediction program and are indicated in Fig. 4(a). The *phuR* and *phuS* transcription-start sites were located 50 and 54 nt upstream of the *phuR* and *phuS* translation-initiation sites. Potential −35 promoter elements in the proper distance of the mRNA start sites were located within Fur box 1, leaving only 3 bp between the −35(*phuS*) and −35(*phuR*) elements (Fig. 4a). Additional riboprobes specific for *phuV* and *phuW* transcripts resulted in hybridization patterns identical to the pattern obtained with the *phuS*-specific probe as far as growth phase and iron-dependent expression were concerned (data not shown). This finding, together with the tight spacing of the genes in the *phuSTUVW* operon and the lack of any potential promoter besides the *phuS*
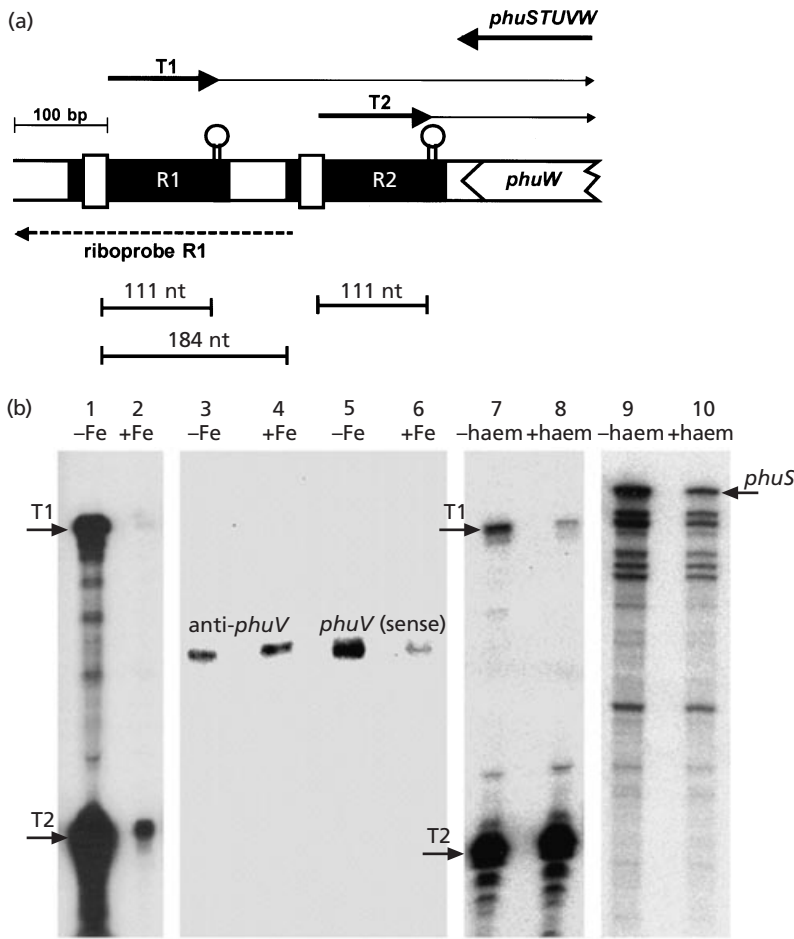
Fig. 5. Characterization of anti-*phuSTUVW* transcripts. (a) Map of the relevant portion of the *phu* locus. The two 154 bp repeats R1 and R2 (black boxes) each contained a Fur-binding site (white boxes) and a stem–loop structure (hairpin). Transcripts T1 and T2 were antisense to the *phuSTUVW* mRNA and partially terminated at the stem–loop structures (thick arrows), but a fraction of T1 and T2 transcripts extended beyond these sites, representing anti-*phuSTUVW* RNAs (thin arrows). The R1 riboprobe (dashed arrow) detected the indicated two major protected fragments of 111 nt and 184 nt, respectively. (b) RNase protection analysis. RNA was isolated from PAO1 grown under low (−Fe) or high (+Fe) iron conditions as indicated in lanes 1–6, or in low-iron medium containing 25 μg haemin ml^{-1} (+haem) as indicated in lanes 7–10. The arrows point to the signals of the relevant transcripts. Lanes 1 and 2, T1 and T2 RNA; lanes 3 and 4, anti-*phuV* RNA (T1 and T2 extended transcripts); lanes 5 and 6, *phuV* mRNA; lanes 7 and 8, T1 and T2 RNA; lanes 9 and 10, *phuS* mRNA.

promoter, strongly suggested that these five genes were indeed organized in an operon. Taken together, it was evident that the *phuR* gene and the *phuSTUV* operon were co-regulated by Fur through binding to the strong Fur box 1 that overlapped their promoters.

Mapping of Fur-regulated transcripts antisense to *phuSTUVW*

The tandem repeats R1 and R2 located downstream of the *phuSTUVW* operon were 154 bp long and 58 bp apart. Each repeat contained a strong Fur-regulated promoter at its 5′ end, and a stem–loop structure of a calculated $\Delta G = -20$ kcal mol^{-1} near its 3′ end (Fig. 5a). The transcripts T1 and T2 driven from the promoters P1 and P2 were detected by RNase protection analysis using a 346 nt riboprobe spanning R1, generated by pPHU-R1 (Fig. 5a). The most prominent band of a protected RNA species was at 111 ± 3 nt, followed by a weaker signal at 184 ± 5 nt: both protected fragments were highly iron regulated (Fig. 5b, lanes 1 and 2). The protected 111 nt fragment corresponded to the expected sizes of T1 and T2 from their transcriptional-start site to the stem–loop structure (Fig.

5a), indicating that the stem–loop within the repeats R1 and R2 acted as transcriptional terminator. The protected 184 nt fragment corresponded to the expected size of a longer T1 transcript from its start site to the start of the riboprobe (Fig. 5a), providing evidence that transcriptional termination at the stem–loop was only partial and that a substantial amount of T1 transcripts extended beyond that site. A riboprobe from pPHU359+ consisting of the plus-strand of a 359 bp internal *phuV* fragment was completely protected, indicating that the T1 and/or T2 transcripts extended at least 1·6 kb further: that is, through the *phuW* and *phuV* genes (Fig. 5b, lanes 3 and 4). However, that protection pattern did not show any level of iron regulation. In contrast, the *phuV* message detected with the minus-strand of *phuV* as a riboprobe from pPHU359− showed iron repression as expected (Fig. 5b, lanes 5 and 6). Clearly, the T1 and/or T2 transcripts were antisense relative to the *phuSTUVW* polycistronic mRNA. However, the T1 or T2 transcripts lacked translational-initiation signals and did not encode any detectable protein when the relevant DNA portion was subjected to a T7 expression and labelling system in *E. coli* (data not shown). A possible regulatory role of the T1 and T2
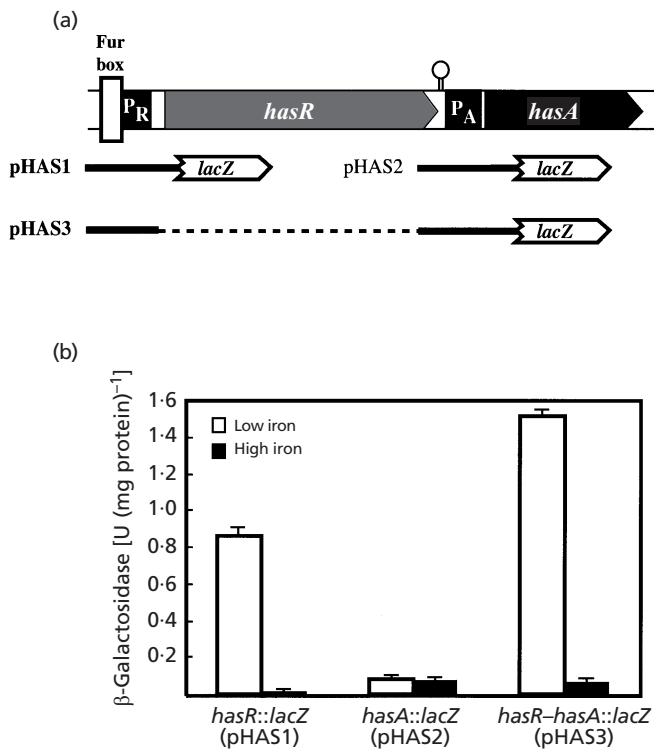
Fig. 6. Characterization of the alternate *hasR–hasA* haem-acquisition system. (a) Map of the *hasR–hasA* locus. Promoters for *hasR* and for *hasA* are shown as black boxes and the location of a Fur box upstream of the *hasR* gene is indicated. Also shown are the DNA portions used to construct the translational *lacZ*-fusion plasmids (pHAS1, pHAS2, pHAS3) for the measurement of *hasR* and *hasA* gene expression. (b) Expression of *hasR*::*lacZ* (pHAS1), *hasA*::*lacZ* alone (pHAS2) or in the presence of the upstream *hasR* promoter (pHAS3). The β-galactosidase activities were measured in soluble protein extracts prepared from triplicate cultures grown for 15 h under low and high iron conditions at 37 °C.

transcripts in response to haem was explored, following a model in which expression of T1 and T2 RNAs would be haem repressible through Fur, possibly resulting in increased *phuSTUVW* translation in the presence of haem. RNA from PAO1 grown in the absence or presence of haem was analysed with riboprobes to detect T1/T2 RNA and *phuS* mRNA. Interestingly, the signal at 184 nt representing the longer T1 transcript was greatly reduced in the presence of haem (Fig. 5b, lanes 7 and 8). However, also the *phuS* mRNA signal was weaker when haem was present (Fig. 5b, lanes 9 and 10). PhuS–*lacZ* activity was also reduced several-fold (data not shown), suggesting that haem was partially relieving the iron starvation.

Fur-mediated iron regulation of the *hasR* gene encoding a second haem receptor

Upstream of the *hasA* gene encoding an extracellular haemophore (Létoffé *et al.*, 1998) we identified the *hasR* gene which encoded a second haem receptor of *P.*

aeruginosa. The 85 bp region between the stop codon of the *hasR* gene and the start codon of the *hasA* gene had a lower G + C content, indicating a potential promoter, although any obvious strong promoter characteristics were undetectable. Also, the *hasR–hasA* intergenic region contained a stem–loop structure (-25 kcal mol^{-1}) as a potential transcriptional terminator for *hasR*. To address the question whether *hasR* and *hasA* form an operon, translational fusions of the putative *hasA* promoter in the absence or in the presence of the upstream *hasR* promoter to the *lacZ* reporter gene were constructed, along with a *hasR–lacZ* fusion (Fig. 6a). The expression of *hasR* was found to be strongly iron regulated, and *hasA* gene expression followed that pattern when the upstream *hasR* promoter and the intergenic stem–loop motif were present, suggesting that *hasR* and *hasA* were co-transcribed. In the absence of the *hasR* promoter, *hasA* expression was low and constitutive, indicating a separate but very weak promoter for *hasA* (Fig. 6b). A putative Fur box that matched the Fur-binding consensus sequence in 13 of 19 bp was detected in the *hasR* promoter region, and a DNase I footprint of 29 bp covering that Fur box was obtained (data not shown).

DISCUSSION

Two distinct haem- and haemoglobin-uptake systems in *P. aeruginosa*

Haem and haemoglobin acquisition has been described for a growing number of Gram-negative micro-organisms. Here we present evidence that *P. aeruginosa* has two distinct haem-uptake systems which enable this opportunistic pathogen to utilize iron from these sources very efficiently. An extracellular protein, HasA, which acts as a haemophore, has recently been isolated from *P. aeruginosa* through immuno cross-reaction with polyclonal antibodies against HasA from *Ser. marcescens* (Létoffé *et al.*, 1998). In that study, the addition of anti-HasA antibodies inhibited the use of haemoglobin iron at concentrations lower than 10^{-6} M. However, *P. aeruginosa* could use free haem iron at a higher concentration (10^{-5} M) and growth inhibition by the specific anti-HasA serum was only partial. These data suggested that the haemophore-dependent haem-uptake system was the unique *P. aeruginosa* high-affinity transport system for haemoglobin-bound haem and that a separate lower-affinity haem-acquistion system existed. The *phu* locus described here is unrelated to the *has* locus and encodes an alternative haem-uptake pathway. The six factors encoded by the *phu* genes show high similarity to the haem-uptake components of *Y. enterocolitica and Y. pestis* in terms of genetic organization and protein identities, while the factors encoded by the *has* system are more closely related to the homologous system in *Ser. marcescens*. To our knowledge, *P. aeruginosa* is now the second micro-organism besides *H. influenzae* type b (Cope *et al.*, 1995; Hanson *et al.*, 1992) known to have two distinct haem-acquisition systems. This finding further stresses the

importance of iron for the preferred aerobic growth of *P. aeruginosa* and may indicate the importance of iron and haem in pathogenesis.

Factors encoded in the *phu* and *has* loci

Most of the systems involved in the uptake of iron chelates consist of an outer-membrane receptor and an ABC transport system. In *P. aeruginosa* the *phu*-encoded ABC transport system consisted of five proteins, while only four proteins are involved in the homologous haem-uptake systems *hem* of *Y. enterocolitica* (Stojiljkovic & Hantke, 1994), *hmu* of *Y. pestis* (Hornung *et al.*, 1996) and in the *fec* ferric dicitrate uptake machinery of *E. coli* (Staudenmaier *et al.*, 1989). In contrast, the *Shi. dysenteriae shu* haem-transport locus consists of seven genes, one of which is a pseudogene and two others do not have any known function (Wyckoff *et al.*, 1998). The cellular locations and functions of the components have been studied in some of these systems. The receptors, including PhuR, contain a typical signal sequence, a TonB box, and are found in the outer membrane. The PhuS component of the uptake system exhibited striking homology to the HemS, HmuS, FecB and ShuS components of the ABC transporters mentioned above, for one of which (FecB) a periplasmic location has been demonstrated (Staudenmaier *et al.*, 1989). HemS has been suggested to have haemin-binding activity and to prevent the accumulation of haem to toxic levels in the cell (Stojiljkovic & Hantke, 1994). However, its homologue in *Shi. dysenteriae*, ShuS, was not required for protection against haem toxicity (Wyckoff *et al.*, 1998). PhuT and the homologues HemT, HmuT and ShuT are assumed to be localized in the periplasm, although this has not been directly demonstrated. The similarities of PhuU and PhuV to HemU and HemV suggest that they represent the permease and ATPase required for the haem transport across the inner membrane. Interestingly, an additional factor, the putative inner-membrane-bound PhuW protein encoded by the last gene of the *phuSTUVW* operon, was necessary for optimal efficiency of haem and haemoglobin utilization.

Mutations in the *phu* or *has* loci affect haem and haemoglobin utilization

The feeding of various iron-containing compounds to iron-starved *phu* mutant cells clearly indicated that the *phu* locus was required for haem and haemoglobin uptake, whereas the utilization of other iron chelates such as transferrin or lactoferrin was not affected. While the haem-uptake defect was very pronounced in the $\Delta phuR$ and $\Delta phuSTUV$ mutants, the $\Delta phuW$ mutant was still capable of growing quite well compared to the $\Delta phuR$ and $\Delta phuSTUV$ mutants with haem as the sole iron source. In agreement with this was the finding that plasmid-borne copies of just the *phuSTUV* genes restored haem utilization to near wild-type levels in the $\Delta phuSTUV$ mutant in which expression of *phuW* was absent due to a polar effect of the deletion.

Surprisingly, a mutant strain affected in the second haem-receptor gene, *hasR*, did not grow efficiently with either haem or haemoglobin as the iron source in spite of a functional *phu* locus. Haem utilization of the $\Delta hasR$ mutant was not restored with plasmid-borne copies of the *hasR* gene. As demonstrated by using translational *lacZ* fusions, *hasR* appeared to be the first gene in a Fur-regulated operon with *hasA*. Only low and constitutive expression of the *hasA* gene was detected from a weak *hasA* promoter. This was in good agreement with an earlier observation that the HasA protein accumulated in culture supernatants preferably under iron-limiting conditions (Létoffé *et al.*, 1998). The insertion of a gentamicin-resistance cartridge into the *hasR* gene had thus a predicted negative polar effect on *hasA*, resulting in very low production of HasA protein from its own promoter. Most likely, these low levels of HasA protein impaired efficient haem uptake even in the presence of a functional *phu* locus such as in the $\Delta hasR$ mutant. Also, the extent of incapacity to grow on haem of the $\Delta phuR$ and $\Delta phuSTUV$ mutants was somewhat surprising, since these mutants still harbour a functional *has* locus. It is intriguing to discuss the possibility that the *phu* and the *has* systems may be interdependent and to entertain a model in which the HasA haemophore may use either the PhuR or the HasR receptor for haem delivery to the cell. Although there is no direct experimental evidence for this model at this point, the phenotypes of the mutant strains studied support the hypothesis of such a cross-talk between the two systems. A $\Delta phuR$ $\Delta hasR$ double mutant grew very poorly with haem-containing compounds as the sole iron source, although some small colonies were formed around a disk containing haemoglobin. This remaining growth may be due to the combined action of extracellular proteases and siderophores as an indirect mechanism to acquire haemoglobin.

Regulation of the *phu* and *has* systems by Fur

Haem- and haemoglobin-acquisition systems in many bacterial species are directly regulated by Fur. Strong Fur boxes are present in the promoter regions of the *Y. enterocolitica hemRSTUV* operon (14 of 19 matches to the Fur consensus), of the *Y. pestis hmuRSTUV* operon (14 of 19), of the *Ser. marcescens hasR* gene (15 of 19) and of the *Shi. dysenteriae shuA* gene (17 of 19). The *phuR* gene and the *phuSTUV* operon were found to be co-regulated by Fur. Two tandem Fur boxes matching the consensus Fur-binding sequence in 15 of 19 and in 11 of 19 bp were detected and their significance was demonstrated in DNase footprints. The presence of two tandem Fur boxes has been associated with very tight repression of the affected genes. The *P. aeruginosa pvdS* gene encoding a Fur-regulated alternative sigma factor (Ochsner *et al.*, 1996) also harbours two adjacent Fur boxes, resulting in a complete repression of *pvdS* in the presence of iron (H. Barton, unpublished results). The *P. aeruginosa hasR* gene was under direct control of Fur binding to a single weaker Fur box (13 of 19 matches) in the *hasR* promoter region, suggesting that the two

haem-uptake systems of *P. aeruginosa* may be derepressed sequentially upon encountering iron limitation.

The role of the two Fur-regulated antisense *phuSTUVW* RNAs originating at the tandem repeats R1 and R2 downstream of the *phuSTUVW* operon was explored in terms of a regulatory mechanism in response to haem. Since haem binding of Fur has been observed *in vitro* for *E. coli* Fur (Smith *et al.*, 1996), a model was tested in which the transcripts T1 and T2 would be repressed by haem. However, the addition of haem to otherwise iron-depleted medium did not result in a selective repression of T1/T2, but had a non-discriminative negative effect on T1/T2 as well as on *phuS* expression. An alternative role of the tandem repeats could be to function as excision sites for the haem-uptake locus as a mobile element. Although there is no direct evidence for such a mechanism, a horizontal gene transfer of the *shu* haem transport locus has been proposed for *Shigella* and pathogenic *E. coli*, based on the presence of two short repeats of 12 nt flanking the *shu* locus (Wyckoff *et al.*, 1998).

ACKNOWLEDGEMENTS

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