

Proteobactin and a yersiniabactin-related siderophore mediate iron acquisition in *Proteus mirabilis*

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Summary

Proteus mirabilis causes complicated urinary tract infections (UTIs). While the urinary tract is an iron-limiting environment, iron acquisition remains poorly characterized for this uropathogen. Microarray analysis of *P. mirabilis* HI4320 cultured under iron limitation identified 45 significantly upregulated genes ($P \leq 0.05$) that represent 21 putative iron-regulated systems. Two gene clusters, PMI0229-0239 and PMI2596-2605, encode putative siderophore systems. PMI0229-0239 encodes a non-ribosomal peptide synthetase-independent siderophore system for producing a novel siderophore, proteobactin. PMI2596-2605 are contained within the high-pathogenicity island, originally described in *Yersinia pestis*, and encodes proteins with apparent homology and organization to those involved in yersiniabactin production and uptake. Cross-feeding and biochemical analysis shows that *P. mirabilis* is unable to utilize or produce yersiniabactin, suggesting that this yersiniabactin-related locus is functionally distinct. Only disruption of both systems resulted in an *in vitro* iron-chelating defect; demonstrating production and iron-chelating activity for both siderophores. These findings clearly show that proteobactin and the yersiniabactin-related siderophore function as iron acquisition systems. Despite the activity of both siderophores, only mutants lacking the yersiniabactin-related siderophore have reduced fitness *in vivo*. The fitness requirement for the yersiniabactin-related siderophore during UTI shows, for the first time, the

importance of siderophore production *in vivo* for *P. mirabilis*.

Introduction

Iron, an essential element, is required for the function of many proteins and enzymes involved in diverse biological processes including oxygen transport, gene regulation and nitrogen fixation. Under aerobic conditions and neutral pH, iron exists in the insoluble ferric (Fe^{3+}) form, which can be toxic following interaction with oxygen and oxygen-reduced species. During colonization of the host, pathogens must overcome host iron sequestration to establish infections because free iron is essentially unavailable *in vivo*. To counterbalance iron-limiting conditions and maintain iron homeostasis, bacterial species have evolved various iron transport systems, intracellular iron stores, redox stress resistance systems and iron responsive regulatory elements to control the expression of genes involved in diverse cellular functions (Andrews *et al.*, 2003).

One way bacteria acquire extracellular ferric iron is by secreting ferric chelators known as siderophores that scavenge iron from the environment during iron-limiting conditions. Over 500 siderophores have been described and can be classified into three groups; catecholates, hydroxamates and hydroxycarboxylates (Miethke and Marahiel, 2007). Precursors for siderophore biosynthesis include citrate, amino acids, dihydroxybenzoate and N^5 -acyl- N^5 -hydroxyornithine (Winkelmann, 2002). Often, genes that encode the biosynthetic enzymes for siderophores are clustered with genes for ferri-siderophore transport (Koster, 2001; Crosa and Walsh, 2002; Wandersman and Delepelaire, 2004). The mechanism of siderophore synthesis and transport is tightly regulated by the ferric uptake regulator (Fur) protein in response to iron availability (Ernst *et al.*, 1978; Hantke, 2001; Crosa and Walsh, 2002).

Once synthesized and transported outside the bacterial cell, the siderophore chelates ferric iron with high affinity. The ferri-siderophore, in turn, binds with high specificity to a TonB-dependent outer membrane receptor in Gram-negative bacterial envelopes. Transport into the cell is driven by cytosolic membrane potential mediated by the energy-transducing TonB-ExbB-ExbD system, which

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requires the direct contact of TonB and the outer membrane receptor (Tuckman and Osburne, 1992; Braun, 1995). Once in the periplasmic space, the ferri-siderophore is shuttled by a periplasmic binding protein to a cytosolic membrane ATP binding cassette (ABC) transporter. The ferri-siderophore is delivered to the cytosol of the bacterium where iron is reduced to the ferrous (Fe^{2+}) form and dissociates from the siderophore (Neilands, 1995; Ratledge and Dover, 2000; Faraldo-Gomez and Sansom, 2003; Wandersman and Deleplaire, 2004).

Previous reports have indicated that *Proteus mirabilis*, a species within the *Enterobacteriaceae* and an important etiologic agent of complicated urinary tract infection (UTI) (Warren *et al.*, 1982; Mobley and Warren, 1987), lacks detectable siderophore production (Miles and Khimji, 1975; Marcelis *et al.*, 1978; Massad *et al.*, 1995). The Arnov and Csaky tests failed to detect catechol- or hydroxamate-type siderophores respectively, when testing *P. mirabilis* P18 (Evanylo *et al.*, 1984). These findings were confirmed with gas chromatography in conjunction with mass spectroscopy (Evanylo *et al.*, 1984). However, analysis of the newly annotated *P. mirabilis* HI4320 genome (Pearson *et al.*, 2008) revealed at least two gene clusters with genes related to both siderophore biosynthesis and ABC transport. One of these appears to be a novel non-ribosomal peptide synthetase (NRPS)-independent siderophore (NIS) system, herein termed proteobactin that has not been previously described in any bacterial species. The other gene cluster contains the *nrp* operon, which has been previously described to be upregulated during iron limitation in *P. mirabilis* U6450 (Gaisser and Hughes, 1997). Recently, the *nrp* operon has been shown to be encoded within the high-pathogenicity island (HPI) in *P. mirabilis* HI4320 that has homology to the HPI of *Yersinia* spp. (Flannery *et al.*, 2009). In addition to the possibility that *nrp* has a function related to iron limitation, the metabolite, α -hydroxyisovaleric acid, has been identified as a possible siderophore (Evanylo *et al.*, 1984). α -keto acids produced by amino acid deaminases and α -hydroxycarboxylic acids have also been postulated as possible siderophores in *Proteus*, *Providencia* and *Morganella* species (Drechsel *et al.*, 1993; Massad *et al.*, 1995). Despite these studies, none of these metabolites nor those produced by the *nrp* gene products have been shown to possess iron-chelating properties.

Studies on uropathogenic *Escherichia coli* have shown that the urinary tract is iron-limited and that iron acquisition by outer membrane receptors is important during UTI (Torres *et al.*, 2001; Snyder *et al.*, 2004; Alteri and Mobley, 2007; Hagan and Mobley, 2009). Consistent with this, multiple outer membrane proteins of *P. mirabilis* are upregulated in both human urine and iron-limiting

medium (Shand *et al.*, 1985) and three outer membrane proteins induced by iron-starvation are involved in haem uptake in *P. mirabilis* 6515 (Piccini *et al.*, 1998). One of these, was shown to function as a haem receptor that contributes to the uropathogenesis of *P. mirabilis* 6515 (Lima *et al.*, 2007). Using signature-tagged mutagenesis (STM), we previously identified five genes (PMI3120; putative TonB-dependent receptor, PMI2605; putative 4'-phosphopantetheinyl transferase *nrpG*, PMI2959; putative iron ABC transporter permease, PMI0842; putative TonB-dependent receptor, PMI0030; biopolymer transport protein *exbD*) associated with iron acquisition in *P. mirabilis* HI4320 that when mutated attenuated the bacterium (Burall *et al.*, 2004; Himpf *et al.*, 2008).

Since iron acquisition remains poorly defined for *P. mirabilis*, we sought to define the global response to iron limitation by first using microarray analysis. This approach allowed for the identification of 21 putative iron acquisition systems based upon the 45 significantly upregulated genes during *in vitro* iron limitation. Molecular and biochemical characterization of two of these iron acquisition systems verified that these genes are transcriptionally upregulated during iron limitation, repressed by iron and are responsible for siderophore production in this human pathogen.

Results

Differential expression of P. mirabilis genes during iron limitation

Gene expression of *P. mirabilis* HI4320 cultured under iron limitation was analysed by microarray. A concentration of 15 μM Desferal, an iron-chelator, inhibited the growth rate of *P. mirabilis* in Luria–Bertani (LB) medium (Fig. 1). Microarray analysis of cells cultured in LB medium with and without 15 μM Desferal showed that 1017 genes were differentially expressed: 872 were upregulated and 145 were downregulated. Fold change for all 1017 genes was \geq twofold and calculated based upon the ratio of average spot intensity between these conditions. Of these genes, significance analysis of microarray (SAM) identified 45 significantly upregulated genes (Table S1) and 43 significantly downregulated genes (Table S2).

The 45 significantly upregulated genes represent 21 putative iron acquisition systems. These systems include the energy-transducing complex TonB–ExbB–ExbD, genes for haem uptake (PMI0409, PMI1424 and *hmuR1R2STUV*), an aerobic ferrous iron uptake system (*sitABCD*) (Fisher *et al.*, 2009), two putative ferri-siderophore systems (*nrpSUTABG* and PMI0229-0239) and three putative ferri-siderophore transporters (*ireA*, PMI0331 and PMI2957-2960) (Table 1). Other genes

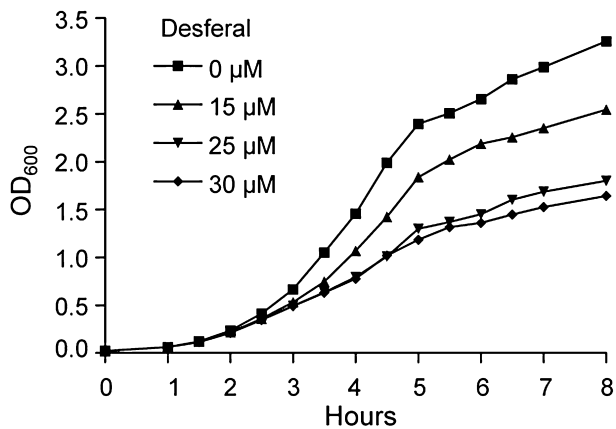


Fig. 1. The growth rate of *P. mirabilis* HI4320 is decreased following culture in LB medium containing Desferal. *P. mirabilis* HI4320 was cultured in LB medium containing 0, 15, 25 or 30 μM Desferal, an iron-chelating agent. A concentration of 15 μM Desferal in LB medium decreases the growth rate of wild-type in comparison with wild-type grown in LB medium alone.

induced during iron limitation are a putative iron utilization protein PMI1437, two putative TonB-dependent systems that may be involved in colicin uptake, PMI1551-1548 and PMI0842, and systems with potential iron-sulfur clusters, *sufABCDSE*, *bfd* and PMI0176-0172. As expected, in contrast to *sitABCD* the *P. mirabilis* ferrous iron uptake system *feoAB* was not upregulated \geq twofold during aerobic iron limitation. Genes repressed during iron limitation include predicted iron storage proteins and iron-metalloproteins.

To validate the results of the microarray, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on select genes that were iron responsive *in vitro*. A total of six individual upregulated genes (*exbD*, TonB-dependent receptor PMI0842, *sitA*, *hmuR1*, *glpD/glyD*, *bfd*) and one significantly downregulated gene (*ftnA*) were verified by qRT-PCR (Fig. 2A). As expected, all genes except *glpD/glyD* were upregulated $>$ twofold and *ftnA* was downregulated $>$ twofold during culture of *P. mirabilis* HI4320 in 15 μM Desferal (Fig. 2A). Putative siderophore biosynthesis and ABC transport gene clusters encoding the capacity to synthesize the yersiniabactin-related siderophore, *nrp* (PMI2596-2605) and proteobactin, *pbt* (PMI0229-0239) were also tested by qRT-PCR to verify upregulation (Fig. 2B and C). Putative siderophore TonB-dependent receptor, PMI2596 and all genes in the putative *nrp* operon (PMI2597-2605) were upregulated $>$ twofold (Fig. 2B). Genes of the *pbt* gene cluster (PMI0229-0239) were upregulated $>$ twofold, except *pbtH* (PMI0239) (Fig. 2C). Both *nrp* and *pbt* gene clusters contain consensus sequences for ferric uptake regulator (Fur) binding (Fig. 3) and, consistent with this, the transcription of *pbt* genes are repressed by the addition of 25 μM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ to bacteria cultured in iron-

chelated LB medium (Fig. 2C). Both of these putative siderophore systems are organized as operons as demonstrated using RT-PCR; the *nrp* system consists of two transcripts and the *pbt* system is transcribed in three units (Fig. 3).

Since the urinary tract is iron-limited (Torres *et al.*, 2001; Snyder *et al.*, 2004; Alteri and Mobley, 2007; Hagan and Mobley, 2009) and the *nrp* and *pbt* operons are induced during iron limitation *in vitro*, the expression of one putative siderophore biosynthesis gene from each of the two systems was examined *in vivo*. We assessed the transcription of these genes during infection by comparing expression of *pbtA* (PMI0232) and *nrpR* (PMI2599) using qRT-PCR to quantify RNA purified from wild-type *P. mirabilis* cultured in LB medium and from *P. mirabilis* recovered from the urine of infected mice. Bacteria recovered from urine of infected mice upregulated both siderophore synthetase genes *in vivo* as compared with LB medium. Interestingly, *pbtA* appeared to display a greater level of induction than *nrpR* under these conditions ($P = 0.0002$) (Fig. 2D).

Characterization of the yersiniabactin-related locus in *P. mirabilis*

Previously, we have shown that the *nrp* genes (PMI2597-2605) are located on a pathogenicity island in *P. mirabilis* HI4320 (Flannery *et al.*, 2009). These genes have homology to genes required for synthesis of yersiniabactin, a NRPS siderophore, which is encoded on the HPI of *Yersinia* spp. (Carniel *et al.*, 1996; Flannery *et al.*, 2009). In *P. mirabilis*, the putative yersiniabactin receptor, PMI2596, is annotated as a TonB-dependent receptor and shares 27% amino acid sequence identity with the yersiniabactin/pesticin receptor gene *psn* (Fig. 4A, Table 2). Genes PMI2597-2605 are divergently transcribed from PMI2596 (Fig. 3) and make up an operon encoding a major facilitator superfamily (MFS) transporter, a conserved hypothetical protein, two putative non-ribosomal peptide synthetases including *nrpS*, two putative siderophore biosynthesis genes *nrpU* and *nrpT*, two putative siderophore ABC transport ATP binding/permeases *nrpA* and *nrpB*, and putative 4'-phosphopantetheinyl transferase *nrpG* (Fig. 4A). To maintain consistency with the current nomenclature, the three previously uncharacterized genes that are expressed in this operon are designated *nrpX* (PMI2597), *nrpY* (PMI2598) and *nrpR* (PMI2599).

Despite the homology to yersiniabactin biosynthesis and transport genes in other *Enterobacteriaceae* (Table 2), the yersiniabactin-related siderophore synthesized by *P. mirabilis* HI4320 is predicted to lack modification by salicylate. Genes involved in salicylic acid biosynthesis and incorporation, *pchB*, *pchA* and *irp5* in *Pseudomonas syringae* pv. tomato DC3000 and *ybtE*

Table 1. Putative iron acquisition systems upregulated in *P. mirabilis* HI4320 during iron-limiting microarray^a.

Function ^b	PMI No.	Gene	Description	Log ₂ fold change ^c	
Haem uptake	0409*		putative TonB-dependent haem receptor	6.22	
	1424*		putative hemin uptake protein	8.52	
	1425*	<i>hmuR1</i>	hemin receptor	5.92	
	1426*	<i>hmuR2</i>	hemin receptor	7.89	
	1427*	<i>hmuS</i>	hemin transport protein	6.96	
	1428*	<i>hmuT</i>	hemin binding periplasmic protein	5.02	
	1429	<i>hmuU</i>	hemin transport system; permease protein	3.42	
	1430*	<i>hmuV</i>	hemin transport system; ATP binding protein	5.42	
Ferrous iron uptake	1024	<i>sitD</i>	iron ABC transporter; membrane protein	3.41	
	1025	<i>sitC</i>	iron ABC transporter; membrane protein	4.81	
	1026*	<i>sitB</i>	iron ABC transporter; ATP binding protein	5.49	
	1027	<i>sitA</i>	iron ABC transporter; periplasmic substrate binding protein	5.93	
Ferric citrate transport	3704		exported protease	1.36	
	3705		putative ABC transport binding protein	2.14	
	3706		TonB-dependent receptor	2.45	
	3707		FecR-transcriptional regulator	3.83	
	3708		extracytoplasmic function (EFC)-family σ factor	4.52	
	3709		TonB-like protein	2.92	
Putative siderophore biosynthesis & ABC transport systems	0229*		ABC transporter; permease protein	4.81	
	0230		ATP binding protein	3.94	
	0231*	<i>pbtI</i>	putative citrate lyase β -subunit	6.45	
	0232*	<i>pbtA</i>	putative siderophore biosynthesis protein	6.36	
	0233*	<i>pbtB</i>	putative TonB-dependent siderophore receptor	6.04	
	0234*	<i>pbtC</i>	putative lysine/ornithine decarboxylase	5.30	
	0235*	<i>pbtD</i>	putative pyridoxal-phosphate dependent enzyme	4.77	
	0236	<i>pbtE</i>	putative octopine/opine/taurine dehydrogenase	3.23	
	0237	<i>pbtF</i>	MFS-family transporter	3.31	
	0238	<i>pbtG</i>	putative substrate binding protein	1.76	
	0239	<i>pbtH</i>	conserved hypothetical protein	2.53	
		2596*		putative siderophore TonB-dependent receptor	7.13
		2597	<i>nrpX</i>	MFS-family transporter	5.92
		2598*	<i>nrpY</i>	conserved hypothetical protein	7.93
		2599	<i>nrpR</i>	putative non-ribosomal peptide synthase	5.15
		2600	<i>nrpS</i>	putative non-ribosomal peptide synthase	3.62
		2601	<i>nrpU</i>	putative siderophore biosynthetic protein	2.92
		2602	<i>nrpT</i>	putative siderophore biosynthetic protein (thioesterase)	2.90
		2603	<i>nrpA</i>	put. sid. ABC transporter; ATP binding/permease protein	4.08
		2604	<i>nrpB</i>	put. sid. ABC transporter; ATP binding/permease protein	3.56
	2605	<i>nrpG</i>	putative 4'-phosphopantetheinyl transferase	1.27	
Additional ferri-siderophore transporters	0331*		putative ABC transporter; substrate binding protein	6.49	
	0363		putative TonB-dependent ferri-siderophore receptor	3.52	
	1945*	<i>ireA</i>	putative TonB-dependent ferri-siderophore receptor	6.65	
	2957*		putative iron ABC transporter; substrate binding protein	6.20	
	2958		putative iron ABC transporter; permease component	3.93	
	2959		putative iron ABC transporter; permease	2.99	
	2960		putative iron ABC transporter; ATP binding	3.13	

a. Putative iron acquisition systems of *P. mirabilis* HI4320 listed are composed of genes that were upregulated during *in vitro* iron limitation with a fold change \geq twofold.

b. Genes are grouped by function and listed within putative systems or operons by ascending *P. mirabilis* gene identification number (PMI No.).

c. Log₂ transformation of fold change values determined by taking ratio of transcript level of *P. mirabilis* growth in LB medium and LB medium treated with 15 μ M Desferal averaged across all five microarray experiments.

*Genes found to be significantly upregulated as determined by SAM.

and *ybtS* in *Yersinia pestis* KIM that are essential for yersiniabactin function in these bacteria are absent in the *P. mirabilis* *nrp* locus (Fig. 4A). The *P. mirabilis* *nrp* operon also lacks a linked transcriptional regulator analogous to PSPTO2606 and YbtA (Fig. 4A). Another distinctive product of the *P. mirabilis* *nrp* operon is the

presence of a conserved hypothetical protein encoded by *nrpY* (PMI2598), which has similarity to methyl-transferases found in NRPS systems (Fig. 4A). Additionally, the 4'-phosphopantetheinyl transferase located at the end of the *P. mirabilis* *nrp* operon, *nrpG*, is encoded separately from the yersiniabactin operon on the Y.

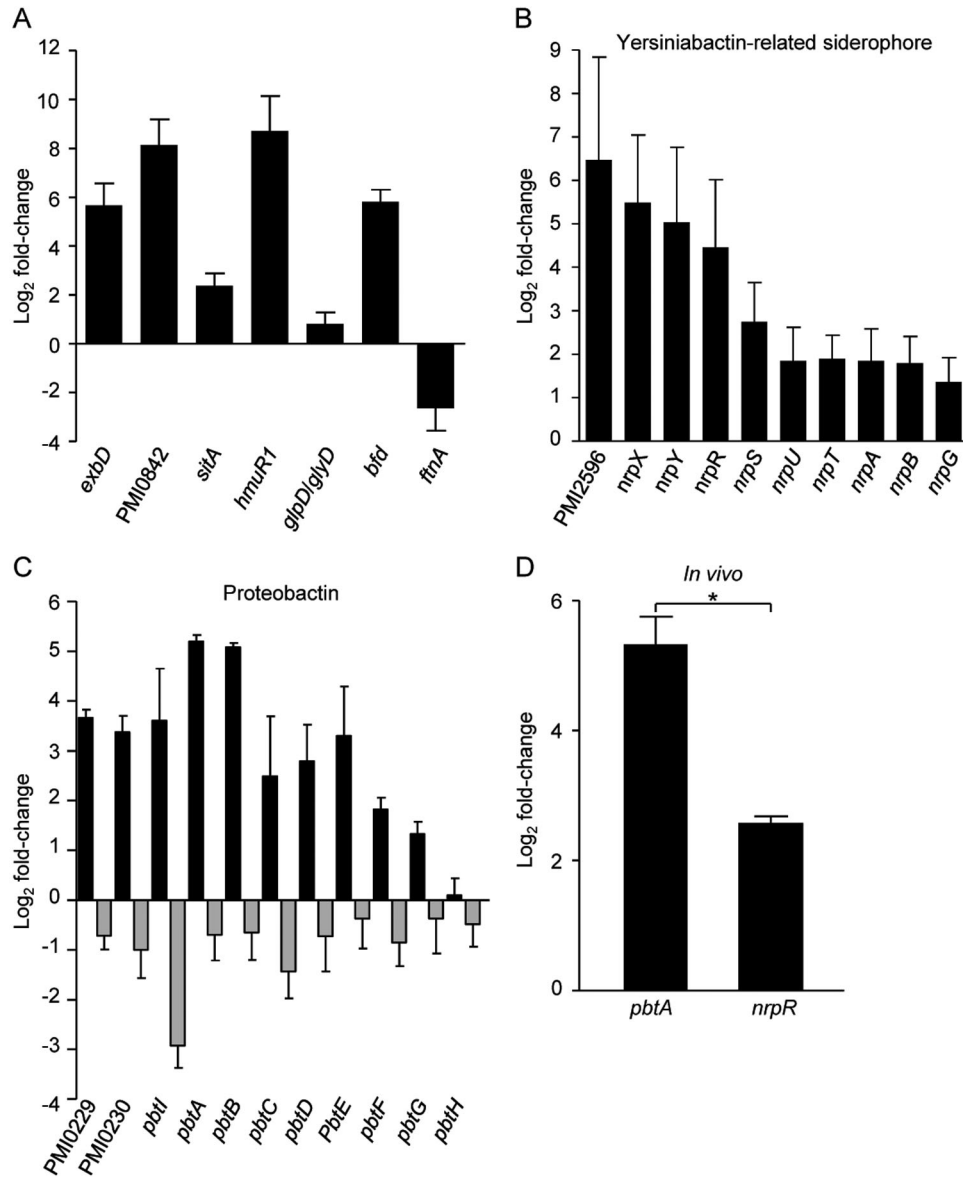


Fig. 2. qRT-PCR of mRNA from *P. mirabilis* HI4320 cultured in LB medium containing 15 μ M Desferal validates microarray results that identified upregulated and downregulated genes in response to iron limitation. Log₂ fold change was determined relative to strain HI4320 cultured in LB medium alone.

A. qRT-PCR validation of select HI4320 genes upregulated or downregulated during iron limitation *in vitro* as determined by microarray.

B. qRT-PCR validation of HI4320 putative yersiniabactin-related siderophore, *nrp* (PMI2596-2605) that was upregulated during iron limitation *in vitro* as determined by microarray.

C. qRT-PCR validation of HI4320 putative siderophore system proteobactin, *pbt* (PMI0229-0239) that was upregulated during iron limitation *in vitro* as determined by microarray (black bars) and downregulated following the addition of 25 μ M FeCl₃·6H₂O to an iron-chelated LB medium culture of bacteria (grey bars).

D. qRT-PCR confirmation of HI4320 siderophore biosynthesis genes *pbtA* (PMI0232) and *nrpR* (PMI2599) expression *in vivo*. mRNA, isolated from bacteria recovered from the urine of experimentally infected CBA/J mice, was subjected to qRT-PCR. A significant difference in expression levels (**P* = 0.0002) was determined using a two-tailed unpaired *t*-test.

pestis chromosome (*ybtD*) (Fig. 4A). Likewise, the 4'-phosphopantetheinyl transferase of *P. syringae* is absent from the yersiniabactin operon (Fig. 4A).

Proteus mirabilis is unable to grow on LB agar containing 25 μ M Desferal. However, in cross-feeding assays, *P. mirabilis* growth is restored (Fig. 5A, Table 3) by uropatho-

genic *E. coli* (UPEC) strain 536 that produces both enterobactin and yersiniabactin (Brzuszkiewicz *et al.*, 2006). This restoration of *P. mirabilis* growth was abolished when cross-fed by a UPEC 536 construct that lacks both enterobactin and yersiniabactin (Fig. 5C, Table 3). Unexpectedly, *P. mirabilis* was incapable of growth on iron-chelator

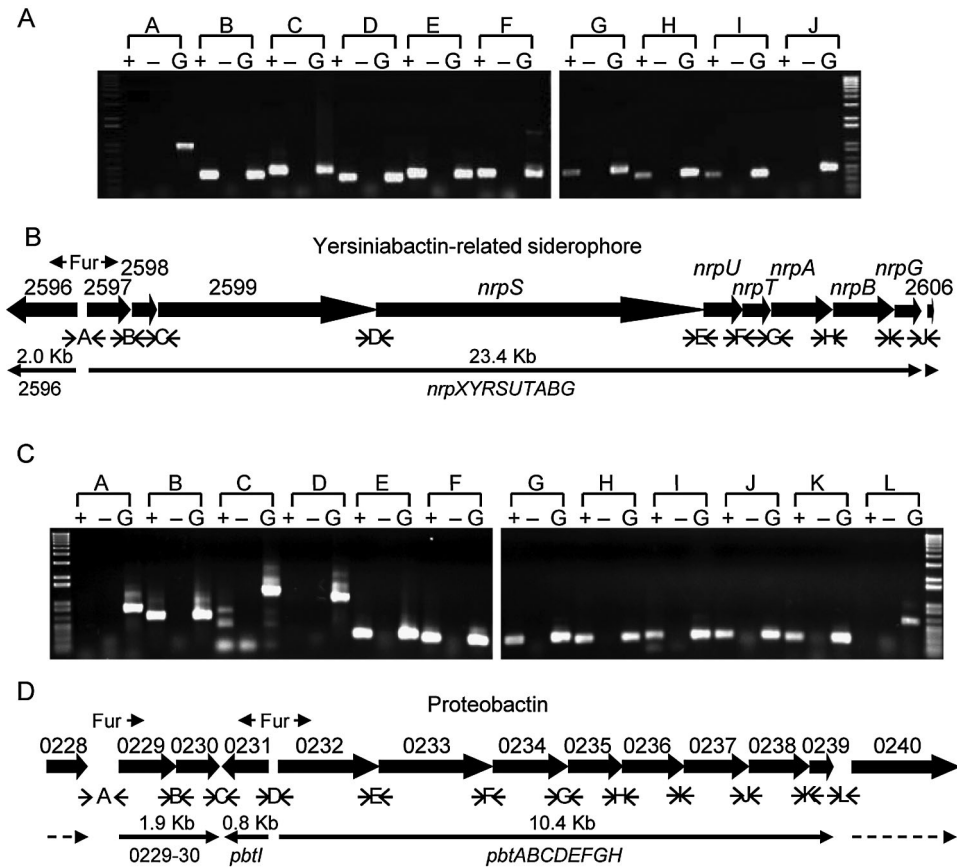


Fig. 3. Transcriptional organization of putative siderophore system PMI2596-2605 and PMI0229-0239 in *P. mirabilis* HI4320. A, C. mRNA for cDNA synthesis was isolated from wild-type HI4320 following culture in LB medium containing 15 μ M Desferal. For PCR reactions: +, reverse transcriptase added to cDNA synthesis reaction; -, no reverse transcriptase added, mRNA used as template; G, HI4320 genomic DNA used as template. B, D. Siderophore biosynthesis and transport gene clusters of HI4320 drawn to scale with primer design for RT-PCR operon mapping. Thick arrows represent open reading frames that indicate direction of transcription. Ferric uptake regulator (Fur) recognition sequences were identified based on homology to those of *P. aeruginosa* using a promoter analysis program in the Virtual Footprint software suite (Munch *et al.*, 2005). Location and direction of Fur recognition sequences are indicated. Intergenic primer pairs used for RT-PCR are represented by alphabetical letters. Thin solid arrows represent direction and span the length of genes that are required to make a complete transcript. Proposed gene nomenclature for transcripts are indicated below thin solid lines and size of transcript are indicated above the solid lines.

when cross-fed by the 536 strain that produces yersiniabactin and lacks only enterobactin (Fig. 5B, Table 3). In addition, UPEC CFT073, a strain incapable of synthesizing yersiniabactin (Bultreys *et al.*, 2006), can also restore the growth defect in *P. mirabilis* in cross-feeding assays (Fig. 5D, Table 3); in this assay, *P. mirabilis* swarms around *E. coli* CFT073. This ability of *P. mirabilis* to utilize enterobactin is independent of both the *npr* and *pbt* iron acquisition systems (Table 3). These surprising findings show that yersiniabactin is not able to be utilized by *P. mirabilis* and suggests that despite the clear evolutionary relationship, the *npr* locus is not involved in the production and uptake of yersiniabactin *per se*.

The lack of yersiniabactin production is also supported by a high-performance liquid chromatography (HPLC) analysis showing the absence of UV absorbance maxima

(310 and 385 nm) characteristic of yersiniabactin (Jones *et al.*, 2007) when concentrated supernatants from *P. mirabilis* cultured under iron limitation were analysed by HPLC and compared directly with concentrated supernatants of *E. coli* 536 mutants *entF::kan* and *entF ybtS::kan* (Fig. 6). Furthermore, extracts of the same experimental *P. mirabilis* HI4320 and control *E. coli* 536 cultures were prepared using previously reported methods (Bultreys *et al.*, 2006; Jones *et al.*, 2007) and analysed by ESI-LC-MS. Monitoring in positive ion mode, *m/z* matching that of a ferri-yersiniabactin species described by Jones *et al.* (2007) could be detected in *E. coli* 536 *entF::kan* (expected: 535.1, observed: 534.8); the same signal could not be detected from *E. coli* *entF ybtS::kan* negative control or *P. mirabilis* HI4320 samples (Fig. 6). These data support the assertion that *P. mirabilis npr*, while highly

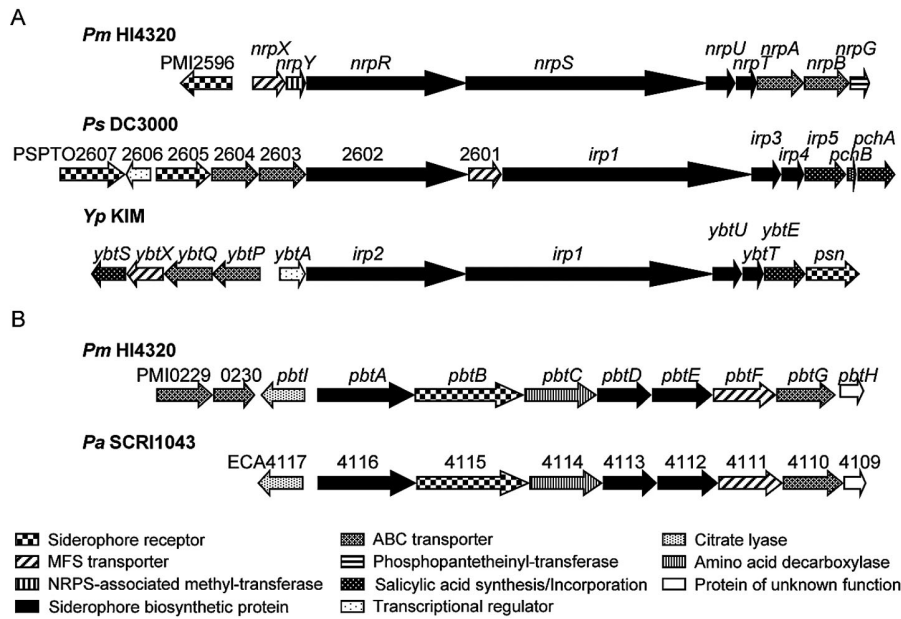


Fig. 4. The yersiniabactin-related siderophore and proteobactin of *P. mirabilis* are similar to siderophore biosynthesis and transport gene clusters in other *Enterobacteriaceae* as shown by gene sequence alignments.

A. *P. mirabilis* yersiniabactin-related siderophore is homologous to that of *P. syringae* DC3000 and *Y. pestis* KIM. The yersiniabactin-related siderophore of *P. mirabilis* lacks the genes involved in salicylic acid biosynthesis and incorporation, *ybtS* and *ybtE* of *Y. pestis* and *irp5*, *pchB* and *pchA* of *P. syringae*. The *nrp* locus of *P. mirabilis* also lacks a transcriptional regulator but encodes a NRPS-associated methyl-transferase and a phosphopantetheinyl-transferase, which are not located within the yersiniabactin operons of *P. syringae* and *Y. pestis*.

B. The newly identified NIS system of *P. mirabilis*, proteobactin, is similar to identical gene clusters found in *P. carotovora* ssp. *atroseptica* (shown) and *P. carotovora* ssp. *carotovora*.

similar to the yersiniabactin operon of other species, is incapable of producing a molecule identical to yersiniabactin.

Identification of a novel NIS system that synthesizes proteobactin

The second siderophore biosynthesis operon of *P. mirabilis* HI4320 encodes a novel NIS pathway that is predicted to produce a hydroxycarboxylate siderophore. The first two genes, PMI0229–0230, are predicted to encode an ABC transport permease protein and an ABC transport ATP binding subunit, and are expressed as a two gene transcript (Fig. 3C and D). *pbtI* (PMI0231) encodes a putative citrate lyase β -subunit and is transcribed as a single mRNA from the opposite coding strand (Fig. 3C and D). Citrate lyase β activity converts citric acid to oxaloacetate, which is a key biosynthetic precursor and could be the preferred substrate of the putative siderophore biosynthesis protein encoded by *pbtA* (PMI0232). Because oxaloacetate is a closely related metabolic intermediate to α -ketoglutarate, a well known substrate for NIS type B synthetases (Challis, 2005), it is probable that *pbtA* also belongs to the type B subfamily of NIS synthetases. The polycistron that contains the putative NIS synthetase

also codes for a putative TonB-dependent siderophore receptor, a putative lysine/ornithine decarboxylase, a putative pyridoxal-phosphate dependent enzyme, a putative octopine/opine/taurine dehydrogenase, a MFS-family transporter, a substrate binding protein and a hypothetical protein (Fig. 3C and D).

All known NIS biosynthetic pathways utilize at least one siderophore synthetase with homology to hydroxamate synthesis enzymes *lucA* (NIS type A) and *lucC* (NIS type C) (Challis, 2005). In line with this, the siderophore synthetase from this operon in *P. mirabilis* has 21% and 25% identity to *lucA* and *lucC* of UPEC respectively. Upon further examination, the closest homologue to this *P. mirabilis* hydroxamate synthetase is found in an uncharacterized siderophore system of the phytopathogens *Pectobacterium* (formerly *Erwinia*) *carotovora* ssp. *atroseptica* and *P. carotovora* ssp. *carotovora* (54% identity to both). The primary siderophore biosynthetic enzyme used by these systems and the *P. mirabilis* synthetase encoded by *PbtA* have only 27–28% identity to the achromobactin biosynthetic enzyme *AcsD*, which has recently been classified as the prototype member of a new family of enzymes, NIS type B, that synthesize a hydroxycarboxylate NIS in *Pectobacterium chrysanthemi* (Munzinger *et al.*, 2000; Franza *et al.*, 2005; Schmelz *et al.*, 2009).

Table 2. Similarity search between *P. mirabilis* and *Y. pestis*, *E. coli*, *P. syringae* and *P. luminescens*.

Gene (No. of amino acids)		% Identity (% similarity)	No. of amino acids
PMI2596 (662)	<i>Yp</i> KIM <i>psn</i>	27 (48)	673
	<i>Ec</i> 536 ECP_1947	27 (49)	673
	<i>Ps</i> DC3000 PSPTO2605	27 (47)	685
	<i>Pl</i> TT01 Plu2316	30 (46)	668
PMI2597 <i>nrpX</i> (409)	<i>Yp</i> KIM <i>ybtX</i>	39 (58)	467
	<i>Ec</i> 536 <i>ybtX</i>	38 (58)	462
	<i>Ps</i> DC3000 PSPTO2601	25 (46)	408
	<i>Pl</i> TT01 Plu2317	31 (53)	414
*PMI2598 <i>nrpY</i> (245)	<i>Yp</i> KIM y0042	34 (51)	260
	<i>Ec</i> 536 ECP_3773	34 (51)	260
	<i>Ps</i> DC3000 <i>argS</i>	26 (44)	578
	<i>Pl</i> TT01 Plu2237	40 (59)	315
PMI2599 <i>nrpR</i> (2034)	<i>Yp</i> KIM <i>irp2</i>	40 (59)	2035
	<i>Ec</i> 536 HMWP2	40 (59)	2035
	<i>Ps</i> DC3000 PSPTO2602	42 (59)	2057
	<i>Pl</i> TT01 Plu2320	41 (58)	2049
PMI2600 <i>nrpS</i> (3071)	<i>Yp</i> KIM <i>irp1</i>	50 (66)	3163
	<i>Ec</i> 536 HMWP1	50 (66)	3163
	<i>Ps</i> DC3000 <i>irp1</i>	52 (67)	3173
	<i>Pl</i> TT01 Plu2321	39 (56)	3908
PMI2601 <i>nrpU</i> (362)	<i>Yp</i> KIM <i>ybtU</i>	42 (64)	386
	<i>Ec</i> 536 <i>ybtU</i>	42 (64)	366
	<i>Ps</i> DC3000 <i>irp3</i>	44 (62)	360
	<i>Pl</i> TT01 Plu2322	38 (57)	365
PMI2602 <i>nrpT</i> (257)	<i>Yp</i> KIM <i>ybtT</i>	47 (58)	218
	<i>Ec</i> 536 <i>ybtT</i>	44 (57)	262
	<i>Ps</i> DC3000 <i>irp4</i>	48 (61)	271
	<i>Pl</i> TT01 Plu2323	47 (57)	258
PMI2603 <i>nrpB</i> (588)	<i>Yp</i> KIM <i>ybtP</i>	30 (53)	600
	<i>Ec</i> 536 <i>ybtP</i>	30 (53)	570
	<i>Ps</i> DC3000 PSPTO2604	45 (67)	593
	<i>Pl</i> TT01 Plu2318	31 (52)	572
PMI2604 <i>nrpA</i> (575)	<i>Yp</i> KIM <i>ybtQ</i>	32 (56)	600
	<i>Ec</i> 536 <i>ybtQ</i>	32 (57)	600
	<i>Ps</i> DC3000 PSPTO2603	46 (68)	581
	<i>Pl</i> TT01 Plu2319	31 (52)	600
*PMI2605 (249)	<i>Yp</i> KIM y1938	35 (52)	410
	<i>Ec</i> 536 ECP_1982	38 (62)	62
	<i>Ps</i> DC3000 <i>bphP</i>	43 (56)	745
	<i>Pl</i> TT01 Plu4193	31 (46)	237

**P. mirabilis* gene listed has homology to genes located outside of the yersiniabactin operon of other *Enterobacteriaceae*.

To determine which NIS synthetase subfamily the synthetase of proteobactin represents, 92 protein sequences homologous to siderophore synthetase PbtA, identified by BLASTP, were aligned using ClustalW (Thompson *et al.*, 1994) and used for phylogenetic analysis of the related NIS enzymes (Fig. 7). The resulting phylogeny clearly

identified the three known NIS subfamilies as distinct clades and placed PbtA within the same clade as Y4xN (Fig. 7), a siderophore synthetase of *Sinorhizobium fredii* described to be a type B NIS synthetase (Challis, 2005). This phylogenetic analysis suggests that the NIS of *P. mirabilis* is a type B NIS synthetase and the genes that

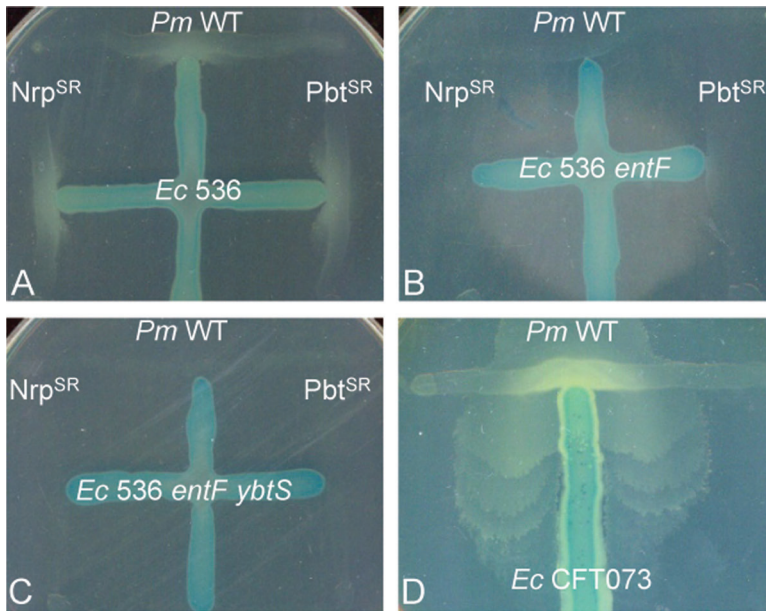


Fig. 5. *P. mirabilis* is unable to utilize yersiniabactin produced by UPEC 536. LB medium was supplemented with 25 μ M Desferal, the minimum concentration of chelator required to completely suppress growth of *P. mirabilis*. Lactose-fermenting *E. coli* are distinguished from *P. mirabilis* by the using the chromogenic indicator Xgal. A. Growth of wild-type *P. mirabilis*, the proteobactin synthesis/receptor mutant (Pbt^{SR}), and the yersiniabactin-related siderophore/receptor mutant (Nrp^{SR}) can be restored when cross-fed by *E. coli* 536 that produces both enterobactin and yersiniabactin. B, C. Restoration of *P. mirabilis* growth for all strains tested is abolished when cross-fed by the either *E. coli* 536 enterobactin mutant, *entF::kan*, or the enterobactin/yersiniabactin double mutant, *entF ybtS::kan*. D. *P. mirabilis* growth can be restored using *E. coli* CFT073, an enterobactin producing strain incapable of synthesizing yersiniabactin.

encode for the capacity to produce this NIS, proteobactin, are referred to as *pbtABCDEFGHI*, for proteobactin synthesis (Fig. 4B).

Siderophore production by *P. mirabilis* HI4320

To detect siderophore production by both the yersiniabactin-related and proteobactin systems, we examined *P. mirabilis* on chrome azurol S (CAS) agar and by the CAS assay. Following growth of HI4320 on CAS agar, a colour change of CAS from blue to orange, indicating iron chelation, was observed as a halo surrounding the bacteria. This effect was less pronounced than that caused by UPEC CFT073 (Fig. 8A). The iron chelation effect produced by wild-type HI4320 on CAS agar is similar to that of either proteobactin (*pbtA*) or yersiniabactin-related (*nrpR*) biosynthesis mutants (Fig. 8A). However, a double mutant

in both siderophore systems (*nrpR pbtA*) is unable to chelate iron from the dye (Fig. 8A). This iron-chelating defect in the double mutant is similar to *E. coli* CFT073 *entF::kan iucB::cam*, which does not produce siderophores (Torres *et al.*, 2001) (Fig. 8A).

To quantify the observed iron binding properties of both *P. mirabilis* proteobactin and the yersiniabactin-related siderophore, a soluble CAS assay was performed using 50-fold concentrated *P. mirabilis* supernatants from log-phase cells cultured in MOPS defined medium (Neidhardt *et al.*, 1974) with and without 0.1 mM $FeCl_3 \cdot 6H_2O$. Results of this quantitative CAS assay clearly demonstrate that when cultured under iron limitation, *P. mirabilis* produces and releases one or more molecules that chelate iron from the ferri-CAS complex ($P = 0.0001$) (Fig. 8B). This same result was seen for each *P. mirabilis* single siderophore biosynthesis mutant; presence of either proteobactin or the yersiniabactin-related siderophore was sufficient to chelate iron during iron limitation (indicated by a decrease in A_{630}) ($P < 0.0001$) (Fig. 8B). In contrast, significant iron chelation by the *P. mirabilis* double siderophore biosynthesis mutant was not observed during iron limitation (Fig. 8B). This is consistent with the iron-chelating defect seen with the *E. coli* CFT073 enterobactin/aerobactin double siderophore mutant *entF::kan iucB::cam*. Interestingly, iron chelation by either single synthetase mutant was modestly increased as compared with wild-type HI4320 ($P < 0.0001$) (Fig. 8B). Despite our best efforts to standardize bacterial cultures by optical density, it is possible that variation and siderophore stability during sample preparation could be responsible for this result. qRT-PCR analysis determined this phenotype is not due to the upregulation of one

Table 3. Cross-feeding of *P. mirabilis* wild-type and siderophore mutants by *E. coli* 536, 536 siderophore mutants and CFT073.

	Ability to cross-feed <i>P. mirabilis</i> strains			
	<i>E. coli</i> 536	<i>E. coli</i> 536 <i>entF::kan</i>	<i>E. coli</i> 536 <i>entF ybtS::kan</i>	<i>E. coli</i> CFT073
<i>P. mirabilis</i>	+	–	–	+
<i>P. mirabilis</i> Pbt^{SR}	+	–	–	+
<i>P. mirabilis</i> Nrp^{SR}	+	–	–	+

Strain noted as synthetase^S and receptor^R mutant in the proteobactin (*Pbt*) and/or yersiniabactin-related (*Nrp*) siderophore system. +, growth; –, no growth.

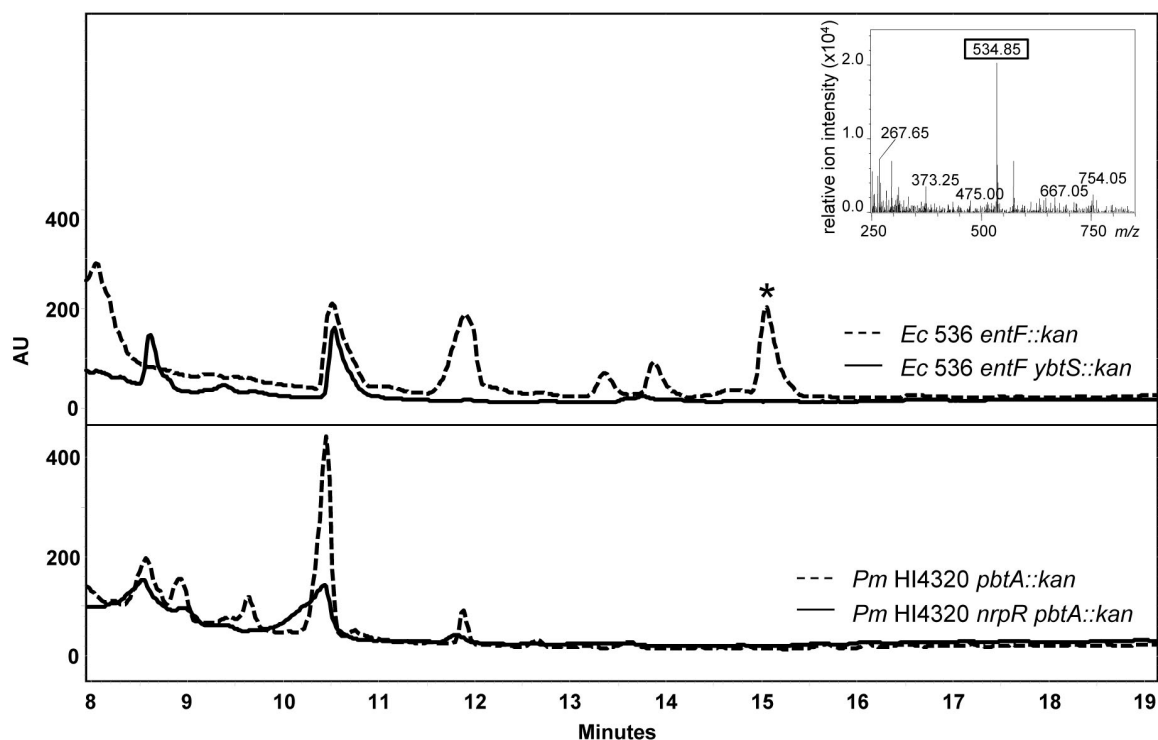


Fig. 6. Preparative HPLC and LC-MS analysis demonstrates *P. mirabilis* HI4230 is incapable of producing yersiniabactin. Preparative HPLC chromatogram (upper traces) performed on crude supernatant extracts from yersiniabactin-producing *E. coli* 536 *entF::kan* (dashed line) and yersiniabactin-deficient *entF ybtS::kan* (solid line) cultures to demonstrate production/lack of production of yersiniabactin. The lower traces represent chromatograms using the same HPLC conditions from *P. mirabilis* HI4230 *pbtA::kan* (dashed line) and *nrpR pbtA::kan* (solid line). The y-axis represents arbitrary units (AU) to offset sample traces. The inset indicates the LC-MS (positive ion mode) analysis of *E. coli* yersiniabactin present in the dominant peak (the asterisk) isolated at 15 min by HPLC. Iron-bound yersiniabactin is known to have an ion intensity at *m/z* of 535.1.

synthetase gene in the absence of the other synthetase gene (data not shown).

To determine whether the yersiniabactin-related siderophore and proteobactin are common in other *P. mirabilis* isolates, 10 urine isolates and 10 non-UTI isolates were screened by PCR for the presence of siderophore biosynthesis genes *pbtA* and *nrpR*. As expected, 100% (10/10) of the *P. mirabilis* urine isolates contain both *pbtA* and *nrpR* while only 50% (5/10) of the *P. mirabilis* non-UTI isolates contain both genes. Therefore, urine isolates are more likely to have both *pbtA* and *nrpR* ($P = 0.0325$) compared with *P. mirabilis* non-UTI isolates.

In vivo contribution of siderophore systems during ascending UTI

To determine the role of siderophores during murine ascending UTI, siderophore synthetase and TonB-dependent receptor mutants of proteobactin or the yersiniabactin-related siderophore were transurethrally inoculated into the bladders of CBA/J mice in independent and cochallenge competition experiments with wild-type

HI4320. In independent infections, the loss of the proteobactin siderophore system alone or a double mutant in both siderophore synthetase genes did not attenuate HI4320 (Table 4). During independent infection, the yersiniabactin-related synthetase/receptor mutant (Nrp^{SR}) appeared to colonize better than wild-type HI4320 ($P < 0.05$) (Table 4) where the median colony-forming units per gram (cfu g⁻¹) was less than one-half log greater for Nrp^{SR} than HI4320 (Fig. S2). In cochallenge competition experiments, the strain lacking only proteobactin did not have a fitness defect and loss of Pbt^{SR} may slightly increase fitness in the kidneys ($P = 0.034$) (Table 5). Interestingly, the yersiniabactin-related synthetase/receptor mutant (Nrp^{SR}) was significantly out-competed by wild-type in the bladder ($P = 0.004$) and kidneys ($P = 0.027$) (Table 5). Similarly, the yersiniabactin-related synthetase and proteobactin synthetase/receptor mutant (Nrp^SPbt^{SR}) was significantly out-competed by wild-type in the bladder ($P = 0.019$) (Table 5). These data, from both independent and cochallenge infections, consistently indicate that the yersiniabactin-related siderophore had a greater contribution for *P. mirabilis* fitness *in vivo*.

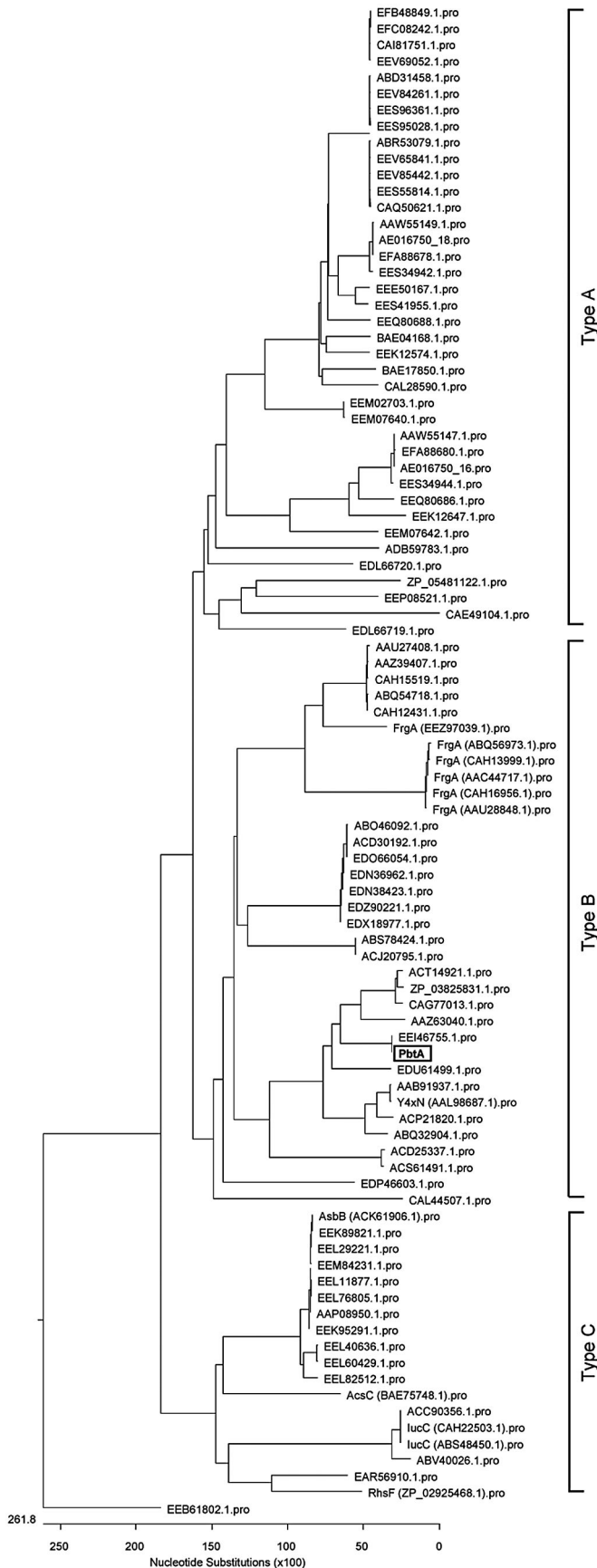


Fig. 7. Phylogenetic analysis of 92 homologous protein sequences to NIS synthetase PbtA involved in the assembly of proteobactin. The NIS synthetase enzymes are split into three subfamilies A, B and C. Proteobactin synthetase, PbtA (boxed) is placed with the type B subfamily clade of NIS synthetases, which includes Y4xN, a siderophore synthetase previously found to be homologous to other NIS synthetases of this group.

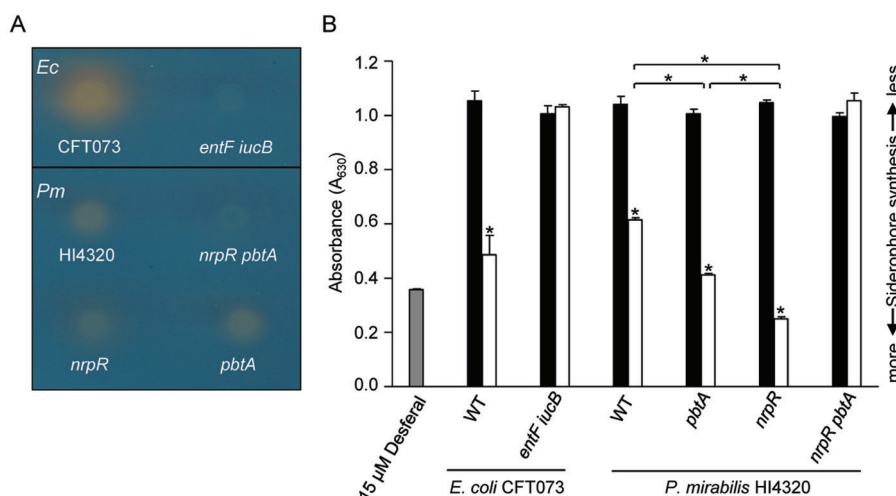


Fig. 8. Siderophore production by *P. mirabilis* HI4320.

A. Detectable siderophore production on CAS agar of overnight LB bacterial cultures spotted in 5 μl volumes. Blue to orange colour change of agar indicates siderophore production.

B. CAS assay depicting siderophore production from filtered bacterial supernatants following culture in MOPS defined medium with (black bars) and without (white bars) 0.1 mM FeCl₃·6H₂O. Only supernatants of *P. mirabilis* samples were concentrated 50-fold. Supernatant of *E. coli* CFT073 and double mutant, *entF::kan iucB::cam*, were not concentrated. Positive control of phosphate buffered saline containing 15 μM Desferal (grey bar). A lower absorbance (A₆₃₀) indicates greater siderophore production. Significant differences in absorbance (**P* ≤ 0.0022) was determined using a two-tailed unpaired *t*-test.

Discussion

It is well known that the urinary tract is iron-limited. Despite this, little is known about iron acquisition by the common uropathogen *P. mirabilis*. To improve our understanding of iron acquisition and identify potential siderophore systems utilized by this pathogen; we examined the genome-wide transcriptome from bacteria cultured under iron-replete and iron-deficient conditions. As expected, many genes predicted to encode iron acquisition systems or proteins were upregulated during iron limitation including *exbD* and three additional genes that have been previously identified by our STM screens (Burall *et al.*,

2004; Himpf *et al.*, 2008). The iron acquisition systems identified in the present study are grouped into functional classes that include genes required for haem uptake, aerobic ferrous iron uptake (Fisher *et al.*, 2009), ferric citrate transport, and siderophore biosynthesis and transport. These microarray findings may not comprehensively demonstrate expression of every iron acquisition system employed by *P. mirabilis* HI4320; however, this study has been useful to propose a model of iron acquisition and transport strategies utilized by this human pathogen (Fig. 9).

Numerous unsuccessful attempts to detect siderophores in *P. mirabilis* have led to speculation that *P. mira-*

Table 4. *In vivo* virulence of siderophore biosynthesis mutants following independent challenge experiments.

Strain defect ^a	Bladder		Kidneys	
	Median cfu	<i>P</i> -value ^b	Median cfu	<i>P</i> -value ^b
Wild-type	3.77 × 10 ⁶		4.01 × 10 ⁵	
Pbt ^{SR}	5.17 × 10 ⁷	0.208	2.72 × 10 ⁶	0.068
Wild-type	2.26 × 10 ⁷		6.29 × 10 ⁵	
Nrp ^{SR}	6.25 × 10 ⁷	0.045	2.28 × 10 ⁶	0.036
Wild-type	3.04 × 10 ⁷		2.15 × 10 ⁶	
Nrp ^{SR} Pbt ^{SR}	2.70 × 10 ⁷	0.741	3.00 × 10 ⁵	0.106

a. Strain defect noted as synthetase^S and/or receptor^R mutant in the proteobactin (Pbt) and/or yersiniabactin-related (Nrp) siderophore system.

b. *P*-value determined by Mann–Whitney *U*-test. Significant *P*-values are bolded.

Table 5. *In vivo* fitness of siderophore biosynthesis mutants following cochallenge competition experiments with *P. mirabilis* HI4320.

Strain defect ^a	Bladder		Kidneys	
	CI ^b	<i>P</i> -value ^c	CI ^b	<i>P</i> -value ^c
Pbt ^{SR}	0.195	0.850	0.040	0.034
Nrp ^{SR}	7.900	0.004	30.95	0.027
Nrp ^{SR} Pbt ^{SR}	2.069	0.019	1.470	0.051

a. Strain defect noted as synthetase^S and/or receptor^R mutant in the proteobactin (Pbt) and/or yersiniabactin-related (Nrp) siderophore system.

b. Competitive index, determined by dividing the ratio of the cfu of wild-type to mutant at 7 days post-infection by the ratio present in the input inoculum. Significant CI > 1 indicates mutant has a fitness defect.

c. CI *P*-value as determined by Wilcoxon signed-rank test. Significant *P*-values are bolded.

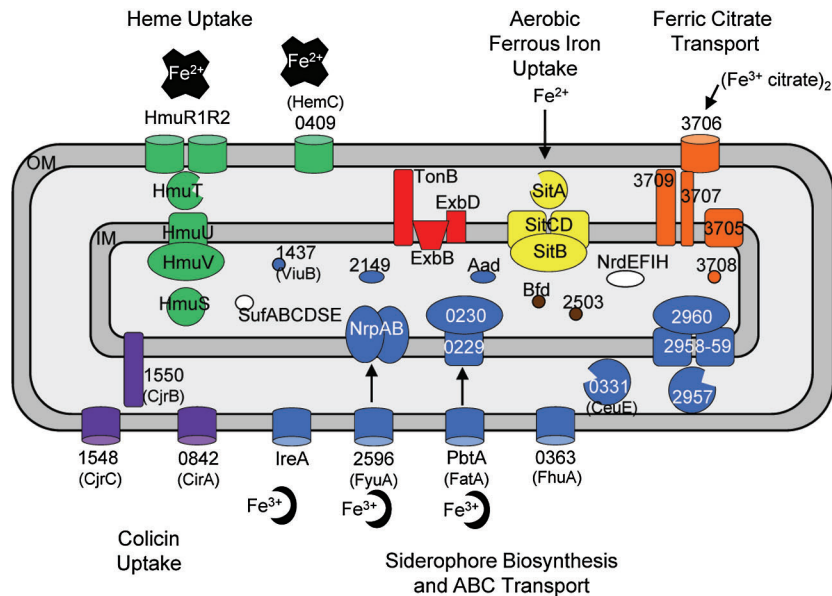


Fig. 9. A proposed model for iron acquisition systems in *P. mirabilis*. Hypothetical functions of the proteins listed are based on upregulation of their genes under iron limitation, homology with genes from other bacterial species, and functional studies conducted in this report and related bacterial species. Outer membrane (OM) receptors are dependent on the energy-transducing complex TonB-ExbB-ExbD (shown in red) for intracellular transport of iron. Haem uptake systems are displayed in green. Haem uptake may also be carried out by the OM receptor, HemC. Import of periplasmic ferrous iron is performed by the ABC transporter SitABCD (shown in yellow) located on the inner membrane (IM). A putative ferric citrate system (shown in orange) encodes a TonB-dependent receptor PMI3706, and accessory proteins. Proteins involved in siderophore biosynthesis and transport of ferri-siderophores are shown in blue. Ferri-siderophores are transported by OM receptors IreA, FyuA, FatA and FhuA. Ferri-siderophores transported to the periplasm are shuttled to IM transport proteins, NrpAB and PMI0229-0230. Additional putative ferri-siderophore transport proteins include the periplasmic binding protein, CeuE, and IM transport proteins, PMI2957-2960. Cellular proteins, Aad, PMI2149, ViuB and, may be involved in siderophore synthesis and the regulation and utilization of iron of ferri-siderophore. Putative TonB-dependent receptors possibly involved in colicin uptake are shown in purple. Cytoplasmic iron-containing proteins include *nrdEFIH* and *sufABCDSE* (shown in white). Bfd, an iron removal protein, and PMI2503 involved in iron metabolism are shown in brown.

bilis is a poor siderophore producer (Miles and Khimji, 1975; Marcelis *et al.*, 1978; Evanylo *et al.*, 1984; Massad *et al.*, 1995). However, the results of the microarray indicated the upregulation of a number of genes related to siderophore synthesis during iron limitation including a known and unknown amino acid deaminase *aad* and PMI2149 both of which could possibly chelate iron (Drechsel *et al.*, 1993; Massad *et al.*, 1995). Also, genes homologous to the transport and metabolism of ferri-siderophores, such as putative ABC substrate binding protein PMI0331, ABC transport system PMI2957-2960, putative iron utilization protein PMI1437 and putative esterase PMI2503 were upregulated during iron limitation. A total of four putative TonB-dependent ferri-siderophore receptors were upregulated during iron limitation: *ireA*, PMI0363, PMI2596 and PMI0233. Two of these ferri-siderophore receptors, PMI0233 and PMI2596, are located in regions that encode for both siderophore biosynthesis and ABC transport, which is a common feature of known ferri-siderophore systems (Koster, 2001; Crosa and Walsh, 2002; Wandersman and Delepelaire, 2004).

One of these *P. mirabilis* siderophore systems encodes the *nrp* operon and is closely related to the synthesis, uptake and ABC transport of yersiniabactin, a NRPS

siderophore encoded on the HPI of *Yersinia* spp. (Flanery *et al.*, 2009). Despite apparent homology and organization to yersiniabactin, cross-feeding assays indicate that *P. mirabilis* is unable to utilize yersiniabactin produced by UPEC strain 536 (Table 3, Fig. 5). This finding suggests that the *nrp* locus of *P. mirabilis* is functionally distinct from yersiniabactin. Indeed, the *nrp* synthesis and ABC transport genes of *P. mirabilis* differ equally from the known yersiniabactin synthesis and ABC transport genes of *Y. pestis* KIM, *E. coli* 536, *P. syringae* pv. tomato DC3000 and *Photobacterium luminescens* TT01 (Table 2). Previously, three yersiniabactin loci evolutionary groups have been described in *Y. pestis*, *P. syringae* and *P. luminescens* (Bultreys *et al.*, 2006). The *nrp* locus of *P. mirabilis* may likely encode a fourth yersiniabactin evolutionary group. Interestingly, the genes for salicylic acid biosynthesis and incorporation required for yersiniabactin synthesis in these *Enterobacteriaceae* are absent from the *P. mirabilis* *nrp* operon. Despite the absence of these genes, we have shown that the *nrp* system of *P. mirabilis* is able to chelate iron suggesting the salicylic acid biosynthesis genes may be located elsewhere on the chromosome or perhaps the *nrp* locus has evolved to synthesize a novel yersiniabactin-related siderophore that does not require

this modification. Furthermore, efforts to chemically detect yersiniabactin from *P. mirabilis* were unsuccessful (Fig. 6), supporting the possibility of an evolutionary divergence from producing traditional yersiniabactin.

In addition to the *nrp*/yersiniabactin-related system, *P. mirabilis* also has a previously uncharacterized NIS system. This newly identified NIS system, proteobactin, has similarity to NIS group A (*lucA*) and NIS group C (*lucC*) synthetases. Closer scrutiny reveals the proteobactin synthetase, PbtA, is most closely related to a predicted synthetase that is part of an uncharacterized siderophore system found in the phytopathogens, *P. carotovora* ssp. *atroseptica* and ssp. *carotovora*. Proteobactin and the molecules produced by these putative systems are distinct from achromobactin, a recently identified NIS system in *P. chrysanthemi* (Munzinger *et al.*, 2000; Franza *et al.*, 2005; Schmelz *et al.*, 2009). Furthermore, both these subspecies of *P. carotovora* and *P. mirabilis* do not possess the *acs* genes required for achromobactin synthesis suggesting proteobactin represents a new NIS system (Franza *et al.*, 2005). Phylogenetic analysis (Fig. 7) places PbtA among the type B subfamily clade of NIS synthetases. Other genes in the NIS operon potentially involved in the synthesis of proteobactin include *pbtD* and *pbtE*. PbtD is a putative pyridoxal-phosphate dependent enzyme and PbtE is a putative octopine/opine/taurpine dehydrogenase. The pyridoxal-phosphate dependent enzyme could be responsible for the incorporation or tailoring of thiol-containing moieties into the proteobactin hydroxycarboxylate siderophore (Kessler, 2006). The octopine/opine/taurpine dehydrogenase could be involved in modification of the primary amine of a siderophore precursor by condensing it to an enzymatically reduced pyruvate or similar small ketone. This may function in incorporating an additional chelating carboxylate to siderophore biosynthesis intermediates (Mihara *et al.*, 2005). Future biochemical studies will be useful to discern the molecular basis and biochemical function for the NIS genes and enzymes (Oves-Costales *et al.*, 2009).

Initial studies verified that proteobactin and the *nrp* genes were upregulated during *in vitro* iron limitation of *P. mirabilis* HI4320. Likewise, proteobactin and the yersiniabactin-related siderophore biosynthesis genes *pbtA* and *nrpR* were upregulated following experimental UTI of mice with HI4320, not only validating our *in vitro* findings but supporting prior evidence that the urinary tract is an iron-limiting environment and confirming that both siderophores are expressed *in vivo*. Interestingly, *P. mirabilis* urine isolates were significantly more likely to have both *pbtA* and *nrpR* ($P = 0.0325$) compared with *P. mirabilis* non-UTI isolates. This finding suggests that both siderophore systems are significantly more prevalent in *P. mirabilis* strains that infect the urinary tract than those that colonize other body sites.

The universal chemical assay to detect siderophore production independent of structure (Schwyn and Neilands, 1987) clearly shows that the yersiniabactin-related siderophore and proteobactin of *P. mirabilis* HI4320 chelate iron *in vitro* (Fig. 8). However, we speculate that iron chelation in this assay is inefficient compared with other species, such as *E. coli* CFT073. Unlike the robust conversion from blue to orange observed for *E. coli*, *P. mirabilis* generates a less intense, but nevertheless significant, colour change from blue to orange on CAS agar (Fig. 8A). Similarly, in quantitative liquid CAS assays, comparable levels of siderophore activity to *E. coli* CFT073 could only be detected when supernatant from *P. mirabilis* was concentrated 50-fold. A mutant with defects in both proteobactin and yersiniabactin-related biosynthesis (but not single mutants) abolished iron chelation, suggesting that both systems are producing functional molecules that chelate iron *in vitro* (Fig. 8A and B). Additional experiments determined that this phenotype was not due to a growth defect, as inactivation of both siderophore synthesis systems did not affect the *in vitro* growth of *P. mirabilis* in LB medium alone or in LB medium with 15 μ M Desferal (Fig. S1). During growth, the presence of other iron transport systems as identified by microarray could compensate for the loss of one or both siderophore biosynthesis systems (Table 1).

It is likely that the compensatory action of other iron acquisition and uptake systems in *P. mirabilis*, which were identified in the iron-limiting microarray play a role during uropathogenesis and will be the focus of future studies. In support of this, results of the cross-feeding assays depict *E. coli* CFT073, an enterobactin producing, yersiniabactin-negative strain, able to cross-feed *P. mirabilis* (Fig. 5D, Table 3). This suggests that *P. mirabilis*, while incapable of producing enterobactin, encodes a putative TonB-dependent receptor capable of importing enterobactin. Both the *P. mirabilis* NIS type B proteobactin synthesis/receptor mutant (Pbt^{SR}) and the yersiniabactin-related synthesis/receptor mutant (Nrp^{SR}) could utilize enterobactin (Fig. 5A, Table 3). These results show that *P. mirabilis* does possess a TonB-dependent receptor or other mechanism capable of importing enterobactin. Microarray findings support this conclusion, as at least two other putative TonB-dependent receptors, PMI0842 and *IreA*, were upregulated during the iron limitation. The TonB-dependent receptor *Iha* has been shown to function as a catechol siderophore receptor that mediates uptake of enterobactin (Leveille *et al.*, 2006). Both PMI0842 and *IreA* of *P. mirabilis* have 33% and 34% identity to *Iha* of *E. coli* respectively. It is likely that enterobactin is being taken up by these putative TonB-dependent receptors in *P. mirabilis*.

To investigate the role of these siderophore systems *in vivo*, independent and cochallenge competition experi-

ments were performed with the mutant strains and wild-type HI4320. Independent challenge experiments showed that loss of both siderophore synthesis genes did not attenuate virulence. During independent challenge the yersiniabactin-related synthetase/receptor mutant (Nrp^{SR}) was recovered at modestly higher levels than wild-type HI4320 in the bladder ($P = 0.045$) and kidneys ($P = 0.036$) (Table 4). This small difference in the median cfu g⁻¹ may not be biologically relevant as the ability of both Nrp^{SR} and wild-type to colonize the urinary tract was nearly indistinguishable (Fig. S2). Despite the lack of attenuation during independent infections we found that cochallenge competition experiments between mutant and wild-type strains revealed key differences in fitness. In these experiments the proteobactin synthetase/receptor mutant (Pbt^{SR}) was able to slightly out-perform wild-type HI4320 ($P = 0.034$) where a small difference in cfu was observed. However, these findings may also suggest the yersiniabactin-related system is predominant *in vivo* as this strain only produces the yersiniabactin-related siderophore. Consistent with this, only mutants lacking the yersiniabactin-related siderophore reduce *P. mirabilis* fitness *in vivo* (Table 5). This also agrees with a previous study from our lab, where a STM screen identified another yersiniabactin-related gene, *nrpG*, as being out-competed by wild-type HI4320 (Himpfl *et al.*, 2008).

This study is the first genome-wide view of the *P. mirabilis* HI4320 response to iron limitation. These iron-limiting microarray findings identified systems important in ferri-siderophore uptake, haem uptake, aerobic ferrous iron uptake (Fisher *et al.*, 2009) and ferric citrate transport (Fig. 9). This study is also the first report to demonstrate siderophore production both *in vitro* and *in vivo* by two distinct iron acquisition systems in *P. mirabilis* HI4320. Together, proteobactin and the yersiniabactin-related siderophore system, along with the numerous potential iron acquisition and transport systems identified by our microarray analysis shows that *P. mirabilis* employs a general strategy of encoding multiple systems with similar function like other extraintestinal pathogens, including UPEC.

Experimental procedures

Bacterial strains and culture conditions

Proteus mirabilis HI4230 and 10 additional *P. mirabilis* caUTI isolates were isolated from urine samples from patients presenting with bacteriuria during long-term catheterization (Warren *et al.*, 1982; Mobley and Warren, 1987). *P. mirabilis* colonizing strains isolated from the anterior nares, oropharynx, groin or wound sources (non-UTI isolates) have been previously described (Mody *et al.*, 2007). *E. coli* CFT073 was isolated from the blood and urine of a patient with acute pyelonephritis (Mobley *et al.*, 1990). The *E. coli* CFT073

enterobactin and aerobactin mutant, *entF::kan iucB::cam*, has been previously described (Torres *et al.*, 2001). *E. coli* 536 has been previously described (Berger *et al.*, 1982). *E. coli* 536 *entF::kan* and *entF ybtS::kan* deletion mutants (E.C. Hagan and H.L. Mobley, unpublished) were constructed using the lambda Red recombinase system (Datsenko and Wanner, 2000). Bacteria were routinely cultured in LB medium (per litre, 0.5 g NaCl, 5.0 g yeast extract, 10.0 g tryptone) or on LB agar (15.0 g l⁻¹ agar). Iron-limiting medium was prepared by adding 15 µM deferoxamine mesylate (Desferal) (Sigma) to LB medium. Desferal was chosen for this study based upon previous findings in which *P. mirabilis* urinary tract isolates were found to be highly susceptible to this agent (Lowy *et al.*, 1984). We determined a concentration of 15 µM Desferal in LB medium that decreased the growth rate of wild-type in comparison with wild-type grown in LB medium alone (Fig. 1). Growth curves were performed using a Bioscreen growth curve analyser (Growth Curves) following culture of bacterial samples in triplicate. Error bars were calculated by the standard error of the mean. Iron-limited LB agar supplemented with 25 µM Desferal (Sigma) restricted *P. mirabilis* growth for siderophore cross-feeding assays. Neidhardt MOPS Minimal Medium (Neidhardt *et al.*, 1974) with and without 0.1 mM FeCl₃·6H₂O was used for culture of bacterial strains. For growth of *P. mirabilis*, Neidhardt MOPS Minimal Medium was supplemented with 1 ml 1% nicotinic acid, 1 ml 1 M MgSO₄·7H₂O, 10 ml 20% glycerol and 1 ml 20% chelex-treated casamino acids per litre. CAS agar and CAS shuttle solution was prepared as previously described (Schwyn and Neilands, 1987). Antibiotics were added as necessary at the following concentrations: kanamycin (25 µg ml⁻¹), tetracycline (15 µg ml⁻¹), ampicillin (50 µg ml⁻¹) and chloramphenicol (20 µg ml⁻¹).

Microarray analysis

70-mer oligonucleotides representing open reading frames within the genome of *P. mirabilis* HI4320 were spotted (Microarrays) in triplicate onto UltraGAPSII slides (Corning) (Flannery *et al.*, 2009; Pearson *et al.*, 2010). Overnight LB medium cultures of HI4320 were diluted 1:100 in fresh LB medium or LB medium containing 15 µM of Desferal and incubated for three hours at 37°C, or until the culture reached an OD₆₀₀ of 1.0. Cultures were treated with 2 volumes of RNAprotect (Qiagen), incubated at room temperature for five minutes to stabilize RNA and centrifuged for (10 min, 8000 r.p.m., 25°C). RNA (4 µg) was purified (Qiagen) and DNase-treated (Ambion) as described above. First strand cDNA synthesis reactions, labelling of cDNA with CyDyes and hybridization to the microarray slides were performed following the protocols; ftp://ftp.jcvi.org/pub/data/PFGRC/pdf_files/protocols/M007.pdf and ftp://ftp.jcvi.org/pub/data/PFGRC/pdf_files/protocols/M008.pdf, found on The Institute for Genomic Research website. The microarray was scanned by a ScanArray Express Microarray Scanner (Perkin Elmer) at 10 µm resolution.

Five independent RNA samples were subjected to microarray analysis. The cyanine 3 or cyanine 5 dye was swapped for each condition for two runs. Spot intensities were extracted and LOWESS normalized with ScanArray Express v 4.0 (Perkin Elmer) software, spot replicates were merged

with Microarray Data Analysis System software and statistically analysed using Multiexperiment Viewer (MeV) software (Saeed *et al.*, 2003). Using MeV software, a one-class unpaired test was performed by SAM to identify genes significantly upregulated or downregulated in response to *in vitro* iron limitation (Tusher *et al.*, 2001; Saeed *et al.*, 2003). The False Discovery Rate was manually set to zero. Fold change of gene expression is presented as the \log_2 transformation of the ratio of the transcript level of *P. mirabilis* growth in LB medium and LB medium with Desferal, averaged across the five experiments. Microarray data are accessible through the Gene Expression Omnibus database at with Accession No. GSE18051.

RNA preparation and qRT-PCR

Overnight bacterial cultures were diluted 1:100 in 5 ml of fresh medium and incubated at 37°C until an OD₆₀₀ of 1.0 was reached. A 500 µl volume of cells was treated with 62.5 µl of 5% phenol-ethanol stop solution and incubated at room temperature for 10 min. For RNA from *in vivo* urine samples, urine was collected into RNeasy Protect (Qiagen) following manufacturer's recommendation. Bacterial suspensions were centrifuged (5 min, 8000 r.p.m., 4°C) and RNA was purified using an RNeasy (Qiagen) column following the manufacturer's protocol. Contaminating genomic DNA was removed with TURBO DNA-free (Ambion) following the manufacturer's protocol. Lack of genomic DNA contamination was determined by PCR analysis using primers to amplify the housekeeping gene *rpoA* prior to cDNA synthesis.

cDNA synthesis of total RNA was performed using Super-Script II first strand synthesis kit (Invitrogen) using 50 ng of random hexamers per 5 µg total RNA following the manufacturer's protocol. cDNA was purified on a QIAquick column (Qiagen) according to the manufacturer's protocol and eluted in 30 µl of the supplied elution buffer. Purified cDNA was quantified using a NanoDrop spectrophotometer. Transcripts were quantified on a MX3000P real-time PCR machine (Stratagene) using Brilliant SYBR green QPCR master mix (Stratagene) in 25 µl reactions containing up to 30 ng of total cDNA. Real-time oligonucleotide sequences of genes were designed using the PrimerQuest program [Steve Rozen, Helen J. Skaletsky (1996, 1997, 1998)] on the Integrated DNA Technologies website and are listed in Table S3. Transcript levels were normalized to the housekeeping gene *rpoA* and \log_2 fold change was determined relative to an experiment-specific calibrator using the MXPro v 4.1 software package (Stratagene). Error bars were calculated by the standard error of the mean and statistical analysis was performed using an unpaired *t*-test.

RT-PCR

Transcriptional organization of siderophore biosynthesis and ABC transport gene clusters, PMI0229-0239 and PMI2596-2605, was examined by RT-PCR from mRNA isolated following culture of *P. mirabilis* HI4320 in LB medium with 15 µM Desferal. RNA purification, DNase-treatment and cDNA synthesis was performed as previously described. A total of 30 ng of cDNA or genomic DNA was used in 25 µl reactions.

The intergenic regions spanning genes within these systems were amplified to identify contiguous genes on the same transcript.

Mutant construction

To test siderophore production and colonization in the murine model of ascending UTI, single mutations and a double mutation of PMI0232 and PMI2599, a putative siderophore biosynthesis protein and a putative non-ribosomal peptide synthase respectively, were constructed using the TargeTron Gene Knockout System (Sigma) following a modified protocol (Pearson and Mobley, 2007). Oligonucleotides for mutant design were created using the TargeTron Design Site (Sigma) and are listed in Table S1. PCR confirmation of mutants was performed using oligonucleotides designed with the PrimerQuest program as previously described and are listed in Table S1. Mutants constructed are identified as *pbtA::kan*, *nrpR::kan*, *nrpR pbtA::kan* and *nrpR 2596::kan*; the last two designations for these mutants refer to the *nrpR* insertion mutant with the kanamycin marker deleted and the *pbtA* or PMI2596 insertion mutant with the kanamycin marker retained. The kanamycin resistance cassette was removed from the intron by IPTG induction of the cre recombinase in pQL123 (Liu *et al.*, 1998).

Detection of siderophore production

Bacteria, cultured in LB medium with aeration at 37°C to stationary phase, were spotted in 5 µl volumes onto CAS agar plates (Schwyn and Neilands, 1987) and incubated at 30°C for 18 h. A visible colour change of agar from blue to orange around bacterial growth indicates siderophore production. *E. coli* CFT073 and *E. coli* CFT073 *entF::kan iucB::cam*, an enterobactin aerobactin double siderophore biosynthesis mutant, were used as positive and negative controls respectively.

For the CAS assay, overnight bacterial cultures in LB medium were diluted 1:100 into MOPS defined medium with 0.1 mM FeCl₃·6H₂O. Cells were grown to stationary phase, harvested by centrifugation (5 min, 10 000 r.p.m., 4°C) and washed before diluting 1:100 into fresh MOPS buffer with and without 0.1 mM FeCl₃·6H₂O. Following growth to OD₆₀₀ = 1.0, cells were centrifuged (20 min, 5000 r.p.m., 4°C), supernatant was collected and passed through a 0.22 µm filter (Millipore). Wild-type and mutant samples of *P. mirabilis* were lyophilized in 50 ml volumes and subsequently resuspended in 1 ml of phosphate buffered saline. A total of 150 µl of siderophore-containing supernatant was mixed with 150 µl of CAS shuttle solution as previously described (Schwyn and Neilands, 1987). Phosphate buffered saline was used for resuspension of lyophilized supernatant and phosphate buffered saline containing 15 µM Desferal served as a reference for *P. mirabilis* samples and a positive control respectively. Following one hour incubation at room temperature, samples were measured at an absorbance of 630 nm using a Micro-Quant Spectrophotometer (Bio-Tek). Each sample was measured in triplicate. Absorbance readings were calculated by dividing the samples' averaged absorbance by the absorbance of the reference. Error bars were calculated by the

standard error of the mean and statistical analysis was performed using an unpaired *t*-test.

Presence of *pbt* and *nrp* genes in other Enterobacteriaceae

Proteus mirabilis isolates were screened by PCR for the presence or absence of genes, PMI0232 and PMI2599, a putative siderophore biosynthesis protein and a putative non-ribosomal peptide synthase respectively, using genomic DNA (Flannery *et al.*, 2009). Forward and reverse qRT-PCR primers, *pbtA* and 2599 (Table S3), were used to amplify within the open reading frame of both genes using 60 ng of gDNA as template. Primers for amplification of PMI3255 were used as a positive DNA control as previously described (Flannery *et al.*, 2009). Associations were determined by using the Fisher exact test (GraphPad Prism).

For sequence analysis of the proteobactin synthesis protein PbtA, protein sequences homologous to PbtA were identified by BLAST (Altschul *et al.*, 1990). The resulting 92 sequences were input into MegAlign (DNASTAR) and subjected to multiple alignment using ClustalW (Thompson *et al.*, 1994) using a BLOSUM protein weight matrix and a gap penalty of 10.00. The results of the multiple alignment were used to generate a phylogenetic cladogram where the branch distances correspond to sequence divergence. The cladogram was visualized by bootstrap analysis comparing tree constructs using the neighbour-joining methodology performed with 10 000 trials initialized with a random seed.

Cross-feeding assays

Cross-feeding assays were performed to determine whether yersiniabactin produced by other bacterial species could cross-feed *P. mirabilis* HI4320. LB agar was supplemented with 25 μ M Desferal, an iron-limited condition in which *P. mirabilis* was unable to grow. For cross-feeding assays, wild-type *P. mirabilis*, the proteobactin synthesis/receptor and mutant (Pbt^{SR}), and the yersiniabactin-related synthesis/receptor mutant (Nrp^{SR}) were inoculated from overnight LB medium cultures onto the surface of the iron-limited plate using a sterile 1 μ l loop. *E. coli* CFT073, *E. coli* 536 and *E. coli* 536 mutant strains *entF::kan* and *entF ybtS::kan* were cross-struck from LB medium perpendicular to *P. mirabilis* strains. Xgal (40 μ g ml⁻¹) was added to the iron-limited plates to distinguish growth of the *lacZ* positive, *E. coli* 536 and CFT073, and *lacZ* negative, *P. mirabilis*, strains. Following incubation at 37°C for 18 h plates were scored for promotion of growth of the *P. mirabilis* strains.

Chromatographic detection of yersiniabactin

A final concentration of 100 μ M FeCl₃·6H₂O was added to supernatants of previously described MOPS iron-depleted cultures of *E. coli* 536 mutants *entF::kan* and *entF ybtS::kan* and *P. mirabilis* mutants *pbtA::kan* and *nrpR pbtA::kan*. 50 ml of each sample were then evaporated to dryness and redissolved in 2 ml of methanol. 500 μ l of this concentrated preparation was injected onto a Beckman-Coulter System Gold

HPLC in line with a Waters XBridge C₁₈ (5 μ m, 10 \times 250 mm) column running the following method: 5% acetonitrile (MeCN) in ddH₂O for 1 min followed by a linear increase to 90% MeCN over 20 min and a final 3 min 100% MeCN wash step. Peaks not shared between all samples were collected for further investigation. Prior to LC-MS analysis, concentrated supernatant samples were diluted with an equal volume of additional methanol and all insoluble material was centrifuged out of solution. 2 μ l of the remaining supernatant was injected onto a Shimadzu electrospray ionization (ESI) LCMS-2010A mass spectrometer in line with an HPLC system using a Phenomenex Luna C₁₈ (1.5 \times 250 mm) column and the following elution method: 5% (MeCN) in ddH₂O for 10 min (over which time material was diverted from the detector) followed by a jump to 20% MeCN and increasing by a linear gradient to 95% MeCN over 30 min. Detection occurred in positive scanning and selected ion monitoring modes. Collected peaks from previous HPLC analysis were evaporated under N₂, redissolved in methanol and analysed by LC-MS in an identical manner.

CBA/J mouse model of ascending UTI

The CBA mouse model of ascending UTI (Hagberg *et al.*, 1983; Johnson *et al.*, 1987) was used to assess the contribution to fitness and virulence of the siderophore biosynthesis and ABC transport systems. For cochallenge competition (to determine fitness) experiments, 6–8 week old female CBA/J mice (20–22 g; Jackson Laboratories) were transurethrally infected with a 50 μ l bacterial suspension of 5 \times 10⁷ cfu containing a 1:1 ratio of *P. mirabilis* HI4320 and *P. mirabilis* antibiotic-resistant mutant. For independent challenge (to determine virulence) mice were infected with wild-type HI4320 alone or mutant alone. Urine was collected and pooled from mice infected with wild-type *P. mirabilis* HI4320 at 24 h, 48 h and 5 days post-infection for transcriptional analysis. Mice were euthanized 7 days post infection. Bladder and kidneys were collected, homogenized and plated on plain LB agar and/or LB agar with antibiotic (Auto-plate 4000, Spiral Biotech). Viable colony counts were enumerated using a QCount (Spiral Biotech) and expressed as cfu g⁻¹ of tissue. For cochallenge competition experiments, wild-type infection was determined by subtracting the number of colonies on LB agar containing antibiotic from the number of colonies on plain LB agar. Competitive indices (CI) were calculated by dividing the ratio of the cfu of wild-type to mutant recovered from mice following infection by the ratio of the cfu of wild-type HI4320 to the cfu of mutant present in the input. A CI > 1 indicates the wild-type out-competes the mutant strain and a CI < 1 indicates the wild-type is out-competed by the mutant. The Mann–Whitney *U*-test was used to determine the statistical significance of data obtained in independent challenge experiments and cochallenge competition experiments were analysed using the Wilcoxon signed-rank test.

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Supporting information

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