Analysis of the aerobactin and ferric hydroxamate uptake systems of *Yersinia pestis*

Stanislav Forman, Michal J. Nagiec,[†] Jennifer Abney, Robert D. Perry and Jacqueline D. Fetherston

Department of Microbiology, Immunology and Molecular Genetics, University of Kentucky, Lexington, KY 40536-0298, USA

Correspondence
Stanislav Forman
standa@uky.edu

Yersinia pestis genomes contain genes homologous to the aerobactin receptor (*iutA*) and biosynthetic genes (*iucABCD*) as well as the ferric hydroxamate uptake system (*fhuCDB*) of *Escherichia coli*. However, *iucA* is disrupted by a frameshift mutation. An *E. coli* strain carrying the cloned *Y. pestis* aerobactin region was unable to produce aerobactin, but could use the siderophore as an iron source. Repair of the frameshift mutation in *iucA* did not allow aerobactin production in *E. coli* or *Y. pestis*. In contrast, a *Y. pestis* strain with a plasmid encoding the *iucABCD-iutA* genes from *Shigella flexneri* or pColV-K30 did produce and secrete the siderophore. In addition, *Yersinia pseudotuberculosis* PB1, which encodes the *iucABCD-iutA* locus without the *Y. pestis*-specific frameshift mutation, also failed to produce aerobactin. The *Y. pestis* *fhuCDB* operon, encoding an ABC transporter for a range of hydroxamate siderophores, was able to complement a strain of *E. coli* with a transposon insertion in *fhuC*, allowing utilization of aerobactin and ferrichrome. *Y. pestis* KIM6, a strain deficient in the production of the siderophore yersiniabactin, was able to use both the ferrichrome and the aerobactin siderophores as a source of iron. Mutations in *iutA* or the *fhu* operon abolished the ability of KIM6 to use aerobactin. Mutations in the *fhu* operon, but not in *iutA*, affected the ability of KIM6 to use ferrichrome. This demonstrates that *Y. pestis* uses both ferrichrome and aerobactin, but has lost the ability to synthesize aerobactin.

Received 13 November 2006
Revised 5 February 2007
Accepted 22 February 2007

INTRODUCTION

Like many other bacterial species (Crosa *et al.*, 2004), *Yersinia pestis* contains a plethora of iron/haem transport systems (Perry, 2004; Perry & Fetherston, 2004). There are two haem transport systems, designated Hmu and Has. The *Y. pestis* Has system resembles the well-characterized Has haemophore transport system of *Serratia marcescens* (Debarieux & Wandersman, 2004). However, the *Y. pestis* Has system does not appear to be functional (Rossi *et al.*, 2001). The Hmu system is virtually identical to the Hem haem transport system that was originally identified and characterized in *Yersinia enterocolitica* (Stojiljkovic & Hantke, 1992, 1994), and is the haem transport system in *Y. pestis* responsible for utilization of haem and haemoproteins (Hornung *et al.*, 1996; Thompson *et al.*, 1999). There are five *Y. pestis* systems that have been shown to transport iron: Ybt, Yfe, Yfu, Feo and Yiu. The

yersiniabactin (Ybt) system, which has been extensively studied, synthesizes and transports the siderophore yersiniabactin. Feo appears to transport ferrous iron in a number of organisms, including *Y. pestis* (Perry *et al.*, 2007). The Yfe, Yfu and Yiu systems are all ABC transporters that were originally identified by their ability to enhance the growth of an *Escherichia coli* enterobactin-biosynthetic mutant under iron-deficient conditions and were subsequently shown to function in *Y. pestis* as well (Bearden & Perry, 1999; Bearden *et al.*, 1998; Gong *et al.*, 2001; Hantke, 2004; Kirillina *et al.*, 2006; Perry, 2004; Perry & Fetherston, 2004).

Here we examine the functionality of two additional systems, one homologous to the aerobactin system of *E. coli* and another to the ferric hydroxamate uptake (Fhu) system of *E. coli*. The *E. coli* aerobactin biosynthetic operon consists of four genes, *iucABCD*, with the receptor encoded by a separate gene, *iutA* (Payne & Mey, 2004). In *Y. pestis*, *iucA* has undergone a frameshift, generating two separate open reading frames [*iucA1* (y3380) and *iucA2* (y3381) in strain KIM10+] (Deng *et al.*, 2002). The remaining genes are intact and the proteins encoded share 67–74% similarity with their *E. coli* counterparts (Fig. 1). Our results indicate that the *Y. pestis* aerobactin

[†]Present address: Interdisciplinary Program in Biomedical Sciences, UNC-Chapel Hill, CB#7100, Chapel Hill, NC 27599-7100, USA.

Abbreviations: DIP, 2,2'-dipyridyl; FBS, Fur-binding site.

The list of the oligonucleotides used in this study and alignments of aerobactin receptor and biosynthesis proteins are available as supplementary data with the online version of this paper.

Fig. 1. Genetic organization of the *Y. pestis iuc* and *fhu* loci. (a) Map of the *Y. pestis* aerobactin locus. Arrows denote the location and size of ORFs. The *Clal* sites used to generate a deletion within *iutA* are shown. The asterisk indicates the *Clal* site that is not digested due to methylation. The upstream ORF y3378, designated *shiF* in *E. coli* CFT073 (Welch *et al.*, 2002), is also included. Although homologues of this ORF are upstream of aerobactin loci in other organisms, there is no proven function for this gene product. The small, annotated y3379 ORF is 180 bp. (b) Map of the *fhu* operon of *Y. pestis*. Arrows indicate the location and size of putative ORFs for *fhuCDB*. The *EcoRV* sites were used to create a deletion within the *fhu* operon. Comparisons of *Y. pestis* (*Yp*) and *E. coli* (*Ec*) ORF lengths and percentage similarities are shown below each genetic map. Predicted amino acid sequences are from the genome sequence of *Y. pestis* KIM10+ (Deng *et al.*, 2002). The *E. coli* aerobactin and *Fhu* sequences are from pColV-K30 (Herrero *et al.*, 1988; Krone *et al.*, 1985; Martinez *et al.*, 1994) and *E. coli* K-12 MG1655 (Blattner *et al.*, 1997), respectively.

biosynthetic gene products are unable to produce aerobactin or complement an *E. coli iucB* : : *cam* mutant. The Fhu system of *Y. pestis* consists of three genes, *fhuCDB*, which closely resemble the corresponding genes in *E. coli* (Braun *et al.*, 2004). *Y. pestis* lacks a gene homologous to *fhuA*, the *E. coli* receptor for ferrichrome, but does contain a gene (y2556) that is 85 % identical to the ferrichrome receptor of *Y. enterocolitica* (FcuA) (Koebnik *et al.*, 1993). The *Y. pestis* aerobactin receptor (*IutA*) as well as the putative FcuA ferrichrome receptor (y2556) and the Fhu ABC transporter are functional, since we show here that *Y. pestis* can utilize exogenous aerobactin and ferrichrome.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Unless indicated otherwise, *E.*

coli strains were grown in liquid or solidified Luria broth (LB). *Y. pestis* strains were grown on tryptose blood agar (TBA) plates. Retention of the *pgm* locus was confirmed for *Y. pestis* by culture on Congo red plates at 26–30 °C (Jackson & Burrows, 1956).

Plasmid construction. Sequences of all oligonucleotides used in this study are presented in Supplementary Table S1 (available with the online version of this paper). Primers Iuc-3 and Iuc-4 were used to amplify a 539 bp digoxigenin (DIG)-labelled fragment from the aerobactin locus of KIM6+ genomic DNA. Colony blot hybridization of the DIG-labelled fragment to a KIM6+ genomic DNA library (Perry *et al.*, 1990) resulted in the identification of clone 1985 (renamed pAero1985; Table 1). Based upon restriction enzyme analysis and DNA sequencing, pAero1985 contains a 9035 bp insert that encompasses the putative aerobactin biosynthetic genes (*iucA1*-*A2*-*B*-*C*-*D*) and the outer-membrane receptor (*iutA*) (data not shown). The ends of the insert in subclone pAeroYp1 (Table 1) were sequenced (Northwestern University Biotechnology Laboratory) using primers HCW and SCCW, which bind to sequences in the pBR322 vector.

In *Y. pestis* strains KIM10+, CO92, 91001, Angola, Antiqua and Nepal516, *iucA* has undergone a frameshift mutation (Deng *et al.*, 2002; Parkhill *et al.*, 2001; Song *et al.*, 2004). The frameshift mutation in *Y. pestis iucA1/iucA2* is the result of a single base deletion in the L179 codon. To correct this, primers IucA(G)01 and IucA(G)02 in combination with outside primers IucA(*SalI*) and IucA(*BsrGI*) with pAeroYp1 as a template were used to generate a PCR fragment with a corrected *iucA* gene. We used a two-step PCR cloning procedure (Forman *et al.*, 2006) to construct and introduce the repaired *SalI*-*BsrGI* fragment into pAeroYp1, generating pAeroYp4 (*iucA*-*D-iutA*⁺).

Comparison of the *Y. pestis* IucA amino acid sequence with that of *Yersinia pseudotuberculosis* IucA, which does not have a frameshift in any of the three genomes sequenced, showed a substitution of the first base in the codon (TAT→CAT), which results in a Y225H change in IucA2 of *Y. pestis*. To reverse this change, a two-step PCR cloning procedure, with pAeroYp1 as the primary target DNA and the primer set IucA(Tyr)01/IucA(Tyr)02 and the outside primers IucA(*SalI*)/IucA(*BsrGI*), was used to generate a PCR fragment with an altered *iucA*-H225Y. The resulting *SalI*-*BsrGI* product was ligated into pAeroYp4 to generate pAeroYp5. This plasmid encodes the entire *iucA*-*D-iutA* locus with an *iucA*-H225Y similar to the *Y. pseudotuberculosis iucA* gene. All final *iuc* locus constructs were sequenced by Elim Biopharmaceuticals to confirm that the sequences were correct.

The 139 bp *iuc* promoter region, between divergent ORFs *iucA* and y3378, was amplified by PCR from pAeroYp1 using primers iucP1 and iucP2. The PCR products were digested with *Ascl* and cloned into the *Ascl* site of pNEB193. The *Ascl* fragment of one clone (pNEBiucP) with the correct nucleotide sequence was inserted into the *Ascl* site of pEU730. The iucP1 and M13(−40) primers were used to identify clones in which *lacZ* transcription was under the control of the *iuc* promoter. One clone with the proper orientation was named pEUiucP. To obtain a reporter plasmid containing the *iuc* promoter region inserted in the opposite orientation, pNEBiucP was digested with *Asp718* and *Pacl*. The promoter fragment was gel-purified and cloned into the same sites in pEU730, resulting in plasmid pEUiucP-Op.

Primers Fhu-1 and Fhu-2 were used to generate a 302 bp DIG-labelled probe that hybridized to clone 1310 (renamed pFhuYp1310) in the KIM6+ genomic library (Perry *et al.*, 1990). This clone contains an approximately 8.3 kb insert that includes the *Y. pestis fhuCDB* operon. An approximately 5.4 kb subclone yielded pFhuYp1 (Table 1).

Table 1. Bacterial strains and plasmids used in this study

All *Y. pestis* and *Y. pseudotuberculosis* strains used are avirulent from the absence of the low-calcium response virulence plasmid pCD1 (Lcr⁻). *Y. pestis* strains with a plus sign possess an intact 102 kb *pgm* locus. *Y. pestis* Δ *pgm* strains no longer make or use yersiniabactin (Ybt⁻). KIM10+ is Pla⁻ from the absence of pPCP1, which encodes the plasminogen activator (*pla*) and the bacteriocin pesticin. Ap^r, Cm^r, Km^r, Sm^r, Spc^r and Tc^r: resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, spectinomycin and tetracycline, respectively.

Strain or plasmid	Relevant characteristics	Source/Reference
<i>Escherichia coli</i>		
1017	<i>ent</i> : : Tn5 Km ^r	S. Payne; Daskaleros <i>et al.</i> (1991)
CFT073-ENT/IUC	<i>iucB</i> : : <i>cam entF</i> : : <i>kan</i>	A. Torres; Torres <i>et al.</i> (2001)
LG1315	(pColV-K30); aerobactin producer	J. Crosa; Williams (1979)
LG1522	(pColV-K30) <i>iuc</i>	J. Crosa; Carbonetti & Williams (1984)
RK4375	<i>fhuC</i> : : Tn10	<i>E. coli</i> Genetic Stock Center; Kadner <i>et al.</i> (1980); Ó Cuív <i>et al.</i> (2004)
<i>Yersinia pestis</i>		
KIM6	Ybt ⁻ (Δ <i>pgm</i>) Lcr ⁻ ; Δ <i>pgm</i> derivative of KIM6+	Fetherston <i>et al.</i> (1992); Sikkema & Brubaker (1987)
KIM6-2030	Ybt ⁻ (Δ <i>pgm</i>) Fur ⁻ (<i>fur</i> : : <i>kan-9</i>) Lcr ⁻	Staggs <i>et al.</i> (1994)
KIM6-2046.1	Ybt ⁻ (<i>irp2</i> : : <i>kan2046.1</i>) Lcr ⁻ Km ^r	Fetherston <i>et al.</i> (1995)
KIM6-2114	Ybt ⁻ (Δ <i>pgm</i>) Δ <i>fhuCD2114</i> Lcr ⁻	This study
KIM6-2113	Ybt ⁻ (Δ <i>pgm</i>) Δ <i>iutA2113</i> Lcr ⁻	This study
KIM10+	Ybt ⁺ Pla ⁻ Lcr ⁻ ; derivative of KIM6+ cured of pPCP1 (Pla ⁻)	Perry <i>et al.</i> (1990)
<i>Yersinia pseudotuberculosis</i>		
PB1-2046.1/0	Ybt ⁻ (<i>irp2</i> : : <i>kan2046.1</i>) Lcr ⁻ Km ^r	Perry <i>et al.</i> (1999)
Plasmids		
pACYC184	4.2 kb cloning vector; Cm ^r Tc ^r	Chang & Cohen (1978)
pAero1985	16.3 kb, 9.035 kb <i>Sau3A</i> fragment from KIM10+ ligated into the <i>Bam</i> HI site of pLG338, Km ^r	This study; Perry <i>et al.</i> (1990)
pAeroYp1	~13.8 kb, ~10.1 kb <i>Sall</i> - <i>Eco</i> RI fragment from pAero1985 ligated into pBR322, Ap ^r	This study
pAeroYp2	~6.8 kb, ~4.1 kb <i>Bgl</i> II- <i>Hind</i> III fragment ligated into pNEB193, Ap ^r	This study
pAeroYp3	~5.9 kb, Δ <i>iutA</i> , 0.93 kb <i>Clal</i> fragment deleted from pAeroYp2, Ap ^r	This study
pAeroYp4	~13.8 kb, ~1.5 kb <i>Sall</i> - <i>Bsr</i> GI PCR fragment replacing the original fragment in pAeroYp1, Ap ^r ; <i>iucA1/iucA2</i> frameshift repaired, <i>iucA-D</i> ⁺ - <i>iutA</i> ⁺	This study
pAeroYp5	~13.8 kb, ~1.5 kb <i>Sall</i> - <i>Bsr</i> GI PCR fragment replacing the same fragment in pAeroYp1, Ap ^r ; <i>iucA-H225Y</i> , <i>iucB-D</i> ⁺ - <i>iutA</i> ⁺	This study
pBGL2	4.8 kb cloning vector, Ap ^r Tc ^r	Perry <i>et al.</i> (1990)
pBR322	4.4 kb cloning vector; Ap ^r Tc ^r	Ausubel <i>et al.</i> (1987)
pColV-K30	<i>iucA-D-iutA</i> ⁺ ; from <i>E. coli</i> LG1315	Williams (1979)
pColV-K30 Δ <i>shiF</i> : : <i>cam</i>	<i>iucA-D-iutA</i> ⁺ ; Δ <i>shiF</i> : : <i>cam</i> ; Cm ^r	This study
pEU730	15.2 kb low-copy-number vector with promoterless <i>lacZ</i> ; Spc ^r	Froehlich <i>et al.</i> (1994)
pEUiucP	15.3 kb, low-copy-number vector with <i>iucA</i> : : <i>lacZ</i> reporter; 139 bp <i>Ascl</i> fragment from pNEBiucP ligated into pEU730; Spc ^r	This study
pEUiucP-Op	15.3 kb, low-copy-number vector with <i>y3378</i> : : <i>lacZ</i> reporter; 139 bp <i>Ascl</i> - <i>PacI</i> fragment from pEUiucP ligated into pEU730; Spc ^r	This study
pFhuYp1	~9.0 kb, ~5.4kb <i>Hind</i> III- <i>Sall</i> fragment from pFhuYp1310 ligated into pACYC184, Cm ^r	This study
pFhuYp2	~8.5 kb, Δ <i>fhuCD2114</i> , 0.47 kb <i>Eco</i> RV fragment deleted from pFhuYp1, Cm ^r	This study
pFhuYp1310	~15.6 kb, ~8.3 kb <i>Sau3A</i> fragment from KIM10+ ligated into the <i>Bam</i> HI site of pLG338, Km ^r	This study; Perry <i>et al.</i> (1990)
pKLS711	cloned <i>S. flexneri</i> aerobactin locus; Cm ^r Tc ^r	E. Wycoff & S. Payne; Lawlor <i>et al.</i> (1987)
pKNG101	6.8 kb suicide vector, <i>sacB</i> ⁺ , R6K origin, Sm ^r	Kaniga <i>et al.</i> (1991)
pKNG Δ FhuCD	~9.1 kb, Δ <i>fhuCD2114</i> , ~2.7 kb <i>Xba</i> I- <i>Hpa</i> I fragment from pFhuYp2 ligated into the <i>Xba</i> I and <i>Sma</i> I sites of pKNG101, Sm ^r	This study
pKNG Δ IutA	~9.8 kb, ~3.0 kb <i>Sca</i> I- <i>Eco</i> RV fragment pAeroYp2 containing Δ <i>iutA2113</i> ligated into the <i>Sma</i> I site of pKNG101, Sm ^r	This study
pNEB193	2.7 kb cloning vector; Ap ^r	New England Biolabs
pNEBiucP	2.8 kb, 139 bp <i>Ascl</i> PCR product from pAeroYp1 ligated into pNEB193; <i>iuc</i> promoter region; Ap ^r	This study

Construction of $\Delta iutA$, $\Delta fhvCD$ and $shiF::cam$ mutations. To generate a deletion within the *flu* operon of *Y. pestis*, pFHUYp1 was digested with *EcoRV* to remove a 467 bp fragment that encompasses the 3' end of *fluC* and the 5' end of *fluD*, creating pFHUYp2 (see Fig. 1b). An approximately 2.7 kb fragment containing the deletion was subcloned from pFHUYp2 into pKNG101, yielding pKNG Δ FhvCD (Table 1), and was introduced into the chromosome of KIM6 by allelic exchange. Strains in which the deleted genes had replaced the wild-type copies were identified by PCR using primers Δ Fhu-1 and Δ Fhu-2. One mutant was selected and named KIM6-2114 ($\Delta fhvDC2114$).

To construct a deletion within the *iutA* gene of *Y. pestis*, an approximately 3.0 kb *ScaI-EcoRV* fragment containing a *Clal* deletion, which removes 930 bp from the *iutA* gene (see Fig. 1a), was subcloned from pAeroYp3 into the suicide vector pKNG101, resulting in pKNG Δ IutA (Table 1). This plasmid was electroporated into *Y. pestis* KIM6 and transformants were selected on TBA plates containing streptomycin (50 μ g ml⁻¹). As previously described (Bearden & Perry, 1999), cells grown overnight in Heart Infusion broth (Difco) without antibiotic selection were used to select sucrose-resistant isolates that had completed the allelic exchange. Strains in which the wild-type gene was replaced by the deleted version were identified by PCR using primers Iut-1 and Iut-2. One isolate was selected and named KIM6-2113 ($\Delta iutA2113$).

The sequence for the *shiF* gene from *E. coli* CFT073 (Welch *et al.*, 2002) was used to generate primers for mutagenesis of the *shiF* gene in pColV-K30. The primers Shi-cat1 and Shi-cat2 (Supplementary Table S1) were used to amplify the chloramphenicol cassette from pKD3 (Datsenko & Wanner, 2000). The PCR product was electroporated into *E. coli* strain 1017 (pColV-K30, pKD46) and the cells were plated on LB plates containing 15 μ g chloramphenicol ml⁻¹. The allelic exchange, which deletes 930 bp from the *shiF* homologue in pColV-K30, was verified by PCR with primers ShiF-1 and ShiF-2 (Supplementary Table S1). The resulting plasmid was named pColV-K30 Δ shiF::cam (Table 1).

β -Galactosidase assays. Reporter plasmids were electroporated into *Y. pestis* KIM6 and KIM6-2030 (*fur::kan-9*) strains to analyse transcriptional regulation of the *iucA* and *y3378* promoters. *Y. pestis* strains containing the reporter plasmid were acclimated to iron-deficient or iron-surplus conditions [~six generations in the defined deferrated medium PMH2 without added iron or with 10 μ M FeCl₃ (Gong *et al.*, 2001)] and harvested during exponential growth at 37°C. β -Galactosidase activities from whole-cell lysates were measured spectrophotometrically with a Genesys 5 spectrophotometer (Spectronic Instruments) following cleavage of *o*-nitrophenyl β -D-galactopyranoside (ONPG) and the results are expressed in Miller units (Miller, 1992). Data presented are the means and standard deviations derived from two independent experiments.

Protein expression analysis. *In vitro* transcription/translation assays were used to examine protein expression from the various cloned aerobactin loci. Purified and concentrated template DNA at approximately 2 μ g per reaction was incubated with T7 S30 *E. coli* extract (Promega) and Trans35S-label [50 μ Ci (1.85 MBq) of [³⁵S]-methionine (~70%) and [³⁵S]-cysteine (~15%) per reaction; MP Biomedicals] for 3 h at 37 °C, following the manufacturer's specifications. Polypeptides in boiled and unboiled samples were separated by SDS-PAGE using 12% acrylamide. Gels were dried and newly synthesized polypeptides visualized by exposure to Blue Lite autoradiography film (ISC BioExpress). [¹⁴C]-methylated molecular mass markers (range 14 000–220 000; Sigma) were used to estimate the molecular masses of the polypeptides.

Siderophore bioassays. pColV-K30 was isolated from *E. coli* LG1315 and electroporated into *E. coli* strain 1017. Cells containing

the plasmid were selected on LB agar plates containing 500 μ M ethylenediamine-di(*o*-hydroxyphenyl-acetic acid). *E. coli* 1017 cells containing pColV-K30 were grown overnight at 37°C in nutrient broth (NB) containing 50 μ M 2,2'-dipyridyl (DIP). All other *E. coli* strains were grown in NB with 25 μ M DIP. The cells from these cultures were pelleted and the supernatants filtered through a 0.22 μ m filter. Assuming 1 ml of culture at an OD₆₂₀ of 1.0 is ~7.5 × 10⁸ cells, approximately 10⁵ cells ml⁻¹ were incorporated into NB agar plates containing either 50 μ M DIP for plates with LG1522 or 75 μ M DIP for plates containing 1017(pAeroYp1). Filtered supernatants to be tested for siderophore activity were added to wells in the plates, which were incubated overnight at 37 °C.

Y. pestis strains were grown through two transfers at 37 °C in deferrated PMH2 (Gong *et al.*, 2001). Two hundred microlitres of a KIM6-2046.1 culture with or without pBGL2 at an OD₆₂₀ of 0.2 were incorporated into PMH2 agar plates containing 75 μ M DIP. Filtered supernatants from iron-deficient cultures were placed into wells and the plates were incubated at 37 °C overnight.

The ability of *E. coli* RK4375 derivatives to use ferrichrome was tested in a plate assay. RK4375 cultures were grown overnight at 37 °C in LB medium containing 100 μ M DIP and incorporated at a concentration of 10⁵ cells ml⁻¹ into LB plates supplemented with 500 μ M DIP. A 25 μ l aliquot of a 1 mM solution of ferrichrome (Sigma) was added to wells in the agar and the plates were incubated overnight at 37 °C. To test for ferrichrome use by *Y. pestis*, strains were grown through two transfers in deferrated PMH2 at 37 °C and 0.04 OD₆₂₀ units of the cultures were included in solidified PMH2 containing 0.5 mM Na₂CO₃, 100 μ M MnCl₂, 4 mM CaCl₂ and 50 μ M DIP. One millimolar ferrichrome (25 μ l aliquot) was added to wells and the plates were incubated at 37 °C overnight. To visualize growth of *Y. pestis* around the wells, the plates were overlaid with TBA containing 0.05 % aesculin and 0.05 % ferric citrate.

Growth curves in the presence of ferrichrome. KIM6 and KIM6-2114 ($\Delta fhvCD$) cells were grown on TBA slants at 30 °C and used to inoculate deferrated PMH2 to an OD₆₂₀ of 0.1. After overnight growth at 37 °C, the cultures were diluted to an OD₆₂₀ of 0.1 into deferrated PMH2 containing or lacking 10 μ M ferrichrome and incubated at 37 °C. Optical density readings using a Genesys 5 spectrophotometer (Spectronic Instruments) were taken at hourly intervals. PMH2 is a defined medium (Gong *et al.*, 2001). Chelex 100 (Bio-Rad) was used to remove contaminating iron as previously described (Staggs & Perry, 1991).

RESULTS AND DISCUSSION

Sequence analysis of the aerobactin locus

Analysis of the *Y. pestis* KIM10+ database (Deng *et al.*, 2002) revealed the presence of an operon with genes homologous to *E. coli iucABCD* and *iutA*. The gene order in the *Y. pestis* aerobactin operon is the same as in *E. coli*. In all of the *Y. pestis* strains sequenced to date (CO92, KIM10+, 91001, Antiqua, Nepal516 and Angola) (Chain *et al.*, 2006; Deng *et al.*, 2002; Parkhill *et al.*, 2001; Song *et al.*, 2004), *iucA* contains a mutation which splits the gene into two ORFs [Fig. 1; *iucA1* (*y3380*) and *iucA2* (*y3381*) in KIM] with the corresponding proteins IucA1 being 62 % similar to residues 1–184 and IucA2 72 % similar to residues 219–575 of *E. coli* IucA, respectively (Fig. 1). The predicted amino acid sequences of the remaining *Y. pestis* aerobactin biosynthetic genes have from 67 to 74 %

similarity to their *E. coli* counterparts. The putative *Y. pestis* aerobactin receptor, IutA, is 73 % similar and 67 % identical to *E. coli* IutA (Supplementary Fig. S1, available with the online version of this paper). In contrast, none of the three *Y. pseudotuberculosis* genome sequences analysed has the *iucA* frameshift mutation and they show 98–100 % identity with the *Y. pestis* Iuc/Iut amino acid sequences (Supplementary Figs S2–S5).

A potential Fur-binding site (FBS) is located 44 bp upstream of the putative start of *iucA1*. This FBS matches the *E. coli* FBS consensus sequence (Braun & Hantke, 1991) in 16 out of 19 bases. The FBS starts 2 bp upstream of a putative –10 region and overlaps the putative –35 element. This element may also regulate a divergent promoter for an ORF, Y3378, with similarity to major facilitator superfamily efflux proteins.

Sequence analysis of the *fhuCDB* operon

In a number of enteric organisms, aerobactin is transported across the inner membrane by the Fhu system (Payne & Mey, 2004). The *Y. pestis* genomes contain genes homologous to the *E. coli* *fhuCDB* operon (Fig. 1). Unlike *E. coli*, the *Y. pestis* operon does not contain the gene encoding the ferrichrome receptor, FhuA. In another region of the *Y. pestis* chromosome, a putative ferrichrome receptor (*y2556*) is encoded which is more closely related to FcuA, the ferrichrome receptor of *Y. enterocolitica* (85 % identity and 88 % similarity) (Koebnik *et al.*, 1993).

Regulation of *iuc* genes and *iutA*

Generally, transcription of genes encoding iron uptake systems is repressed by iron-surplus conditions. To test whether the 139 bp region upstream of *iucA* is an iron- and Fur-regulated promoter, we constructed an *iucA* : : *lacZ* transcriptional fusion in a single-copy vector, pEU*iucP*. Cells of KIM6(pEU*iucP*) and KIM6-2030(pEU*iucP*) (*fur* : : *kan*-9) were grown at 37 °C in iron-deficient PMH medium (Staggs & Perry, 1991) in the presence or absence of added iron and assayed for β -galactosidase activity. The expression of *iucA* : : *lacZ* in KIM6 grown with surplus iron was repressed 23-fold compared to that of the iron-deficient culture. In the absence of Fur, the *iucA* : : *lacZ* promoter was no longer repressed by surplus iron in the medium (Fig. 2). Thus, the *iucA* promoter requires a functional Fur protein for repression by iron. To determine whether this region serves as a divergent promoter for *y3378*, an ORF with similarity to ORFs upstream of *iucA* in other bacteria encoding the aerobactin system (termed *shiF* in *Shigella*), the 139 bp between *y3378* and *iucA* was cloned in the opposite orientation (pEU*iucP*-Op) as well. β -Galactosidase activity from this reporter exhibited a 10-fold repression by iron in a Fur⁺ strain. However, iron-deficient expression levels were ~8-fold lower for this orientation than for the *iucA* promoter orientation. Although weaker than the activity for the *iuc* locus, this

Fig. 2. β -Galactosidase activity of *Y. pestis* strains carrying either an *iucA* : : *lacZ* (pEU*iucP*) or *y3378* : : *lacZ* (pEU*iucP*-Op) reporter plasmid. Cells were harvested during exponential growth at 37 °C after approximately six generations in deferrated PMH2 containing no added iron or supplemented with 10 μ M FeCl₃. Enzyme activities are expressed in Miller units (Miller, 1992). The error bars indicate standard deviations from means of replicate samples from two or more independent cultures. Fur⁺ and – indicate the parental KIM6 and *fur* : : *kan* KIM-2030 strains of *Y. pestis*, respectively.

region appears to serve as an iron- and Fur-regulated promoter for expression of *y3378* as well (Fig. 2). Similar *shiF*-like genes lie upstream of the aerobactin locus in a number of bacteria, suggesting a possible role in the aerobactin system. Annotations of *shiF*-like genes indicate it is a member of the COG0477 permeases of the major facilitator superfamily. However, we found no experimental studies on the function of these proteins. COG0477 has no recognized conserved domain and is defined simply as proteins with 12 to 14 transmembrane domains arranged as two groups of six or seven. Analysis of Y3378 with TMHMM (Sonnhammer *et al.*, 1998) predicts only 11 transmembrane domains with no division into two groups. While it is possible that Y3378 serves as an aerobactin exporter, an insertion in a similar ORF in pColV-K30 (pColV-K30 Δ *shiF* : : *cam*; Table 1) still allowed aerobactin secretion in *E. coli* (data not shown). This indicates either that ShiF is not essential for the functioning of the aerobactin system, or that another system encoded within the *E. coli* genome can also function to export aerobactin.

Y. pestis does not synthesize aerobactin

A recombinant plasmid, pAero1985, containing the aerobactin region was identified from a *Y. pestis* genomic library (Perry *et al.*, 1990). The insert of pAero1985 includes 373 bp of DNA upstream of the putative start codon for *iucA1*, containing the putative promoter elements and the *iucA2*, *iucB*, *iucC*, *iucD* and *iutA* genes. A subclone of the *Y. pestis* aerobactin region, pAeroYp1 (Table 1), was introduced into the *E. coli* strain 1017. *E. coli* 1017 is a derivative of HB101 with a transposon

insertion within the enterobactin biosynthetic operon and is thus unable to produce enterobactin (Daskaleros *et al.*, 1991). Supernatants isolated from *E. coli* 1017(pAeroYp1) cells grown under iron-deficient conditions were unable to feed LG1522, an *E. coli* strain carrying a pColV-K30 plasmid with a mutation in the aerobactin biosynthetic genes (Carbonetti & Williams, 1984; Valvano *et al.*, 1986). However, LG1522 was fed by supernatants isolated from an *E. coli* 1017 strain carrying wild-type ColV-K30. In addition, supernatants from KIM6, which encodes the *iucA1-D-iutA* locus but lacks the *ybt* locus which encodes the yersiniabactin iron transporter, were unable to restore growth of KIM6-2046.1 (*irp2-2046::kan*), a strain unable to synthesize the Ybt siderophore, on PMH2 plates containing 75 μ M DIP (data not shown). These results suggest that *Y. pestis* genes *iucA1*, *iucA2*, *iucB*, *iucC* and *iucD* do not produce enzymes capable of aerobactin synthesis. To determine if the *Y. pestis* genes downstream of the *iucA1/iucA2* frameshift are functional, pAeroYp1 was electroporated into *E. coli* strain CFT073-ENT/IUC. CFT073-ENT/IUC is a uropathogenic strain of *E. coli* with kanamycin and chloramphenicol gene cassettes disrupting *entF* and *iucB*, respectively. Thus, this strain does not synthesize either enterobactin or aerobactin, although it can use both of these siderophores (Torres *et al.*, 2001). Supernatants isolated from CFT073-ENT/IUC(pAeroYp1) grown under iron-deficient conditions were unable to feed LG1522 (data not shown). This suggests that the *Y. pestis* *iucBCD* genes could not replace their *E. coli* counterparts in CFT073-ENT/IUC or were not expressed, and therefore did not restore aerobactin synthesis.

In vitro transcription/translation analysis using pAeroYp1 showed a polypeptide with a molecular mass similar to that predicted for the IutA OM receptor (Fig. 3). However, no polypeptides with masses similar to that predicted for IucB, IucC or IucD were apparent (Fig. 3). This suggests that the downstream *iucB-D* genes may not be translated due to the frameshift mutation in *iucA1/iucA2*.

***Y. pestis* utilizes exogenous aerobactin as an iron source**

To determine if the aerobactin receptor gene of *Y. pestis* is functional, we tested the ability of supernatants isolated from *E. coli* 1017(pColV-K30) cells grown overnight in NB with 50 μ M DIP to stimulate the growth of iron-starved *Y. pestis* cells. The 1017(pColV-K30) supernatant restored growth at 37 °C to *Y. pestis* KIM6 cells plated on PMH2 containing 50 μ M DIP. In contrast, *Y. pestis* KIM6-2113 (Δ *iutA2113*) was unable to use aerobactin as an iron source (Fig. 4a). However, KIM6-2113 cells carrying pAeroYp1, which encodes the *Y. pestis* aerobactin locus (*iucA1-A2-B-C-D-iutA*), were able to use aerobactin as an iron source (data not shown). Thus, *Y. pestis* does produce a functional aerobactin receptor (IutA). These results are in contrast to an earlier study which indicated that *Y. pestis* could not use aerobactin as an iron source (Perry & Brubaker, 1979).

Fig. 3. Autoradiograph of *in vitro* transcription/translation products separated by SDS-PAGE. Samples (boiled, lanes 1–3, or unboiled, lanes 4–6) generated from pBR322 (vector control; lanes 1 and 4), pAeroYp1 (*iucA1-D-iutA*; lanes 2 and 5) and pAeroYp5 (*iucA-H225Y-iucB-D-iutA*; lanes 3 and 6) were separated by SDS-PAGE (12% acrylamide). Lines and numbers indicate molecular mass markers in kDa. Labelled arrows designate polypeptide bands unique to pAeroYp1 and/or pAeroYp5 where the expected vs observed molecular masses (in kDa) for the indicated proteins are as follows: IutA (80.4/78.9), IucC (66.6/63.5), IucA (66.9/60.8), IucD (50.4/54.6) and IucB (36.9/33.9). The observed molecular masses were calculated with GelScape (Young *et al.*, 2004). The putative IucB polypeptide was apparent only in unboiled samples.

While that study used a different *Y. pestis* biotype (strain Kuma+), one other likely explanation is that the concentration of conalbumin used to inhibit growth was too high to be overcome by exogenous aerobactin under the growth conditions tested. Supernatants from *E. coli* 1017(pColV-K30) also stimulated the growth of *E. coli* 1017(pAeroYp1) on NB plates containing 75 μ M DIP, while supernatants from *E. coli* 1017(pBR322) did not (data not shown).

The frameshift-corrected *Y. pestis* *iuc* locus does not produce aerobactin

We corrected the frameshift mutation generating a recombinant *iucA-D-iutA* locus with a full-length *iucA_{Yp}* in pAeroYp4 (Table 1). This is similar to the *Y. pseudotuberculosis* *iucA-D-iutA* loci in strains IP32953 (Chain *et al.*, 2004) and PB1 except for a Y225H change in the *Y. pestis* locus. Consequently, we altered this residue to Y225 in pAeroYp5. *In vitro* transcription/translation products from pAeroYp5 yielded polypeptides with molecular masses similar to those predicted for IutA, IucA, IucB, IucC and IucD (Fig. 3). For reasons that are unclear, the polypeptide probably corresponding to IucB was only apparent in unboiled samples. Nevertheless, these results indicate that pAeroYp5 expresses the *Y. pestis* Iuc proteins, providing

Fig. 4. Siderophore use and aerobactin production by various *Y. pestis* strains. (a) After growth in deferrated PMH2 at 37 °C, *Y. pestis* KIM6 (*iutA*⁺ *fhuCDB*⁺), KIM6-2113 (Δ *iutA*) and KIM6-2114 (Δ *fhuCD*) were incorporated into iron-deficient solidified PMH2 medium containing 75 μ M DIP. Solutions or cell-free culture supernatants were added to wells on the plates. After overnight incubation at 37 °C, the plates were overlaid with TBA containing aesculin and ferric citrate to visualize growth around the wells. Solutions: 1, ferrichrome; 3, water; 5, FeCl₃. Iron-deficient culture supernatants from: 2, *E. coli* 1017(pColV-K30); 4, *E. coli* 1017. (b) After growth in deferrated PMH2 at 37 °C, cells of *Y. pestis* KIM6(pBR322) were incorporated into iron-deficient solidified PMH2 medium containing 75 μ M DIP. Cell-free supernatants from iron-deficient cultures were added to wells on the plates. After overnight incubation at 37 °C, the plates were overlaid with TBA containing aesculin and ferric citrate to visualize growth around the wells. All cultures contained ampicillin to retain the various recombinant plasmids. Iron-deficient culture supernatants from: 1, KIM6(pBR322); 2, KIM6(pAeroYp4); 3, KIM6(pAeroYp5); 4, KIM6(pKLS711) and 5, KIM6(pColV-K30 Δ *shiF* : : *cam*).

further evidence that the *iucA1/iucA2* frameshift prevents translation of *iucB*, *iucC* and *iucD*, but not *iutA*.

Iron-deficient culture supernatants from *E. coli* 1017 carrying pBR322, pAeroYp4 or pAeroYp5 all failed to stimulate growth of *E. coli* LG1522(pBR322) under iron-chelated conditions, indicating that aerobactin was not produced by any of these recombinant strains. In contrast,

E. coli 1017(pColV-K30) supernatants did stimulate growth (data not shown).

Supernatants from *Y. pestis* KIM6 cultures carrying pBR322, pAeroYp4 or pAeroYp5 also failed to stimulate the growth of *E. coli* LG1522(pBR322) under iron-chelated conditions. Thus, correcting the frameshift mutation and the *IucA*-Y225H alteration failed to lead to aerobactin synthesis by *Y. pestis* (data not shown). However, *Y. pestis* KIM6 carrying either pKLS711 or pColV-K30 Δ *shiF* : : *cam*, which both encode functional aerobactin loci (Table 1), did produce aerobactin. Supernatants from iron-deficient cultures of these strains supported the growth of *Y. pestis* KIM6(pBR322) on PMH2 plates containing 75 μ M DIP, while supernatants from *Y. pestis* strains carrying pBR322 (negative control), pAeroYp4 and pAeroYp5 did not stimulate growth of the indicator strain (Fig. 4b).

We assessed the ability of iron-starved cells of *Y. pestis* KIM6(pBR322) (*Ybt*[−]), KIM6(pKLS711) and KIM6(pColV-K30 Δ *shiF* : : *cam*) to grow on solidified PMH2 containing 75 μ M DIP at 37 °C. Under these iron-chelated conditions *Ybt*⁺ but not *Ybt*[−] cells of *Y. pestis* are capable of growth. Only KIM6(pKLS711) cells grew well on these plates. KIM6(pColV-K30 Δ *shiF* : : *cam*) cells were able to grow on PMH2 plates containing 50 μ M DIP (data not shown). The ability of KIM69(pKLS711) to grow at a higher DIP concentration than KIM6(pColV-K30 Δ *shiF* : : *cam*) is probably due to a higher plasmid copy number for pKLS711 compared to that of pColV-K30. Overall these results indicate that, at least under these *in vitro* conditions, aerobactin can replace the yersiniabactin iron transport system in supplying iron to *Y. pestis* cells.

The *Y. pseudotuberculosis iuc* locus does not produce aerobactin

We also tested supernatants from *Y. pseudotuberculosis* PB1-2046.1/0 cultured in deferrated PMH2. PB1-2046.1/0 is a derivative of the sequenced PB1 strain that has a *kan* insertion in *irp2*, making it unable to synthesize the yersiniabactin siderophore (Perry *et al.*, 1999). The genome sequence of strain PB1 shows an *iucA-D-iutA* locus with no obvious defects and is identical to the same locus in strain IP32953 (Chain *et al.*, 2004). Despite this, the PB1-2046.1/0 supernatants did not support the growth of *E. coli* LG1522, indicating that *Y. pseudotuberculosis* is unable to synthesize aerobactin. This correlates with a survey that failed to demonstrate aerobactin production in five different *Y. pseudotuberculosis* strains (Stuart *et al.*, 1986).

The reason for this lack of aerobactin production by *Y. pseudotuberculosis* and the *Y. pestis* recombinant *iucA*-H225Y-*iucB*-D-*iutA* locus is unclear. However, conference of aerobactin production in *Y. pestis* by *iuc* genes from *Shigella flexneri* or pColV-K30 suggests the defect lies within the *Yersinia iuc* genes. A comparison of predicted amino acid sequences of *IucA*, *IucB*, *IucC* and *IucD*

between the *Yersinia* and pColV-K30 identified a number of residue differences in all four proteins (Supplementary Figs S2–S5, available with the online version of this paper). No study of amino acid residues critical for the enzymic activity of the Iuc proteins has been published. Consequently, any one or multiple non-conserved residues in the *Yersinia* Iuc proteins could be responsible for the inability to synthesize a functional aerobactin molecule.

The Fhu ABC transporter of *Y. pestis* is functional

The ability of *Y. pestis* to utilize exogenous aerobactin suggests that the FhuCDB transporter is functional. To clearly demonstrate this, a clone containing the *Y. pestis* *fhu* operon was identified from the *Y. pestis* KIM10 + genomic library and the region encompassing *fhuCDB* was subcloned, generating pFhuYp1. This recombinant plasmid was introduced into *E. coli* strain RK4375, which contains a Tn10 insertion within *fhuC* and is thus unable to grow in the presence of ferrichrome (Kadner *et al.*, 1980). The cloned *Y. pestis* *fhuCDB* genes were able to complement the defect in RK4375 and restored its ability to use ferrichrome as an iron source (data not shown). A $\Delta fhuCD$ mutation was introduced into *Y. pestis* KIM6 by allelic exchange. The growth of KIM6-2114 ($\Delta fhuCD2114$) in liquid medium was similar to that of the parental strain under iron-deficient conditions in PMH2. However, growth of the mutant was significantly reduced relative to KIM6 when ferrichrome was present (Fig. 5). By plate assays, KIM6 used both ferrichrome and aerobactin as an iron source

while KIM6-2113 ($\Delta iutA2113$) was able to use ferrichrome but not aerobactin, and KIM6-2114 (Δfhu) could use neither (Fig. 4a). When KIM6-2114 was complemented with pFhuYp1, the ability to use aerobactin as an iron source was restored (data not shown).

Concluding remarks

In summary, *Y. pestis* KIM6 possesses a functional ferric hydroxamate uptake system (FhuCDB) with receptors for the use of aerobactin (IutA) and ferrichrome (FcuA). A $\Delta fhuCD$ mutation abrogates the ability of *Y. pestis* to use either aerobactin or ferrichrome as iron sources, while a $\Delta iutA$ mutant is defective in aerobactin but not ferrichrome utilization. Although *Y. pestis* has sequences homologous to *iucABCD*, these gene products do not appear to synthesize aerobactin and are unable to complement defects in the aerobactin biosynthetic pathway in *E. coli*. Although a frameshift mutation in *iucA*, which is conserved across different biotypes of *Y. pestis*, was thought to be the likely cause of this defect, repair of the frameshift failed to enable aerobactin biosynthesis. In addition, *Y. pseudotuberculosis*, which encodes an aerobactin locus with no obvious defects, is also unable to synthesize aerobactin (Stuart *et al.*, 1986; this study). One likely cause of this defect may be amino acid variations in the four *Yersinia* aerobactin biosynthetic enzymes compared to known functional enzymes from *E. coli*, *S. flexneri* and pColV-K30 (Payne & Mey, 2004; Supplementary Figs S2–S5). This suggests that upon acquisition of the pathogenicity island encoding the yersiniabactin iron transport system, the enteric aerobactin system became unnecessary for growth in iron-deficient environments. The *iucA* frameshift in *Y. pestis* strains may save energy by eliminating translation of the downstream ORFs.

ACKNOWLEDGEMENTS

This study was partially funded by the Department of Homeland Security, Office of Research and Development through Interagency Agreement HSHQDC-05-00317. J. D. F. and R. D. P. are supported by Public Health Service grants AI033481, AI057175 and AI53319. We thank J. Crosa, S. Payne, E. Wycoff and A. Torres for providing plasmids and *E. coli* strains as well as Luther Lindler and the DOE Joint Genome Institute for early access to the draft genome sequence of *Yersinia pseudotuberculosis* strain PB1. We thank Luther Lindler and The Institute for Genome Research (<http://www.genomesonline.org/>) for early access to the incomplete genome sequence of *Y. pestis* Angola.

REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987). *Current Protocols in Molecular Biology*. New York: Wiley.
- Bearden, S. W. & Perry, R. D. (1999). The Yfe system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. *Mol Microbiol* **32**, 403–414.

Fig. 5. Growth curve of *Y. pestis* strains in the presence or absence of 10 μ M ferrichrome. Iron-deficient cultures of KIM6 and KIM6-2114 ($\Delta fhuCD$) were incubated in the presence or absence of ferrichrome.

- Bearden, S. W., Staggs, T. M. & Perry, R. D. (1998). An ABC transporter system of *Yersinia pestis* allows utilization of chelated iron by *Escherichia coli* SAB11. *J Bacteriol* **180**, 1135–1147.
- Blattner, F. R., Plunkett, G., III, Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K. & other authors (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474.
- Braun, V. & Hantke, K. (1991). Genetics of bacterial iron transport. In *CRC Handbook of Microbial Iron Chelates*, pp. 107–138. Edited by G. Winkelmann. Boca Raton, FL: CRC Press.
- Braun, V., Braun, M. & Killmann, H. (2004). Ferrichrome- and citrate-mediated iron transport. In *Iron Transport in Bacteria*, pp. 158–177. Edited by J. H. Crosa, A. R. Mey & S. M. Payne. Washington, DC: ASM Press.
- Carbonetti, N. H. & Williams, P. H. (1984). A cluster of five genes specifying the aerobactin iron uptake system of plasmid ColV-K30. *Infect Immun* **46**, 7–12.
- Chain, P. S., Carniel, E., Larimer, F. W., Lamerdin, J., Stoutland, P. O., Regala, W. M., Georgescu, A. M., Vergez, L. M., Land, M. L. & other authors (2004). Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* **101**, 13826–13831.
- Chain, P. S., Hu, P., Malfatti, S. A., Radnedge, L., Larimer, F., Vergez, L. M., Worsham, P., Chu, M. C. & Andersen, G. L. (2006). Complete genome sequence of *Yersinia pestis* strains Antiqua and Nepal516: evidence of gene reduction in an emerging pathogen. *J Bacteriol* **188**, 4453–4463.
- Chang, A. C. Y. & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* **134**, 1141–1156.
- Crosa, J. H., Mey, A. R. & Payne, S. M. (2004). *Iron Transport in Bacteria*, pp. 499. Washington, DC: ASM Press.
- Daskaleros, P. A., Stoeber, J. A. & Payne, S. M. (1991). Iron uptake in *Plesiomonas shigelloides*: cloning of the genes for the heme-iron uptake system. *Infect Immun* **59**, 2706–2711.
- Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.
- Debarieux, L. & Wandersman, C. (2004). Hemophore-dependent heme acquisition systems. In *Iron Transport in Bacteria*, pp. 38–47. Edited by J. H. Crosa, A. R. Mey & S. M. Payne. Washington, DC: ASM Press.
- Deng, W., Burland, V., Plunkett, G., III, Boutin, A., Mayhew, G. F., Liss, P., Perna, N. T., Rose, D. J., Mau, B. & other authors (2002). Genome sequence of *Yersinia pestis* KIM. *J Bacteriol* **184**, 4601–4611.
- Fetherston, J. D., Schuetze, P. & Perry, R. D. (1992). Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. *Mol Microbiol* **6**, 2693–2704.
- Fetherston, J. D., Lillard, J. W., Jr & Perry, R. D. (1995). Analysis of the pesticin receptor from *Yersinia pestis*: role in iron-deficient growth and possible regulation by its siderophore. *J Bacteriol* **177**, 1824–1833.
- Forman, S., Bobrov, A. G., Kirillina, O., Craig, S. K., Abney, J., Fetherston, J. D. & Perry, R. D. (2006). Identification of critical amino acid residues in the plague biofilm Hms proteins. *Microbiology* **152**, 3399–3410.
- Frøehlich, B., Husmann, L., Caron, J. & Scott, J. R. (1994). Regulation of *rms*, a positive regulatory factor for pili of enterotoxigenic *Escherichia coli*. *J Bacteriol* **176**, 5385–5392.
- Gong, S., Bearden, S. W., Geoffroy, V. A., Fetherston, J. D. & Perry, R. D. (2001). Characterization of the *Yersinia pestis* Yfu ABC inorganic iron transport system. *Infect Immun* **67**, 2829–2837.
- Hantke, K. (2004). Ferrous iron transport. In *Iron Transport in Bacteria*, pp. 178–184. Edited by J. H. Crosa, A. R. Mey & S. M. Payne. Washington, DC: ASM Press.
- Herrero, M., de Lorenzo, V. & Neillands, J. B. (1988). Nucleotide sequence of the *iucD* gene of the pColV-K30 aerobactin operon and topology of its product studied with *phoA* and *lacZ* gene fusions. *J Bacteriol* **170**, 56–64.
- Hornung, J. M., Jones, H. A. & Perry, R. D. (1996). The *hmu* locus of *Yersinia pestis* is essential for utilization of free haemin and haem-protein complexes as iron sources. *Mol Microbiol* **20**, 725–739.
- Jackson, S. & Burrows, T. W. (1956). The pigmentation of *Pasteurella pestis* on a defined medium containing haemin. *Br J Exp Pathol* **37**, 570–576.
- Kadner, R. J., Heller, K., Coulton, J. W. & Braun, V. (1980). Genetic control of hydroxamate-mediated iron uptake in *Escherichia coli*. *J Bacteriol* **143**, 256–264.
- Kaniga, K., Delor, I. & Cornelis, G. R. (1991). A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* **109**, 137–141.
- Kirillina, O., Bobrov, A. G., Fetherston, J. D. & Perry, R. D. (2006). A hierarchy of iron uptake systems: Yfu and Yiu are functional in *Yersinia pestis*. *Infect Immun* **74**, 6171–6178.
- Koebnik, R., Hantke, K. & Braun, V. (1993). The TonB-dependent ferrichrome receptor FcuA of *Yersinia enterocolitica*: evidence against a strict coevolution of receptor structure and substrate specificity. *Mol Microbiol* **7**, 383–393.
- Krone, W. J. A., Stegehuis, F., Koningstein, G., van Doorn, C., Roosendaal, B., de Graaf, F. K. & Oudega, B. (1985). Characterization of the pColV-K30 encoded cloacin DF13/aerobactin outer membrane receptor protein of *Escherichia coli*; isolation and purification of the protein and analysis of its nucleotide sequence and primary structure. *FEMS Microbiol Lett* **26**, 153–161.
- Lawlor, K. M., Daskaleros, P. A., Robinson, R. E. & Payne, S. M. (1987). Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. *Infect Immun* **55**, 594–599.
- Martinez, J. L., Herrero, M. & de Lorenzo, V. (1994). The organization of intercistronic regions of the aerobactin operon of pColV-K30 may account for the differential expression of the *iucABCD iutA* genes. *J Mol Biol* **238**, 288–293.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Ó Cuív, P., Clarke, P., Lynch, D. & O'Connell, M. (2004). Identification of *rhtX* and *fptX*, novel genes encoding proteins that show homology and function in the utilization of the siderophores rhizobactin 1021 by *Sinorhizobium meliloti* and pyochelin by *Pseudomonas aeruginosa*, respectively. *J Bacteriol* **186**, 2996–3005.
- Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C. & other authors (2001). Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413**, 523–527.
- Payne, S. M. & Mey, A. R. (2004). Pathogenic *Escherichia coli*, *Shigella*, and *Salmonella*. In *Iron Transport in Bacteria*, pp. 199–218. Edited by J. H. Crosa, A. R. Mey & S. M. Payne. Washington, DC: ASM Press.
- Perry, R. D. (2004). *Yersinia*. In *Iron Transport in Bacteria*, pp. 219–240. Edited by J. H. Crosa, A. R. Mey & S. M. Payne. Washington, DC: ASM Press.

- Perry, R. D. & Brubaker, R. R. (1979). Accumulation of iron by yersiniae. *J Bacteriol* **137**, 1290–1298.
- Perry, R. D. & Fetherston, J. D. (2004). Iron and heme uptake systems. In *Yersinia: Molecular and Cellular Biology*, pp. 257–283. Edited by E. Carniel & B. J. Hinnebusch. Norfolk, UK: Horizon Bioscience.
- Perry, R. D., Pendrak, M. L. & Schuetze, P. (1990). Identification and cloning of a hemin storage locus involved in the pigmentation phenotype of *Yersinia pestis*. *J Bacteriol* **172**, 5929–5937.
- Perry, R. D., Balbo, P. B., Jones, H. A., Fetherston, J. D. & DeMoll, E. (1999). Yersiniabactin from *Yersinia pestis*: biochemical characterization of the siderophore and its role in iron transport and regulation. *Microbiology* **145**, 1181–1190.
- Perry, R. D., Mier, I. J. & Fetherston, J. D. (2007). Roles of the Yfe and Feo transporters of *Yersinia pestis* in iron uptake and intracellular growth. *Biomaterials* **20**, 699–703.
- Rossi, M.-S., Fetherston, J. D., Létoffé, S., Carniel, E., Perry, R. D. & Ghigo, J.-M. (2001). Identification and characterization of the hemophore-dependent heme acquisition system of *Yersinia pestis*. *Infect Immun* **69**, 6707–6717.
- Sikkema, D. J. & Brubaker, R. R. (1987). Resistance to pesticin, storage of iron, and invasion of HeLa cells by yersiniae. *Infect Immun* **55**, 572–578.
- Song, Y., Tong, Z., Wang, J., Wang, L., Guo, Z., Han, Y., Zhang, J., Pei, D., Zhou, D. & other authors (2004). Complete genome sequence of *Yersinia pestis* strain 91001, an isolate avirulent to humans. *DNA Res* **11**, 179–197.
- Sonnhammer, E. L., von Heijne, G. & Krogh, A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. *Proc Int Conf Intell Syst Mol Biol* **6**, 175–182.
- Staggs, T. M. & Perry, R. D. (1991). Identification and cloning of a *fur* regulatory gene in *Yersinia pestis*. *J Bacteriol* **173**, 417–425.
- Staggs, T. M., Fetherston, J. D. & Perry, R. D. (1994). Pleiotropic effects of a *Yersinia pestis fur* mutation. *J Bacteriol* **176**, 7614–7624.
- Stojiljkovic, I. & Hantke, K. (1992). Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in Gram-negative bacteria. *EMBO J* **11**, 4359–4367.
- Stojiljkovic, I. & Hantke, K. (1994). Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersinia enterocolitica*. *Mol Microbiol* **13**, 719–732.
- Stuart, S. J., Prpic, J. K. & Robins-Browne, R. M. (1986). Production of aerobactin by some species of the genus *Yersinia*. *J Bacteriol* **166**, 1131–1133.
- Thompson, J. M., Jones, H. A. & Perry, R. D. (1999). Molecular characterization of the hemin uptake locus (*hmu*) from *Yersinia pestis* and analysis of *hmu* mutants for hemin and hemoprotein utilization. *Infect Immun* **67**, 3879–3892.
- Torres, A. G., Redford, P., Welch, R. A. & Payne, S. M. (2001). TonB-dependent systems of uropathogenic *Escherichia coli*: aerobactin and heme transport and TonB are required for virulence in the mouse. *Infect Immun* **69**, 6179–6185.
- Valvano, M. A., Silver, R. P. & Crosa, J. H. (1986). Occurrence of chromosome- or plasmid-mediated aerobactin iron transport systems and hemolysin production among clonal groups of human invasive strains of *Escherichia coli* K-12. *Infect Immun* **52**, 192–199.
- Welch, R. A., Burland, V., Plunkett, G., III, Redford, P., Roesch, P., Rasko, D., Buckles, E. L., Liou, S.-R., Boutin, A. & other authors (2002). Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A* **99**, 17020–17024.
- Williams, P. H. (1979). Novel iron uptake system specified by ColV plasmids: an important component in the virulence of invasive strains of *Escherichia coli*. *Infect Immun* **26**, 925–932.
- Young, N., Chang, Z. & Wishart, D. S. (2004). GelScape: a web-based server for interactively annotating, manipulating, comparing and archiving 1D and 2D gel images. *Bioinformatics* **20**, 976–978.

Edited by: S. C. Andrews