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Genetic and Transcriptional Analysis of the Siderophore Malleobactin Biosynthesis and Transport Genes in the Human Pathogen *Burkholderia pseudomallei* K96243

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Burkholderia pseudomallei **is a gram-negative facultative intracellular pathogen that causes melioidosis, an invasive disease of humans and animals. To address the response of this bacterium to iron-limiting conditions, we first performed a global transcriptional analysis of RNA extracted from bacteria grown under iron-limiting and iron-rich conditions by microarrays. We focused our study on those open reading frames (ORFs) induced under iron limitation, which encoded predicted proteins that could be involved in the biosynthesis and uptake of the siderophore malleobactin. We purified this siderophore and determined that it consisted of at least three compounds with different molecular weights. We demonstrated that ORFs BPSL1776 and BPSL1774, designated *mbaA* and *mbaF*, respectively, are involved in the biosynthesis of malleobactin, while BPSL1775, named *fmtA*, is involved in its transport. These genes are in an operon with two other ORFs (*mbaJ* and *mbaI*) whose transcription is under the control of MbaS, a protein that belongs to the extracytoplasmic function sigma factors. Interestingly, the transcription of the *mbaA*, *fmtA*, and *mbaS* genes is not controlled by the availability of the siderophore malleobactin.**

Burkholderia pseudomallei is a gram-negative facultative intracellular pathogen that is the causative agent of melioidosis. Although this invasive disease is endemic to southeast Asia and northern Australia, its worldwide availability, high rate of mortality, and aerosol infectivity resulted in its classification as a select agent (12, 65). *B. pseudomallei* is also a saprophytic organism that has developed high resistance to many hostile environmental conditions such as acidic environments, dehydration, prolonged nutrient starvation, and antiseptic and detergent solutions (12).

Despite increasing knowledge on the epidemiology of this disease, very little is known on the molecular mechanisms of the infection; only a few virulence factors, i.e., the capsular polysaccharide, lipopolysaccharide, type IV pilus, and a type III secretion apparatus, have been described with animal models (55, 64). This pathogen can invade phagocytic and nonphagocytic cells using the type III secretion apparatus to promote its escape into the cytoplasm, where the bacterium induces polar actin polymerization (56, 57). Intracellular *B. pseudomallei* cells can induce host cell membrane protrusions that may facilitate cell-to-cell spread of the bacteria and induce the fusion of adjacent cells forming giant multinucleated cells (24, 25, 54). Sequencing of the two *B. pseudomallei* chromosomes (22) has generated a significant amount of data expected to yield information on new virulence factors and putative targets for vaccine development.

A potential virulence factor in *B. pseudomallei* that so far has not been studied is the ability to utilize iron, a feature that may play an important role in the pathogenesis of the disease caused by this bacterium. Iron is required for the growth of nearly all microorganisms in the environment, as well as in biological fluids. However, in most environments, its concentration is below the amount required by organisms to grow. Therefore, bacteria have evolved a variety of mechanisms for the acquisition, solubilization, and transport of this metal. A widespread mechanism is the synthesis of low-molecular weight compounds called siderophores, which can bind iron with high affinity. Ferric siderophores are recognized by a specific receptor on the bacterial surface, and the iron (or the complexed ferric siderophore) is transported into the cytoplasm of the cell. It has been known for many years that *B. pseudomallei* is able to synthesize a hydroxamate-type siderophore, named malleobactin, that can remove iron from transferrin and lactoferrin, allowing this bacterium to grow under iron-limiting conditions (69, 70). So far, no studies have been performed to characterize the siderophore and/or the genes involved in the biosynthesis and uptake of ferric malleobactin. In this work, using DNA microarrays, we first characterized the global transcriptional response of *B. pseudomallei* to iron-limiting and iron-rich conditions. From the many open reading frames (ORFs) whose expression was altered under these conditions, we focused on those that might be involved in the biosynthesis and uptake of malleobactin. The results presented in this work clearly prove the functionality of some of these genes and, in addition, that their expression is modulated by a protein that belongs to extracytoplasmic function (ECF) sigma factors.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this work are listed in Table 1. Bacterial strains were grown routinely in brain heart infusion (BHI) broth (*B. pseudomallei*) or LB broth (*Escherichia coli*) supplemented with antibiotics as appropriate. For *B. pseudomallei*, antibiotics were kanamycin, 250 μg/ml; chloramphenicol, 50 μg/ml; and gentamicin, 25 μg/ml. For *E. coli*, antibiotics were kanamycin, 50 μg/ml; chloramphenicol, 30 μg/ml; and ampicillin, 100 μg/ml. For both microorganisms, antibiotics were trimethoprim, 40 μg/ml;

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TABLE 1. Bacterial strains and plasmids[a]

Bacterial strain or plasmid	Characteristics	Source or reference
Bacterial strains		
B. pseudomallei		
K96243	Wild-type Gm^r	S. Songsivilai, Siriraj Hospital
ALE-3	*fmtA*::Tet^r	This work
ALE-7	*mbaA*::Tp^r	This work
ALE-33	*mbaF*::Tp^r	This work
ALE-48	*mbaS*::Tp^r	This work
ALE-A5	*fptA*::Tet^r	This work
ALE-13	*mbaA*::Tp^r *fptA*::Tet^r	This work
ALE-50	*mbaA*::Tp *fmtA*::pJP5603 Km^r	This work
ALE-5	*fmtA*::pJP5603 Km^r	This work
ALE-1	*fmtA*::Tet^r/pMAL18	This work
ALE-34	*mbaA*::Tp^r/pMAL28	This work
ALE-20	*mbaS*::Tp^r/pMAL41	This work
ALE-19	*mbaA*::Tp^r *fmtA*::pJP5603 Km^r/pMAL18	This work
E. coli		
TOP10	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
S17-1 λpir	*thi pro hsdR hsdM*$^+$ *recA RP4-2*-Tc::Mu-Km::Tn*7* λ-*pir*	49
MM294	F^- *endA1 hsdR17 supE44 thi-1* λ^- harboring plasmid pRK2013	Laboratory stock
H1717	*aroB fhuF*::λ*plac*Mu53 *fur*	58
Plasmids		
pCR2.1	Amp^r Km^r	Invitrogen
pDM4	Cm^r	33
pMMB208	Cm^r	34
pJP5603	Km^r	40
p34E-Tp	Amp^r Tp^r	17
pBR325	Km^r Tet^r Cm^r	7
pMP190	Cm^r	53
pMAL18	pMMB208-*fmtA*	This work
pMAL28	pMMB208-*mbaA*	This work
pMAL41	pMMB208-*mbaS*	This work
pMAL20	pDM4-*mbaA*::Tp^r	This work
pAA5	pDM4-*fmtA*::Tet^r	This work
pMAL30	pDM4-*mbaS*::Tp^r	This work
pMAL4	pJP5603 with an internal fragment of *fmtA*	This work
pMAL25	pDM4-*mbaF*::Tp^r	This work
pAA6	pDM4-*fptA*::Tet^r	This work
pTET	pCR2.1 with Tet^r from pBR325	This work
pMbaS	pCR2.1 with *mbaS* promoter	This work
pMbaA	pCR2.1 with *mbaA* promoter	This work
pAA1	pMP190-*mbaJ*::*lacZ*	This work

[a] The strains used as controls harboring the pMMB208 vector are not listed in the table. These strains showed phenotypic characteristics identical to those of the respective strain without the empty vector. The antibiotic resistance markers utilized in this study did not include those that provide resistance to any drugs used in the treatment of melioidosis in the United States, according to the Division of Bacterial and Mycotic Diseases, CDC (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/melioidosis_g.htm). Based upon this information, the Oregon Health and Science University Institutional Biosafety Committee does not believe the introduction of these genes into *Burkholderia pseudomallei* compromises the ability to treat disease. We are also aware that these antibiotic resistance genes are being used throughout the world in genetic work with *Burkholderia pseudomallei.* However, since this work may not be allowed under the U.S. *Code of Federal Regulations* (CFR), title 42, part 73 (42 CFR 73) and 9 CFR 121, we have asked the Division of Select Agents and Toxins, CDC, for clarification and are awaiting a response. While awaiting this decision, we have destroyed the resistant strains and have initiated a process to regenerate the mutations described by allelic exchange methods that do not rely on the use of antibiotic-selectable markers.

and tetracycline, 10 μg/ml. M9 minimal medium (44) was also used, and 1.5% (wt/vol) agar was added when needed. For iron-limiting conditions, M9 medium was incubated with Chelex 100 resin (Bio-Rad, Hercules, CA) and then filter sterilized. In some experiments, iron limitation was achieved with 2,2′-dipyridyl (Sigma, St. Louis, MO) at the concentrations indicated. Iron-rich conditions were obtained by the addition of 100-μg/ml ferric ammonium citrate (FAC) to the Chelex-treated M9 medium. Chrome azurol S (CAS) agar plates (46) were used to detect siderophore production. On these plates, siderophore-producing bacteria form colonies with an orange halo, due to the removal of Fe^{3+} from the original blue CAS-Fe^{3+} complex.

All the *B. pseudomallei* manipulations were performed in the biosafety level 3 containment facility in the Department of Molecular Microbiology and Immunology, Oregon Health and Science University (OHSU), according to National Institutes of Health and Centers for Disease Control and Prevention (CDC) regulations.

Bioassay experiments. Strains were grown overnight in M9 with the appropriate antibiotics and diluted 1/100 in Chelex-treated M9 or Chelex-treated M9 with the addition of FAC as indicated below (see Table 3). In some experiments, we used M9 with the iron chelator 2,2′-dipyridyl, as indicated either in the text or in Table 3. Siderophores were added at the following concentrations: malleobactin, 3.3 μg/ml; ornibactin, 1 μg/ml; and pyochelin, 1 μg/ml. Optical density at 600 nm (OD_{600}) was determined after 24 to 48 h.

DNA manipulations and sequence analysis. Chromosomal DNA was extracted according to previously published procedures (44). Plasmid extractions were

performed with the Miniprep kit (QIAGEN, Valencia, CA). Touchdown PCRs were performed with HotStart *Taq* polymerase (QIAGEN, Valencia, CA) under the following conditions: 95°C for 15 min; 40 cycles, each consisting of 95°C for 30 s, 66°C for 1 min (the temperature of this step was lowered 0.3°C each cycle), and 72°C for 1 min; and a final extension step of 72°C for 10 min. Digestions and ligations were performed according to the manufacturer's instructions (New England Biolabs, Inc., Ipswich, MA). DNA sequencing reactions were carried out by the OHSU Molecular Microbiology and Immunology Research Core Facility, using a model 377 Applied Biosystems, Inc., automated fluorescence sequencer. Sequence similarities were analyzed with BLAST (1); when needed, we used the Sanger Institute web site (www.sanger.ac.uk) to perform BLAST analysis. Promoter sequence analysis was performed with the Prokaryotic Database of Gene Regulation (PRODORIC) (36, 37) using default settings and the Fur box from *Pseudomonas aeruginosa* as an input pattern. Analysis of the domains of the nonribosomal peptide synthetases (NRPSs) was performed as described by Challis et al. (10) using the web site http://www.tigr.org/jravel/nrps/.

Construction and complementation of the *B. pseudomallei* mutant strains. Deletions of the entire coding sequence of genes *fmtA*, *fptA*, and *mbaS* were generated using splicing by overlap extension PCR (47). Upstream and downstream regions (each, approximately 700 bp to 800 bp) flanking each gene were amplified with specific primers, and both fragments were mixed and ligated in a new PCR with primers 1 and 2 for each gene (Table 2). In addition, a unique restriction site for a restriction enzyme that can generate blunt ends (Eco47III for *fptA* or SmaI for *fmtA*) was included in primers 3 and 4 (Table 2). In the case of *mbaS*, a naturally occurring NruI site located 75 bp downstream of the putative start codon of the gene was used to insert the trimethoprim cassette. The amplified fragment was cloned in the pCR2.1 vector (Invitrogen, Carlsbad, CA) and digested with the blunt-end-generating enzyme of interest. The plasmids were ligated either to a cassette that contained the resistance to trimethoprim from p34E-Tp digested with SmaI (*mbaS* gene) or to a cassette that encodes the tetracycline resistance from pTET digested with SspI (*fmtA* and *fptA* genes). Plasmids in which the deleted gene was interrupted with the antibiotic resistance cassette were digested with SpeI and XbaI, and the fragment of interest was cloned in the suicide vector pDM4 that had been previously digested with SpeI. *E. coli* S17-1 λpir transformed with the pDM4 derivatives was conjugated with *B. pseudomallei*. Exconjugants were selected on BHI agar or M9 agar with gentamicin and the antibiotic of interest (trimethoprim or tetracycline). Clones were screened for chloramphenicol sensitivity and sucrose resistance. Deletions within the genes of interest were confirmed by PCR. For the construction of the *mbaA* and *mbaF* mutant strains, fragments containing the gene to be mutated and the upstream and downstream flanking sequences were amplified with primers 59F and ROR (*mbaA*) and FOR and 61R (*mbaF*) (Table 2). The fragments included an endogenous Eco47III restriction site that was used to clone the trimethoprim resistance gene. The constructions were subcloned in pDM4 and conjugated into *B. pseudomallei* as described above.

Since the *mbaA*::Tp mutant does not have the upstream region flanking the *fmtA* gene, we could not use the splicing by overlap extension strategy described above to construct the double *mbaA fmtA* mutant. For this reason, we amplified an internal fragment of the *fmtA* gene with primers OU2 and OR2 (Table 2) and cloned in the suicide vector pJP5603, generating plasmid pMAL4. This plasmid was then mobilized into *B. pseudomallei mbaA*::Tp mutant strain to generate the double *mbaA fmtA* mutant. A *fmtA*::pJP5603 Km^r mutant was also constructed as described above using the wild-type strain as a recipient, and the physiological characteristics of this mutant were compared to those of the *fmtA*::*Tet* mutant as a control. Mutations were confirmed by PCR using primers with specificity to the *fmtA* gene and the pJP5603 plasmid.

For the complementation experiments, each gene was amplified by PCR with primers containing restriction sites as indicated in Table 2. The fragments were cloned in pCR2.1 vector (Invitrogen, Carlsbad, CA), sequenced, and then subcloned in pMMB208 under the control of the *Ptac* promoter. The constructs were transferred to *B. pseudomallei* strains by triparental conjugation using the plasmid helper pRK2013. To induce transcription of the cloned genes, 1 mM IPTG (isopropyl-ā-D-thiogalactopyranoside) was added to the solid and/or broth medium.

RNA extractions. Strains were grown to an OD_{600} of 0.3 to 0.6 in Chelex-treated M9 with FAC added, Chelex-treated M9, or Chelex-treated M9 with malleobactin added. For the microarray analysis, three independent cultures of the cells grown in Chelex-treated M9 and Chelex-treated M9 plus FAC were combined and treated as a single sample for the RNA extraction to minimize culture variation. Two samples per condition were used for the microarray analysis. Cells were centrifuged and the pellets were resuspended in RNAWiz reagent (Ambion, Austin, TX). Total RNA was extracted from each strain according to the manufacturer's instructions.

Microarray and hybridizations. The genome sequence and ORF predictions for *B. pseudomallei* strain K96243 were provided by the Wellcome Trust Sanger Institute (Hinxton, Cambridge, United Kingdom). The *B. pseudomallei* DNA microarray (NimbleGen Systems, Inc., Madison, WI) included 5,430 targets represented on the glass slide by 17 unique probe pairs of 24-mer in situ-synthesized oligonucleotides. 321 ORFs (BPSL3110 to BPSL3431) were not included in this microarray. Each pair consists of a sequence perfectly matched to the ORF and another adjacent sequence with two mismatched (MM) bases was used for background and cross-hybridization determination. cDNA labeling and hybridization were performed according to NimbleGen procedures. Microarrays were scanned with an Axon Genepix 4000B scanner at 532 nm and a resolution of 5 μm. Data normalization was carried out by NimbleGen Systems, Inc. (Madison, WI). Microarrays were normalized by the quantile normalization method and analyzed using the affy package to calculate the robust multiarray average values for each ORF (20, 23). Further analysis of these normalized data was performed with GeneSpring 6.0 (Agilent Technologies, Palo Alto, CA) software. Genes with a >2-fold difference of expression between each condition and a P value of <0.01 were further analyzed and described in the manuscript. The Institute for Genomic Research Comprehensive Microbial Resource was consulted for functional classification of each ORF (41).

RNase protection experiments (RPA). Internal fragments of *mbaA*, *mbaS*, *mbaE*, *mbaJ*, and *fmtA* genes, as well as the 3′ and 5′ end regions of the *mbaI-mbaA*, *mbaA-fmtA*, and *fmtA-mbaF* genes analyzed (see Fig. 4), were amplified by PCR using the primers described in Table 2. Reverse primers were designed containing the recognition sequence for the T7 polymerase. RNA probes were synthesized with the MAXIscript SP6/T7 in vitro transcription kit (Ambion, Austin, TX). RPA experiments were conducted with the RPAIII RNase protection assay (Ambion, Austin, TX) according to the manufacturer's instructions. Urea–6% acrylamide gels were exposed between 4 and 24 h, and films (Kodak) were developed.

RT-PCR. RNA was extracted as described above from cells growing in Chelex-treated M9 and treated with DNase TURBO DNA-free (Ambion, Austin, TX) at 37°C for 1 h. cDNA synthesis was performed using MonsterScript reverse transcriptase (EPICENTRE Biotechnologies, Madison, WI) according to the manufacturer's instructions with primers PASRRPA or IR. PCRs were performed using 2 μl of each reverse transcription (RT) reaction. cDNA obtained with primer PASRRPA was used in PCRs with primers PASRRPA-PASU3SAL to amplify the intergenic region located between the *mbaA* and *mbaI* genes and primers IF and IR that amplify a fragment of 200 bp located 373 bp downstream of the putative start codon of *mbaI* and 5,103 bp from the sequence recognized by PASRRPA. cDNA obtained with primer IF was used in PCRs with primers IJ and JI to amplify a fragment of 221 bp comprising the 5′ end of *mbaI* and the 3′ end of *mbaJ* and primers JF and JR, which amplify a fragment of 177 bp located 220 bp from the putative start codon of *mbaJ* and 9,847 bp from the sequence recognized by JF. PCR parameters were as described above, and a control without reverse transcriptase enzyme in the RT reaction was used in each PCR. Fragments were resolved by electrophoresis on a 2% agarose gel.

RLM-RACE. The RNA ligase-mediated rapid amplification of cDNA ends (RML-RACE) method was used as previously described (6) with modifications. RNA from wild-type K96243 was extracted from cells growing in Chelex-treated M9 and treated with DNase TURBO DNA-free (Ambion, Austin, TX) as described above. The DNA-free RNA was purified using RNeasy spin columns (QIAGEN, Valencia, CA), and 10 μg was used in subsequent reaction mixtures. RNA was treated with tobacco acid phosphatase (TAP), and ligation of 5′ RNA adapters to the 5′-terminal ends was carried out using the FirstChoice RLM-RACE kit (Ambion, Austin, TX) according to the manufacturer's instructions. TAP-untreated control reactions were performed by replacing the TAP with nuclease-free water. Reverse transcription was carried out with primer 61R using 2 μl (each) of the adapter-ligated RNA and SuperScript II (Invitrogen, Carlsbad, CA) reverse transcriptase in a final volume of 20 μl. This cDNA was subsequently used as a template in a PCR with an adapter-specific 5′ RACE outer primer (Ambion, Austin, TX) and a gene-specific primer (PASRRPA). PCRs were performed as described above, and the products were analyzed on 2% agarose gels. The fragments obtained from the TAP-treated and TAP-untreated samples were purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA), cloned in pCR2.1 vector (Invitrogen, Carlsbad, CA), and sequenced.

Fur titration assay (FURTA). Upstream regions from *mbaS* and *mbaA* genes were amplified with specific primers (Table 2) and cloned in the high-copy-number plasmid pCR2.1, yielding plasmids pMbaS and pMbaA, respectively. The *E. coli* H1717 reporter strain was transformed with these plasmids and the empty vector as control. The colonies obtained were streaked onto MacConkey-lactose plates with or without the addition of 30 μM ferric ammonium sulfate, as

TABLE 2. Primers used in this work[a]

Name	Sequence (5′ to 3′)	Used in:
ORRPA	**TAATACGACTCACTATAGG**AGGCTCGGCCCCTTCGCGAGCCA	RPA
OFRPA	GCAGACCCGACGATGGCGACGATG	RPA
ARRPA	**TAATACGACTCACTATAGG**GCGAGCTCCACCTGCCCGTGCGCC	RPA
AFRPA	GCGCTTTCGCGACACGAACTACGC	RPA
FURRPA	**TAATACGACTCACTATAGG**GTCTCGATCTCGGCGTCGAAGAA	RPA
FFUR	GGCCACCCTACCGCGCCTCAA	RPA
SRRPA	**TAATACGACTCACTATAGG**GCGCAGCGTGTCGCGCACGAC	RPA
SF	GTCGCGTACGTGACGCGGATG	RPA
OARPA	**TAATACGACTCACTATAGG**CGCCTCGCCGCCCGGCTGCCGCGC	RPA
AORPA	GACGCAGCCGACACGACCGAATA	RPA
OFRRPA	**TAATACGACTCACTATAGG**GTAGACGAGTTTCTTCTTTGCCAT	RPA
PASRRPA	**TAATACGACTCACTATAGG**CCCGCTTCCGCGAGTCGCACGG	RPA, RT-PCR
EF	GCCGACGCGTCGCTCGCGTGG	RPA
ERPA	**TAATACGACTCACTATAGG**CGCGCCGATCCGCTCGACGTC	RPA
JF	GTCGCGCTGCTCGCGGTGATG	RPA
JRPA	**TAATACGACTCACTATAGG**GTCGAAGCACCGCGCGAAGCG	RPA
IJ	GCCGAGCTTCGCGAGGAACTG	RT-PCR
JI	GCGATCGACCGCGCGCGCGAC	RT-PCR
IF	CAGACGGTGCACGCGCCGATG	RT-PCR
IR	GTGCGTCGTCAGCACGAACCA	RT-PCR
61R	CACCGACAGCGCACGCGCGAC	RT reaction in RLM-RACE
61F	GCGTTCGGCTGGCACCGCGGGATG	*mbaA* mutant
ROR	GTAGTTGCCGACCTGCACGCC	*mbaA* mutant
FMT1	TCGTCGATCGCCCGCTCATCGA	SOE (*fmtA*)
FMT2	CTCGCGCAGGCGCGCCTCGAA	SOE (*fmtA*)
FMT3SMA	GTTGAACAGGTTCGTCGCGTTG*CCCGGG*TAGAACGCCACGCCGGCCGC (SmaI)	SOE (*fmtA*)
FMT4SMA	CGGCCGGCGTGGCGTTCTA*CCCGGG*CAACGCGACGAACCTGTTCAA (SmaI)	SOE (*fmtA*)
FPT1	TGTTTCGCGGCAGCCTCGGATGG	SOE (*fptA*)
FPT2	CGGCGCGCGCCGCGTGCGCGAG	SOE (*fptA*)
FPT3	CATCACGTTGCGCGGCTCCCC*AGCGCT*CCCGCCACCCGTACCGCATT (Eco47III)	SOE (*fptA*)
FPT4	ATGCGGTACGGGGTGGCGGG*AGCGCTG*GGGAGCCGCGCAACGTGATG (Eco47III)	SOE (*fptA*)
FOR	GATCAACCTGTCGAGCTGGCG	*mbaF* mutant
59R	CACGAGTTCGTCGAGGATCACGAG	*mbaF* mutant
S1	GCTCGAAGAGCGGCACGTTGCC	SOE (*mbaS*)
S2	GCCGAAATCGGCCGCGAAGCGCTG	SOE (*mbaS*)
S3	GCATTTTTTTACCGTCCGCGCGCGCGCGAGCTTGACGAGCAT	SOE (*mbaS*)
S4	ATGCTCGTCAAGCTCGCGCGCGCGCGGACGGTAAAAAAATGC	SOE (*mbaS*)
FMTH	*AAGCTT*ACCGAATACGAAGAAGCCGACCC (HindIII)	Complementation (*fmtA*)
FMTE	*GAATTC*GTAGACGAGTTTCTTCTTTGCCAT (EcoRI)	Complementation (*fmtA*)
MBAH	*AAGCTT*CATCGGGCGCATCGATGAACG (HindIII)	Complementation (*mbaA*)
MBAE	*GAATTC*CGTGCTGGTTGCCCAACTCCAT (EcoRI)	Complementation (*mbaA*)
SH	C*AAGCTT*GAAGAGCGGCACGTTGCC (HindIII)	Complementation (*mbaS*)
SB	C*GAATTC*GGGTTGCTGGGCTTGCGTCAT (BamHI)	Complementation (*mbaS*)
PASU3SAL	T*GTCGAC*ACGCAGGCGCGCGATGCCGAT (SalI)	FURTA (*mbaA*), RT-PCR
PASRXBA	T*TCTAGA*CCCGCTTCCGCGAGTCGCAC (XbaI)	FURTA (*mbaA*)
S3XBA	T*TCTAGA*GCGCGCGAGCTTGACGAGCAT (XbaI)	FURTA (*mbaS*)
S13SAL	T*GTCGAC*GCTCGAAGAGCGGCACGTTGCC (SalI)	FURTA (*mbaS*)
TETF	*AATATT*CATGTTTGACAGCTTA (SspI)	pTET construction
TETR	*AATATT*GTTTGCGCATTCACAG (SspI)	pTET construction
JSAL	T*GTCGAC*ACGGGTCAATAAGGGAATCGG (SalI)	*lacZ* fusion
JXBA	T*TCTAGA*GTCGCGCAAGCGCGTGGATTGC (XbaI)	*lacZ* fusion
OU2	AGCTGGCGCGTCGATCCGTACATG	*fmtA* mutation
OR2	GTGCTCCTGCGACGTGCGCGTCCA	*fmtA* mutation

[a] Boldface letters represent the recognition sequence for T7 polymerase. The restriction sites are shown in italics, and the restriction enzyme used with each primer is indicated.

previously described (58). The phenotype of the colonies was checked after 24 to 48 h of incubation at 37°C.

β-Galactosidase assays. The upstream region of the *mbaJ* gene was amplified with primers JSAL and JXBA, cloned in pCR2.1 vector (Invitrogen, Carlsbad, CA), sequenced, and subcloned in the pMP190 vector previously digested with SalI and XbaI, generating plasmid pAA1. Plasmids pMP190 and pAA1 were transferred to *B. pseudomallei* wild-type and mutant strains by triparental conjugation. Bacterial strains harboring either pMP190 or pAA1 plasmids were grown in Chelex-treated M9 or Chelex-treated M9 with FAC to an OD_{600} of 0.3 to 0.6. One milliliter of each culture was centrifuged, resuspended in the same volume of buffer Z, and used in the assay. β-Galactosidase activities were determined as previously described (32).

Siderophore purification and analysis. All siderophore purification procedures were carried out using acid-washed (with 0.1 N HCl) glassware.

For the purification and characterization of the siderophore malleobactin, we used supernatants of the wild-type *B. pseudomallei* K96243 strain grown in Chelex-treated M9. The supernatant was lyophilized and resuspended in 1/10 of the volume in high-performance liquid chromatography (HPLC)-grade water (Mallinckrodt Chemicals), and the insoluble matter was separated by centrifugation. Further purifications were performed using a Sephadex LH-20 (Fluka,

Buchs, Switzerland) manual column (20 by 1 cm; Econo Column Bio-Rad, Hercules, CA) with mobile-phase water containing increasing concentrations of methanol from 0 to 50%. This procedure was repeated twice. The siderophore activity was followed by spotting the fractions onto a thin-layer chromatography (TLC) plate (Merck 1.05748) and then developed using 1% $FeCl_3$ in 0.5 N HCl as specific reagent. The positive fractions (I and II) were tested by bioassays, as described above, to assess siderophore activity; purity was confirmed by HPLC (Beckman Gold HPLC System) monitoring at 230 nm using a C_{18} reverse-phase HPLC column (inner diameter, 150 by 6 mm; AQ-312; YMC). The composition of the mobile phases was as follows: phase A, water and 0.01% (vol/vol) trifluoroacetic acid; phase B, acetonitrile and 0.01% (vol/vol) trifluoroacetic acid. Samples were eluted at a flow rate of 1 ml/min using 100% of mobile phase A for 1 min and then a linear gradient of mobile phase B from 0 to 100% over 30 min. For comparative purposes, malleobactin was also purified by phenol-chloroform extraction as described previously (35), and its purity was confirmed by HPLC as described above. Its hydroxamate characteristics were confirmed by the perchloric acid assay (3).

Nominal masses of the ion species (*m/z*) detected during malleobactin purification were determined by mass spectrometry (MS), carried out at the BioAnalytical Shared Resource Core Facility at OHSU with a Thermo Electron LCQ Advantage ion trap mass spectrometer, equipped with an electrospray ionization source. The full electrospray ionization-mass spectrum data were acquired in a negative mode.

The ornibactins from *Burkholderia cenocepacia* were separated by TLC (catalogue no. 1.05748; Merck) using a mobile phase of butanol:acetic acid:water (3:1:1 [vol/vol]). After being sprayed with $FeCl_3$ as described above, positive spots were scraped from the TLC, and samples were extracted twice with methanol. Sample purity was assessed by HPLC, and their molecular weights (*m/z* 790, 762, and 734) were determined by MS as described above for malleobactin. Siderophore activities were confirmed with *B. cenocepacia* and *B. pseudomallei* as indicator strains.

Pyochelin was purified from *B. pseudomallei* strains grown in Chelex-treated M9 medium. When pyochelin was purified from the *mbaA* or *mbaS* mutant strains, they were grown for 72 h before the extraction. The supernatants were extracted twice with ethyl acetate, and the organic phases were dried under nitrogen flux as previously described (14). The samples were resuspended in methanol and used for further analysis. Pyochelin was identified by retention times as determined by HPLC monitoring at 254 nm, by use of a high-performance TLC plate (HPTLC; catalogue no. 1.11764, Merck), and by its UV characteristics. The solvent used for the HPTLC was chloroform:acetone:ethanol (90:5:2.5 [vol/vol]), and the presence of pyochelin was confirmed by UV fluorescence. The green fluorescent spots were scraped from the HPTLC, extracted twice with methanol, and analyzed by HPLC. Pyochelin purified from *P. aeruginosa* was used as a standard.

RESULTS

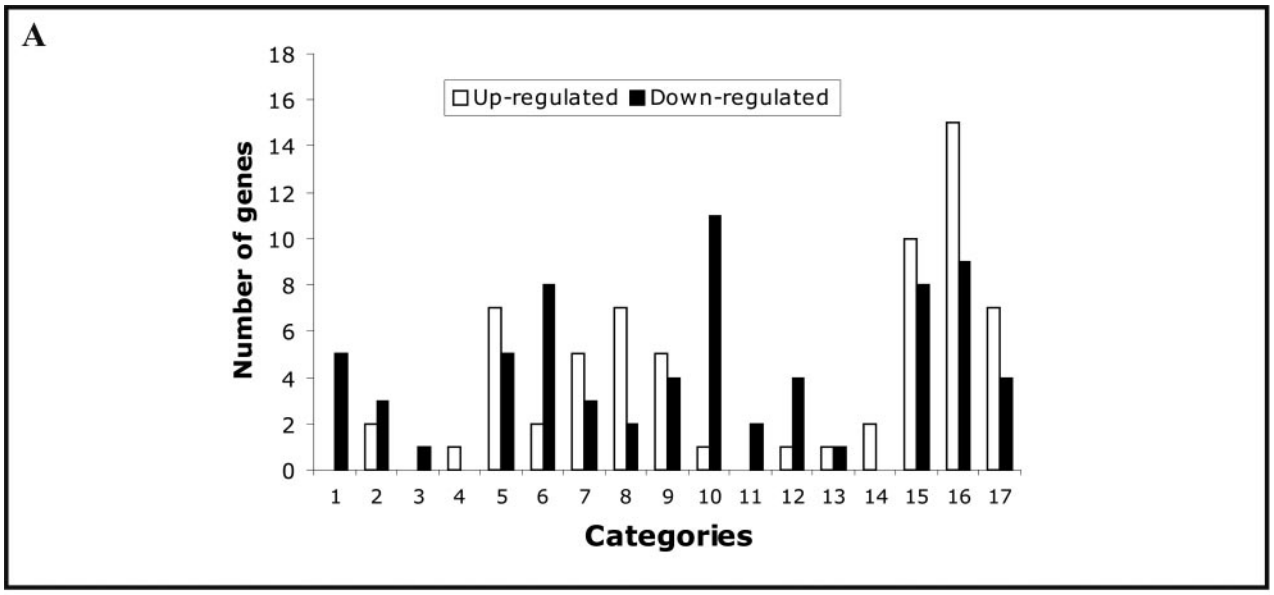
Global transcriptional analysis of *B. pseudomallei* growing under various iron concentrations. To identify genes that are up- or down-regulated under iron-limiting conditions, we performed a global transcriptional analysis of the *B. pseudomallei* K96243 strain grown in Chelex-treated M9 minimal medium (iron limiting) and the same medium supplemented with FAC (iron replete), as described in Materials and Methods. The expression level for each ORF was determined for each condition; those showing a statistically significant change in expression of >2 fold were considered differentially regulated.

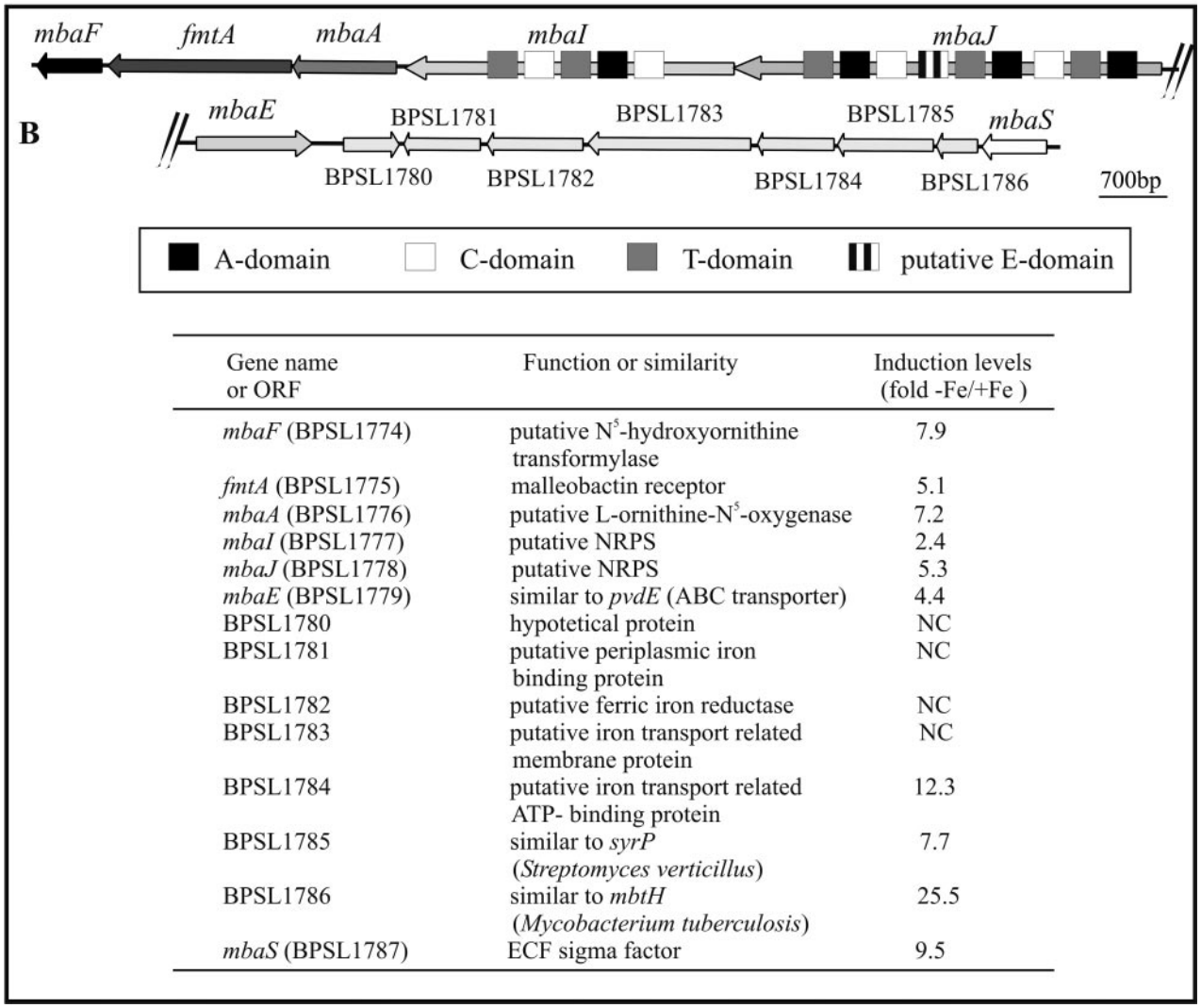
Under iron-limiting conditions, 66 genes were up-regulated, while 69 genes showed a twofold decrease in expression. When all of these genes were sorted into functional classes, it was clear that several biological functions were affected by the iron-limiting conditions (Fig. 1A; also, data available upon request). However, for the sake of simplicity, only a few of these genes will be described below. Under iron-limiting conditions, a cluster of ORFs (BPSL1774 to BPSL1779 and BPSL1784 to BPSL1787) showed a strong induction (Fig. 1B). These ORFs encoded predicted proteins with high similarity to those described for the ornibactin and pyoverdin biosynthesis and transport in *B. cenocepacia* and *P. aeruginosa*, respectively. These ORFs were further analyzed in this work (see below). ORFs BPSS0243, BPSS0244, and BPSS0362 encoded predicted proteins that could be involved in hemin transport. The ORF BPSS0362 showed the highest expression level under iron-limiting conditions (39 fold). The predicted protein encoded by ORF BPSS0244 had similarities to hemin receptors and possessed a corresponding TonB box, suggesting that *B. pseudomallei* could possibly utilize hemin as an iron source. NRPSs are involved in peptide siderophore and antibiotic biosynthesis. Interestingly, in our microarray analysis two ORFs (BPSS1633 and BPSS1634) that encoded predicted NRPSs were induced under iron-limiting conditions, in addition to those present in the gene cluster analyzed in this work. The former ORFs showed similarity to NRPSs from *Pseudomonas syringae* pv. syringae, involved in the biosynthesis of syringomycin, a cyclic lipodepsinonapeptide toxin that causes necrotic symptoms in host plants (5).

Under iron-limiting conditions, ORFs that encode predicted proteins with similarity to succinate dehydrogenases subunits A to D (ORFs BPSS1717 to BPSS1720), aconitase (*acnA*), and those involved in protein biosynthesis (e.g., BPSS1716, BPSL0915, BPSL1943, and BPSL1962) were down-regulated. ORFs BPSS1162 and BPSS1163 showed the widest change in down-regulation; the predicted proteins encoded by these ORFs showed similarity to response regulators containing a CheY-like receiver domain (BPSS1162) and to cyclic AMP-binding proteins (BPSS1163). In addition, the gene *pilA*, involved in virulence in *B. pseudomallei* (19), appeared to be repressed under iron-limiting conditions.

The following sections focus on the identification and characterization of those genes involved in the biosynthesis and transport of the siderophore malleobactin.

Identification of the genes involved in malleobactin biosynthesis and uptake. From our microarray analysis described above, we found that almost all the genes present in the cluster harboring the ORFs BPSL1774 to BPSL1787 were induced under iron-limiting conditions (Fig. 1B). The predicted proteins from these ORFs showed similarity to proteins involved in the biosynthesis and transport of hydroxamate-type siderophores, such as ornibactin and malleobactin. ORFs BPSL1774 and BPSL1776 encoded a protein with similarity to PvdF from *B. cenocepacia* (GenBank accession no. AAG33249; 67% identity [I], 76% similarity [S]) and *P. aeruginosa* (AAX16356; 71% I, 80% S) (51) and the PvdA protein from *B. cenocepacia* (O51940; 77% I, 86% S) and *P. aeruginosa* (AAX16329; 48% I, 65% S), both involved in ornibactin and pyoverdin biosynthesis, respectively (52, 61). ORF BPSL1775 encodes a protein that shows similarity to the ornibactin receptor OrbA from *B. cenocepacia* (AAG33248; 66% I, 78% S) (51). Two other genes, BPSL1777 and BPSL1778, located upstream of BPSL1776, also presented high levels of induction under iron-limiting conditions (Fig. 1B; also, data available upon request); the encoded products showed similarity to the NRPSs family of proteins. The predicted protein, BPSL1778, showed similarity to PvdJ from *P. aeruginosa* (AAX16360; 40% I, 53% S) involved in pyoverdin biosynthesis (27, 42) and to BCAL1696 from *B. cenocepacia* J2315 (55% I, 61% S) (www.sanger.ac.uk), while the predicted protein (BPSL1777) showed similarity to PvdI from *P. aeruginosa* (AAX16327;





Gene name or ORF	Function or similarity	Induction levels (fold -Fe/+Fe)
mbaF (BPSL1774)	putative N^5-hydroxyornithine transformylase	7.9
fmtA (BPSL1775)	malleobactin receptor	5.1
mbaA (BPSL1776)	putative L-ornithine-N^5-oxygenase	7.2
mbaI (BPSL1777)	putative NRPS	2.4
mbaJ (BPSL1778)	putative NRPS	5.3
mbaE (BPSL1779)	similar to *pvdE* (ABC transporter)	4.4
BPSL1780	hypotetical protein	NC
BPSL1781	putative periplasmic iron binding protein	NC
BPSL1782	putative ferric iron reductase	NC
BPSL1783	putative iron transport related membrane protein	NC
BPSL1784	putative iron transport related ATP- binding protein	12.3
BPSL1785	similar to *syrP* (*Streptomyces verticillus*)	7.7
BPSL1786	similar to *mbtH* (*Mycobacterium tuberculosis*)	25.5
mbaS (BPSL1787)	ECF sigma factor	9.5

FIG. 1. Differentially expressed genes under iron-limiting conditions. (A) Genes categorized by functional classification according to TIGRFAM designations (41). Columns: 1, regulatory functions; 2, transcriptional factors; 3, DNA metabolism; 4, mobile and extrachromosomal element functions; 5, cell envelope; 6, energy metabolism; 7, cellular processes; 8, transport and binding proteins; 9, protein fate; 10, protein

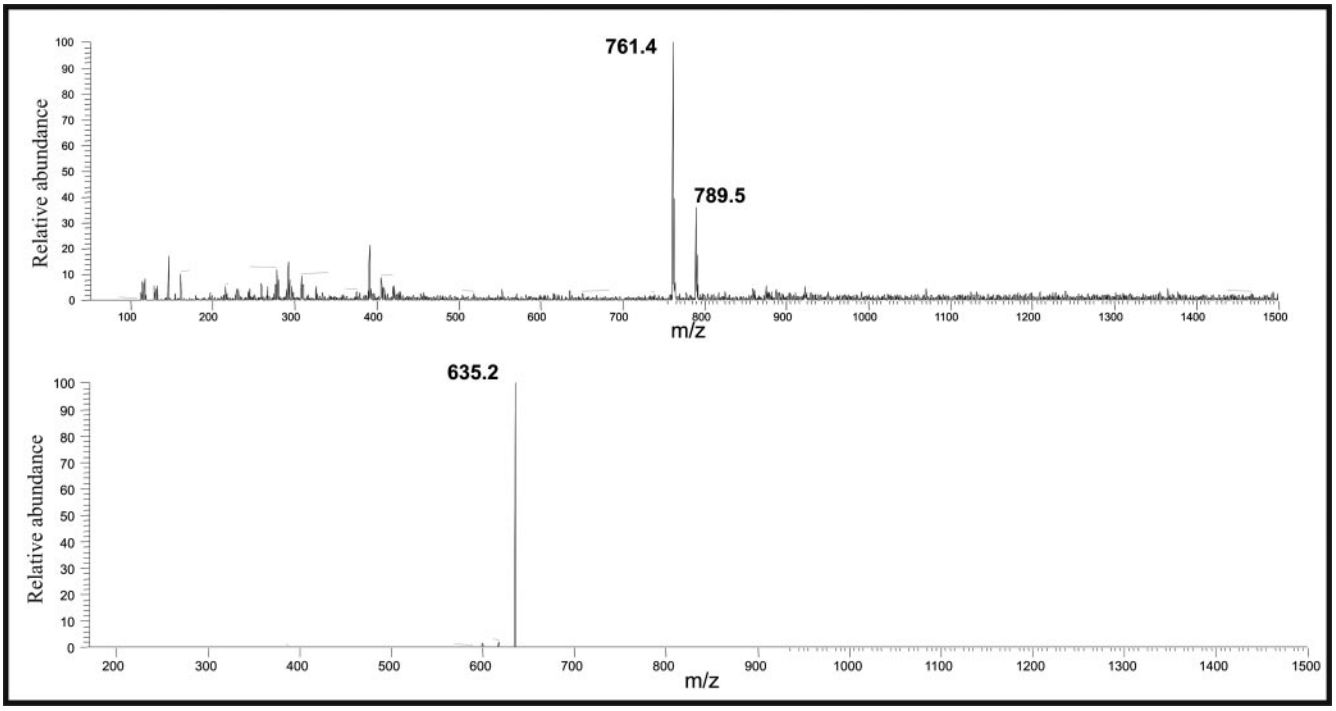


FIG. 2. Negative-mode electrospray ionization mass spectra of the purified siderophore malleobactin from *B. pseudomallei* K96243, identifying three different $[M^- H]^-$ ion species. The fractions containing siderophore activity as assessed by bioassays were fraction I (top) and fraction II (bottom). The nominal masses (*m*/*z*) of the different ion species are indicated in the spectra.

44% I, 56% S) also involved in the pyoverdin biosynthesis (27) and to BCAL1697 from *B. cenocepacia* J2315 (62% I, 66% S) (www.sanger.ac.uk). Prediction of the NRPS domains is depicted in Fig. 1B. In BPSL1778, the second and the third activation (A) domains appear to activate aspartic acid and serine, respectively, while the first A domain does not show any similarity to other known A domains. In the case of BPSL1777, the only A domain present in this protein is predicted to activate N^5-hydroxyornithine. According to the results shown in the manuscript and to simplify nomenclature, ORF BPSL1775 is henceforth designated the ferric malleobactin transport gene A (*fmtA*) and ORFs BPSL1774, BPSL1776, BPSL1777, and BPSL1778 are designated the malleobactin biosynthetic genes (*mbaF*, *mbaA*, *mbaI*, and *mbaJ*, respectively). It should be noticed that the amino acids predicted to be activated by the corresponding A domains of MbaJ and MbaI are not the same as those predicted to be activated in PvdJ and PvdI from *P. aeruginosa* (42).

To determine the function of MbaA and FmtA in the biosynthesis and transport of malleobactin, we constructed two mutant strains in the corresponding genes. To dissect their functionality, it was necessary to first purify the siderophore malleobactin, which until now was known as a 1,000-Da hydroxamate siderophore (70) from *B. pseudomallei* K96243 cells growing under iron-limiting conditions as described in Materials and Methods. By MS, we determined the nominal masses (*m*/*z*) of the purified compounds showing siderophore activities in bioassays. From our MS spectra (Fig. 2), as well as biological experiments (described below), we determined that malleobactin is not a single siderophore with the molecular mass of 1,000 Da, as previously described, but is a mixture of at least three compounds with different molecular masses. The results shown in Fig. 2 indicated that fraction I contained two prominent $[M^- H]^-$ ion peaks in the spectrum, with suggested molecular masses of 790 and 762 Da, while fraction II contained only one peak with a molecular mass of 636 Da. It should be noted that in all the experiments where purified malleobactin was added to the medium, both fractions were used either separately or together with comparable results.

The *mbaA* mutant strain (ALE-7) could not grow in Chelex-

synthesis; 11, fatty acid and phospholipids metabolism; 12, central intermediary metabolism; 13, biosynthesis of cofactors, prosthetic groups, and carriers; 14, amino acid biosynthesis; 15, unclassified; 16, hypothetical proteins; 17, hypothetical conserved proteins. Gene regulation under iron-limiting conditions is shown as up-regulated (white columns) or down-regulated (black columns). (B) Schematic representation of the gene cluster involved in malleobactin biosynthesis and transport in *B. pseudomallei* K96243 and levels of induction under iron-limiting conditions. The ORF number corresponds to the genome annotation, and the proposed name is assigned to those studied in this work. Protein domains are superimposed as boxes on the *mbaJ* and *mbaI* genes that encode predicted NRPSs as follows: activation (A), condensation (C), thiolation (T), and epimerization (E). Induction levels indicate the comparison between the robust multiarray average values obtained under different growth conditions (low iron or high iron) for each transcript, following the analysis described in Materials and Methods. NC, no statistical changes observed for either growth condition.

TABLE 3. Utilization of siderophores by *B. pseudomallei* K96243 wild-type and mutant strains[a]

Strain (genotype)	Medium					
	M9 + FAC	M9	M9 + malleobactin[d]	M9 + ornibactin[e]	M9 + pyochelin[f]	M9 + st *mbaA*[g]
wt[b]	+	+	+	+	ND	ND
mbaA	+	−	+	+	+	+
mbaA/*mbaA*$^+$	+	+	ND	ND	ND	ND
fmtA	+	−	−	−	ND	ND
fmtA/*fmtA*$^+$	+	+	+[c]	+[c]	ND	ND
mbaA fmtA	+	−	−	−	+	+
mbaA fmtA/*fmtA*$^+$	+	−	+	+	ND	ND
mbaF	+	−	+	+	+	+
mbaS	+	−	−	−	+	+
mbaS/*mbaS*$^+$[b]	+	+	+	+	ND	ND
fptA	+	+	ND	ND	ND	ND
mbaA fptA	+	−	+	+	−	−

[a] The strains were grown in Chelex-treated M9 with the addition of the indicated compounds. The growth was evaluated after 24 to 48 h. IPTG (1 mM) was added in the cultures of the complemented strains. FAC, ferric ammonium citrate (100 μg/ml), +, OD_{600} value after 24 to 48 h was at least five times the initial OD_{600} value; −, no growth; ND, not determined.

[b] When siderophore utilization was evaluated, 300 μM 2,2′-dipyridyl was added to the medium.

[c] Growth of *fmtA*/*fmtA*$^+$ strain in these media was higher than the growth observed in the absence of siderophores.

[d] Malleobactin was purified from *B. pseudomallei* K96243. Fractions I and II were used separated and together with identical results.

[e] Ornibactin was purified from *B. cenocepacia.*

[f] Pyochelin was purified from *P. aeruginosa.*

[g] st *mbaA*, ethyl acetate extraction of the supernatant from *B. pseudomallei mbaA* mutant strain.

treated M9 (Table 3) or at 2,2′-dipyridyl concentrations of >100 μM, while the complemented strain grew to OD_{600} values similar to those observed with the wild-type strain. When purified malleobactin was added to the Chelex-treated M9 minimal medium, growth of this mutant was restored (Table 3). When supernatants of the *mbaA* mutant strain were subjected to the purification procedure described to obtain malleobactin, we did not detect the compounds described in Fig. 2. However, we were able to isolate the same compounds described for the wild type in supernatants of the complemented *mbaA* mutant. It was also determined that the latter strain showed a halo similar to that of the wild type in CAS plates, while the *mbaA* mutant had a reduced halo (Fig. 3 and see below).

The *fmtA* mutant strain (ALE-3) cannot grow in Chelex-treated M9 minimal medium with or without 50 μM 2,2′-dipyridyl; however, it could grow in M9 after 30 h of incubation, reaching lower OD_{600} values than the wild type grown in the same medium (Table 3 and data not shown). This strain showed a halo on CAS plates that was consistently larger than that observed for the wild type (Fig. 3). When the *fmtA* mutant strain was complemented with the wild-type gene (strain ALE-12), growth under iron-limiting conditions, as well as the halo size, was restored to levels similar to those of the wild type (Table 3 and Fig. 3). However, when malleobactin was added to the *fmtA* mutant growing in Chelex-treated M9 or M9 with 50 μM 2,2′-dipyridyl, growth was not restored (Table 3). These results suggest that this gene could be involved in ferric malleobactin transport. To corroborate that FmtA was indeed the malleobactin outer membrane receptor, we constructed the double *mbaA fmtA* mutant strain (ALE-50). This double mutant showed a reduced halo on CAS plates, similar to that of the *mbaA* mutant (data not shown), and was not able to grow in Chelex-treated M9 (Table 3) or in M9 with 50 μM 2,2′-dipyridyl. When malleobactin was added to the iron-restricted medium (Chelex-treated M9), the double *mbaA fmtA* mutant was unable to grow, confirming that FmtA was indeed involved in ferric-malleobactin transport. This result was corroborated by complementation of this double mutant with the *fmtA* wild-type gene (ALE-19). In this strain, growth was clearly restored in the presence of malleobactin (Table 3).

The siderophore ornibactin from *B. cenocepacia* was tested by bioassays with all mutant strains. It was clear from these experiments that the FmtA receptor could also recognize this siderophore (Table 3). In addition, each of the three purified ornibactins (*m*/*z* 790, 762, and 734) from *B. cenocepacia* (see Materials and Methods) could cross-feed the *mbaA* mutant but could not restore the growth of the *mbaA fmtA* double mutant unless the *fmtA* gene was present. It was therefore clear from these results and the molecular masses established for two of the malleobactin components identified in this work by MS (790 and 762 Da) that there is a structural similarity between these compounds and the ornibactins. This hypothesis was confirmed because malleobactin (the three identified compounds) could also cross-feed *B. cenocepacia* in bioassays (reference 69 and data not shown). Furthermore, a predictive analysis performed with the A domains of the NRPSs BCAL1696 and BCAL1697 from *B. cenocepacia* J2315 encoded by genes located in a cluster that could be involved in ornibactin biosynthesis confirmed that the same amino acids should be activated as those by MbaJ and MbaI, respectively (data not shown). However, the presence of additional genes in the ornibactin cluster, in comparison with the malleobactin gene cluster described here, suggests differences between the ornibactin and malleobactin molecules.

Since it has been described that *B. cenocepacia* can produce pyochelin (50, 63) and crude supernatants from the *B. pseudomallei mbaA* mutant were able to cross-feed the double *mbaA fmtA* mutant strain in iron-limiting medium (Table 3), we examined whether this result was actually due to pyochelin production. It is worth noticing that we have also observed that the color of the small halo present in the *mbaA* mutant turned reddish after several days (data not shown), a fact that was previously described for pyoverdin mutant strains of *P. aeruginosa* and ascribed to the production of pyochelin (13). Furthermore, from the sequence analysis of the *B. pseudomallei* genome, another cluster of ORFs (BPSS0581 to BPSS0594) was identified that shows similarity to the pyochelin biosynthesis and transport genes present in *P. aeruginosa* (2, 43, 48). ORF BPSS0591 encodes a predicted protein with high similarity to FptA (AAC43213; 63% I, 77% S), the pyochelin receptor from *P. aeruginosa* (2). We named this ORF in *B. pseudomallei fptA* as well. To confirm that this ORF encoded the pyochelin receptor in *B. pseudomallei*, we constructed two new mutant strains: the single mutant *fptA* (ALE-A5) and the double mutant *mbaA fptA* (ALE-13). The single mutant *fptA* did not show any growth defect under iron-limiting conditions (Chelex-treated M9 and M9 with 2,2′-dipyridyl 200 μM) (Table 3), an observation that agrees with malleobactin having a higher affinity for iron than pyochelin, as was described for pyoverdin in *P. aeruginosa* (15). The *mbaA fptA* double mutant was affected in growth under iron limitation to the same extent

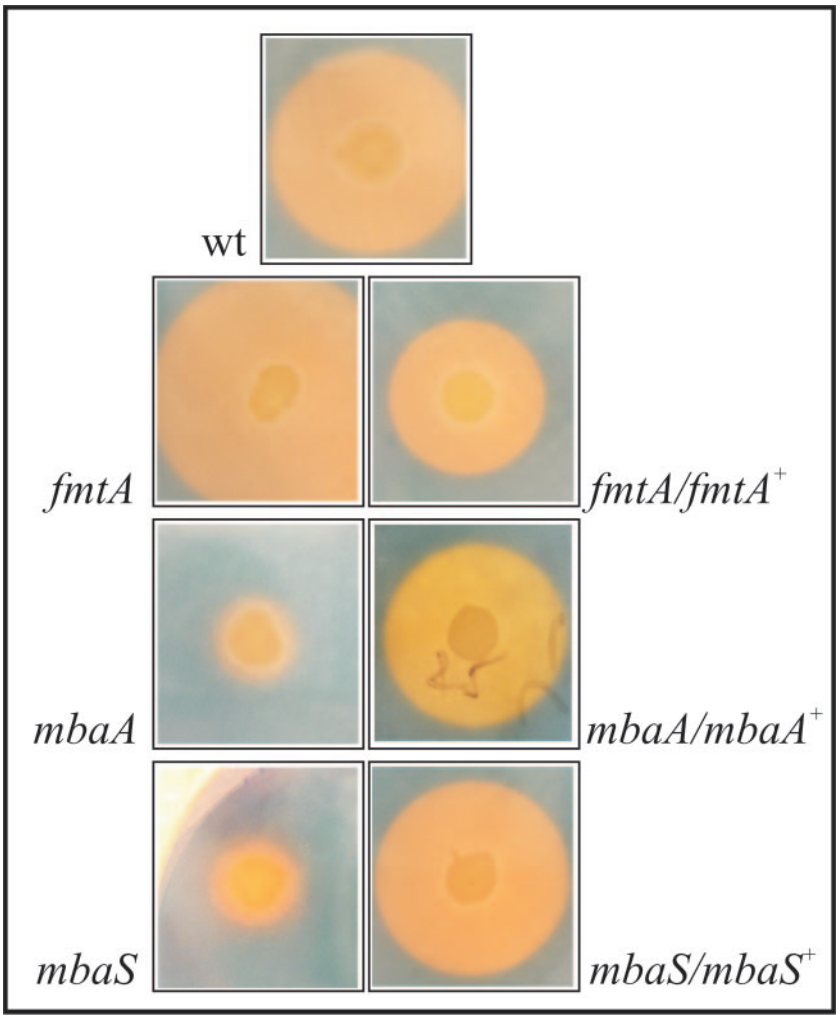


FIG. 3. Detection of siderophore production by *B. pseudomallei* on CAS agar plates. The strains were grown overnight in BHI medium, and 5 μl of the various cultures was spotted onto CAS plates with 1 mM IPTG. The plates were incubated at 37°C for 72 h. The strains are identified as follows: wt, *B. pseudomallei* K96243 with pMMB208; *fmtA*, malleobactin receptor mutant with pMMB208; *fmtA*/*fmtA*$^+$, malleobactin receptor mutant complemented with the wild-type gene; *mbaA*, malleobactin biosynthesis mutant with pMMB208; *mbaA*/*mbaA*$^+$, malleobactin biosynthesis mutant complemented with the wild-type gene; *mbaS*, ECF sigma factor mutant with pMMB208; *mbaS*/*mbaS*$^+$, ECF sigma factor mutant complemented with the wild-type gene.

as the *mbaA* single mutant. The growth of the *mbaA fptA* double mutant could only be restored by the addition of malleobactin but not by pyochelin purified from *P. aeruginosa*, while the single *mbaA* mutant could utilize both siderophores (Table 3). Furthermore, ethyl acetate extracts from supernatants from the *mbaA* mutant growing under iron-limiting conditions were able to cross-feed the *mbaA* and *mbaA fmtA* mutants but not the *mbaA fptA* double mutant, confirming that pyochelin is still synthesized in the *mbaA* mutant (Table 3). Pyochelin production in the *mbaA* strain explains the growth of this mutant in Chelex-treated M9 after 48 to 72 h. Production of pyochelin was also confirmed by HPLC and HPTLC analysis of these extracts, as described in Materials and Methods (data not shown).

The *mbaJIA fmtA* and *mbaF* genes are part of an operon. From the sequence analysis of the gene cluster containing the *fmtA* and *mbaA* genes (Fig. 1), we hypothesized that they could be part of an operon that also harbors three other genes (*mbaF*, *mbaJ*, and *mbaI*). To confirm if these five genes are transcribed together as an operon, we performed RPA analysis. Three probes were designed; one of them, probe a (Fig. 4), spanned the 3′ end of *fmtA* and the 5′ end of *mbaF*; the second probe, b (Fig. 4), spanned the 3′ end of the *mbaA* gene and the 5′ end of the *fmtA* gene; and the third probe, c (Fig. 4), spanned the 3′ end of *mbaI* and the 5′ end of the *mbaA* gene. The results shown in Fig. 4A to C demonstrated that the three probes were protected, indicating that the *mbaI*, *mbaA*, *fmtA*, and *mbaF* genes are cotranscribed as an operon. The smaller fragments observed with the probe from the *mbaA-fmtA* region were also detected with the probe alone, without any RNase treatment, and with increasing exposure time, indicating probe degradation. The analysis of the intergenic region between *mbaI* and *mbaA* indicated that there are at least two other fragments protected together with the full-length probe, suggesting that RNA processing was also taking place (Fig. 4C). A further analysis using the technique described by Bensing et al.

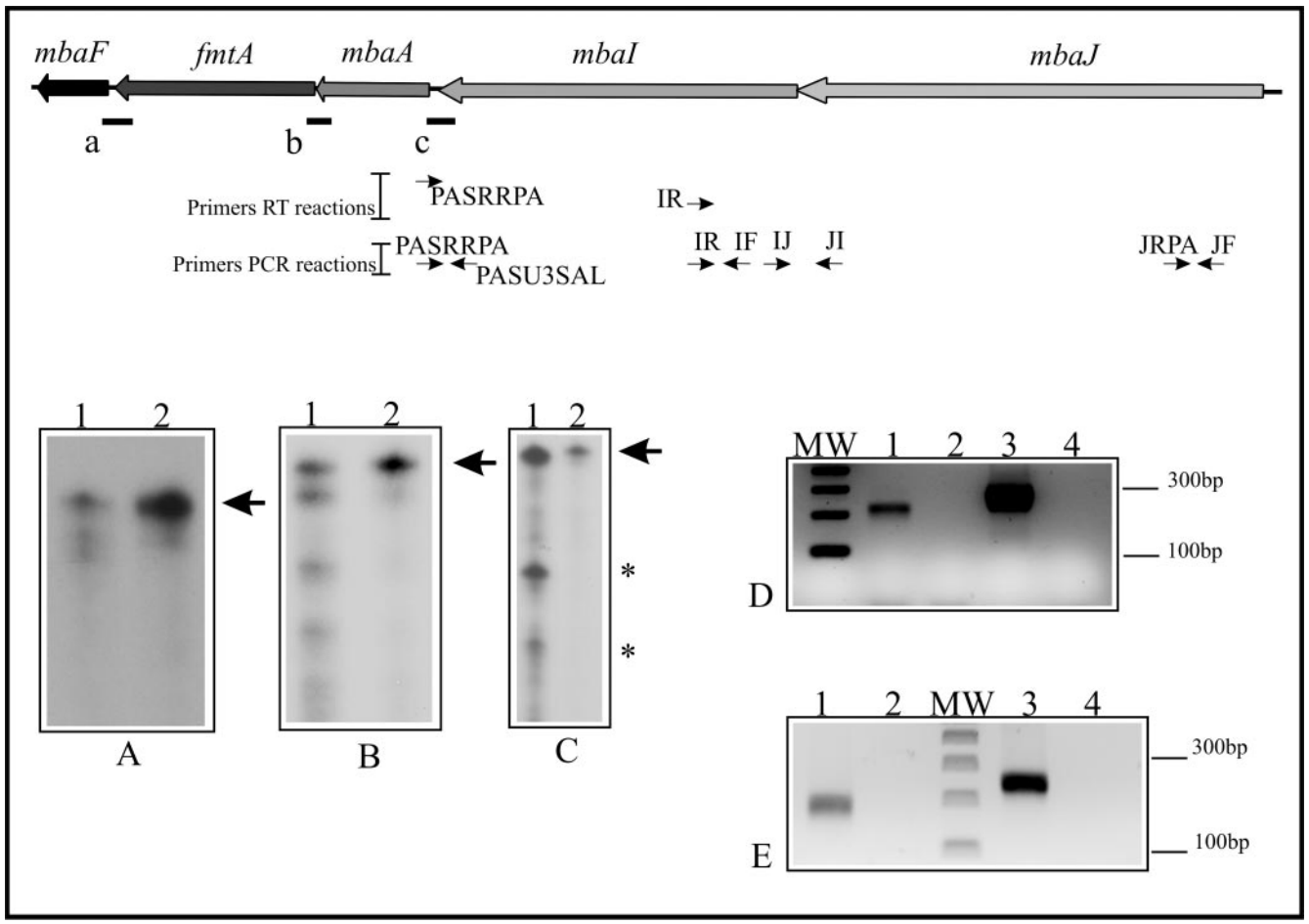


FIG. 4. The *mbaJIA*, *fmtA*, and *mbaF* genes are transcribed together as an operon. (A to C) RNase protection assays performed with total RNA extracted from *B. pseudomallei* K96243 grown in Chelex-treated M9. Lanes 1, RNA from the wild type strain; lanes 2, specific probe without RNase treatment. Specific transcripts for the regions spanning the 3′ end of *fmtA* and the 5′ end of *mbaF* (primers O42 and OFRRPA), the 3′ end of *mbaA* and the 5′ end of *fmtA* (primers AORPA and OARPA), and the 5′ end of *mbaA* and the 3′ end of *mbaI* (primers PASRRPA and PASU3SAL) were detected with riboprobes a, b, and c, respectively. The solid lines below the scheme represent the locations of these riboprobes, the arrow in each panel indicates the full-length probe, and the asterisks (C) indicate the protected fragments (see the discussion in the text). (D and E) Products of RT-PCRs performed as described in Materials and Methods with primers PASRRPA (D) and IR (E). The positions of these primers are depicted below the scheme of the operon. The generated cDNAs were used as templates in PCRs with the indicated primers. (D) Lane 1, amplification with primers IF and IR; lane 3, amplification with primers PASRRPA and PASU3SAL; lanes 2 and 4, same as lanes 1 and 3 but without the addition of RT enzyme in the reaction mixtures. (E) Lane 1, amplification with primers IJ and JI; lane 3, amplification with primers JRPA and JF; lanes 2 and 4, same as lanes 1 and 3 but without the addition of RT enzyme in the reactionmixtures. MW, DNA molecular weight marker.

(6) to discriminate initiated from processed 5′ termini confirmed that there is indeed a processing of the RNA 65 bp upstream of the start codon of *mbaA* (data not shown). RT-PCRs performed using cDNAs obtained with primers located in the 5′ end of *mbaA* (primer PASRRPA) (Fig. 4D) and near the 5′ end of *mbaI* (primer IR) (Fig. 4E) established that the five genes are transcribed as a polycistronic message with a promoter likely located upstream of *mbaJ*.

Regulation of transcription of the malleobactin biosynthesis and transport genes. We intended to establish if the transcription of *mbaJ*, *mbaA*, and *fmtA* genes is regulated by the iron concentration of the medium, as shown from the microarray data (Fig. 1B). Therefore, we performed RPA experiments using total RNA extracted from *B. pseudomallei* wild-type cells growing under iron-rich and iron-limiting conditions with specific RNA probes for *mbaA*, *fmtA*, and *mbaJ* genes. Since in *B. pseudomallei* the *fur* gene is not regulated by the iron levels (29; our own results from the microarray analysis), we used this gene as an internal control in these RPA experiments. As shown in Fig. 5, it is clear that *fmtA*, *mbaA*, and *mbaJ* are induced under iron-limiting conditions. We also included the ORF BPSL1779 in this analysis, which encodes a predicted protein with similarity to PvdE from *P. aeruginosa* (JC5090; 37% I, 59% S) that is a member of the ATP-binding cassette (ABC) family of membrane transporter proteins involved in pyoverdin synthesis (31). We named this gene *mbaE*. As shown in Fig. 5D, this gene appears to be regulated by the iron concentration of the medium. These findings validate our microarray results.

Furthermore, by using a *lacZ* fusion to the *mbaJ* upstream

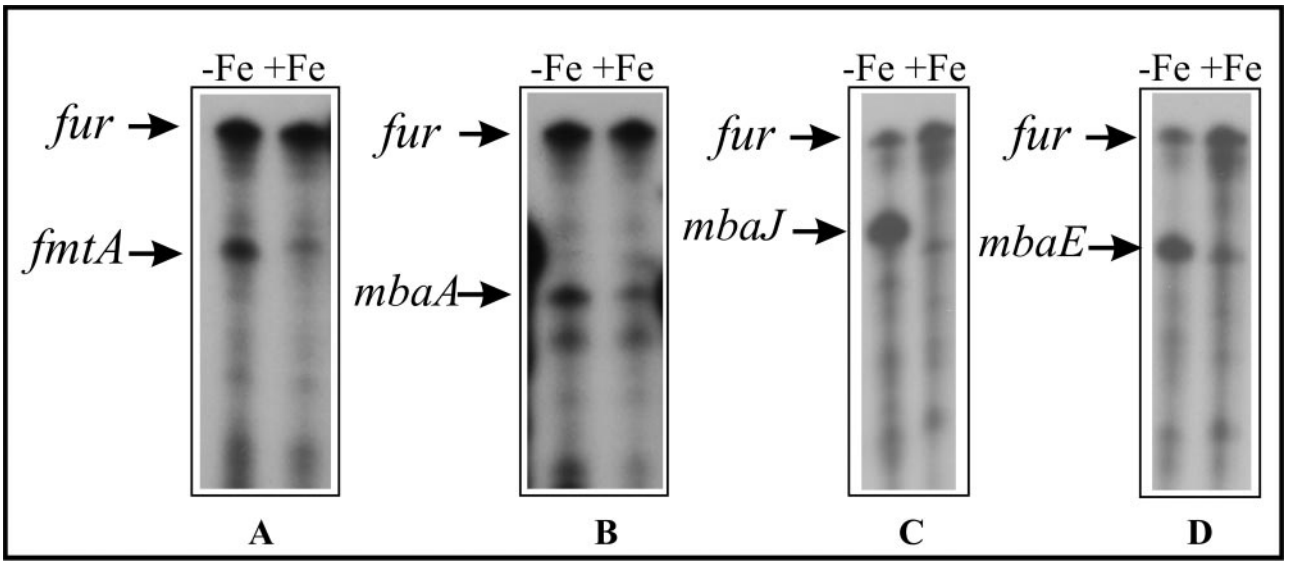


FIG. 5. Expression of the *mbaA*, *fmtA*, *mbaJ*, and *mbaE* genes is regulated by the iron concentration of the medium. RNase protection assays of the *mbaA*, *fmtA*, *mbaJ*, and *mbaE* genes were performed with total RNA extracted from *B. pseudomallei* K96243 grown in Chelex-treated M9 (−Fe) and Chelex-treated M9 plus FAC (+Fe). (A) *fmtA* gene; (B) *mbaA* gene; (C) *mbaJ* gene; (D) *mbaE* gene. The positions of the protected RNAs are indicated by arrows. The *fur* gene was used as a control for RNA loading.

region and measuring β-galactosidase production, we were able to show that iron regulation was indeed at the level of transcription initiation of this region that must contain the promoter (Table 4). We also observed that expression from this putative promoter occurred in the *mbaA* and *fmtA* mutant strains at levels similar to those in the wild type (Table 4).

MbaS, an ECF sigma factor, is involved in the transcriptional control of the *mbaJIA-ftmA-mbaF* operon. Sequence analysis of ORF BPSL1787 revealed that it encodes a predicted protein with similarity to PvdS, an ECF sigma factor from *P. aeruginosa* (AAB09714; 44% I, 60% S) (16). PvdS is involved in the transcriptional activation of the pyoverdin biosynthesis genes, as well as other genes encoding virulence factors such as the endoprotease PrpL and exotoxin A (4, 26, 38, 39, 67). We designated this gene *mbaS* (for malleobactin sigma factor). To analyze the possible involvement of MbaS in malleobactin production and/or transport, we constructed an *mbaS* deletion mutant strain (ALE-48). The phenotypic characteristics of this mutant were similar to those described for the double *mbaA fmtA* mutant. Figure 3 shows that the *mbaS* mutant produces a small halo, as determined by CAS plates, and could not grow under iron-limiting conditions, even in the presence of malleobactin, indicating that the malleobactin transport genes are not being expressed (Table 3). When the mutant strain was complemented with the wild-type *mbaS* gene, malleobactin production and growth under iron-limiting conditions were restored (Table 3). When 2,2′-dipyridyl was added at 300 μM, this complemented strain could not grow, as was also observed for the wild type. However, the addition of malleobactin restored growth, demonstrating the functionality of FmtA in this strain (Table 3). Furthermore, when supernatant of the *mbaS* mutant strain was subjected to the purification procedure described to obtain malleobactin, the extract was unable to cross-feed the *mbaA* mutant. Taken together, all these results suggest that MbaS is indeed involved in the regulation of the malleobactin biosynthesis and transport genes. When supernatants from the *mbaS* strain were extracted by the ethyl acetate protocol used in the pyochelin purification, it was determined that this extract can cross-feed the *mbaA* mutant but not the *mbaA fptA* double mutant. These results demonstrate that pyochelin and its receptor are synthesized in the absence of MbaS.

To examine the transcription of the *mbaS* gene, total RNA from the wild-type strain was extracted and RPA analysis was performed using probes with complementarity to *mbaS*. Figure 6 shows that *mbaS* is induced under iron-limiting conditions, validating our microarray results described in Fig. 1B. When we analyzed the transcription of the *mbaS* gene in the *fmtA* and *mbaA* mutant backgrounds, we observed that iron regulation of *mbaS* transcription was unaffected (Fig. 6).

TABLE 4. Expression of the *mbaJ*::*lacZ* fusion in *B. pseudomallei* wild-type and mutant strains

Strain/plasmid	*lacZ* activity[a]	
	M9[b]	M9 + FAC
wild type/pMP190	<5	<5
wild type/pAA1	551.6 ± 25.7	20.3 ± 8.2
mbaA/pMP190	<5	<5
mbaA/pAA1	336.4 ± 5.0	19.5 ± 1.8
fmtA/pMP190	<5	<5
fmtA/pAA1	464.1 ± 46.5	14.5 ± 2.2
mbaS/pMP190	<5	<5
mbaS/pAA1	<5	<5

[a] Expressed in Miller units. The values represent means ± standard deviation of three independent determinations.

[b] Overnight cultures were diluted 1/100 in Chelex-treated M9 or Chelex-treated M9 plus FAC, and samples were taken at OD_{600} = 0.3 to 0.6. Since the *fmtA* mutant cannot grow in Chelex-treated M9, nontreated M9 was used for this strain.

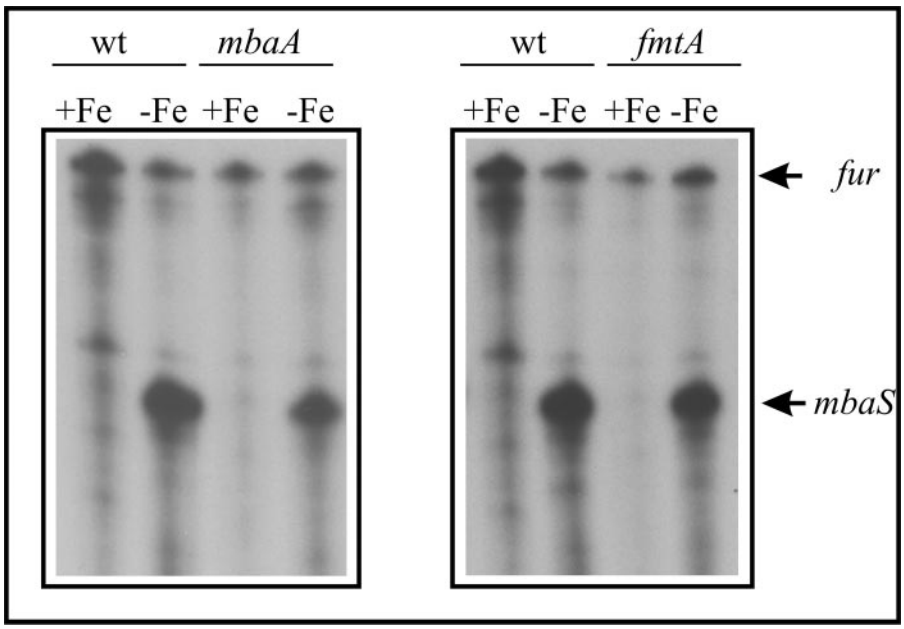


FIG. 6. Expression of the *mbaS* gene is regulated by the iron concentration of the medium. RNase protection assays were performed with total RNA extracted from the *B. pseudomallei* K96243 wild-type strain and the *mbaA* mutant strain, grown in Chelex-treated M9 (−Fe) and Chelex-treated M9 plus FAC (+Fe) (right) and the *B. pseudomallei* K96243 wild-type strain and *fmtA* mutant strain, grown in M9 (−Fe) and M9 plus FAC (+Fe) (left). The arrows indicate the positions of the protected RNAs. The *fur* gene was used as a control for RNA loading.

In the *mbaS* mutant strain, neither *mbaA mbaJ mbaE* nor *fmtA* was expressed, even under iron-limiting conditions (Fig. 7A to D), while transcription was restored upon the addition of IPTG to the medium in the complemented strain in which *mbaS* was under the control of the *Ptac* promoter (Fig. 7A to D). These results clearly demonstrate that the expression of *mbaJ*, *mbaA*, and *fmtA*, as well as *mbaE*, is under the control of MbaS. Furthermore, an *mbaJ-lacZ* fusion in the *mbaS* mutant was not expressed, confirming the involvement of this ECF sigma factor in the transcriptional activation of the *mbaJIAfmtAmbaF* operon (Table 4). Sequence analysis of the upstream region of *mbaJ* revealed a sequence [TAAAA-(N_{16})-CGT] located 106 bp upstream of the start codon of *mbaJ*, showing high similarity to the sequence recognized by PvdS in *P. aeruginosa* [TAAAT-(N_{16})-CGT] (68). Whether this sequence is the consensus sequence for MbaS remains to be determined.

The in silico analysis of the upstream region of *mbaS* using the PRODORIC web site (36, 37) showed a putative Fur box located 136 bp upstream of the putative start codon of the *mbaS* gene (5′-cATAtTGAgAAcgATTcgC-3′; capital letters indicate nucleotides that are identical with those of the 19-mer Fur box consensus) (18), suggesting that transcription in *B. pseudomallei mbaS* could be controlled by the iron concentration in a Fur-dependent manner. Evidence supporting this hypothesis was obtained by FURTA analysis (58) with the promoter region of *mbaS* cloned in a multicopy plasmid. The empty vector and the upstream region of *mbaA* lacking a Fur box were used as controls. Colonies of the *E. coli* H1717 reporter strain with pMbaS showed a red color when they were grown in MacConkey-lactose plates with ferric ammonium sulfate, while both controls showed white colonies as expected. The three strains showed red colonies on plain MacConkey-lactose plates. These results indicate that the *E. coli* Fur protein can recognize a sequence present in the *mbaS* promoter while it cannot interact with the *mbaA* upstream region.

Transcriptional activation of the *mbaA* and *fmtA* genes is not mediated by malleobactin. In the case of siderophores such as pyoverdin and pseudobactin, transcription of the cognate iron transport and biosynthesis genes is activated by the siderophore itself (60, 62). This is also true with *E. coli*, in which diferric dicitrate activates the transcription of its corresponding receptor, FecA (8). In all these systems, signal transduction is mediated by the receptor and a sigma–anti-sigma factor pair (21, 62). An exception is the pyoverdin system, where one anti-sigma factor controls two different sigma factors (4). To analyze the possible involvement of malleobactin in the regulation of its own synthesis and transport, we examined the transcription of the *mbaA*, *fmtA*, and *mbaS* genes under iron-rich conditions (Chelex-treated M9 plus FAC), iron-limiting conditions (Chelex-treated M9), and iron-limiting conditions with the addition of malleobactin to the medium (Chelex-treated M9 plus malleobactin). We used total RNA extracted from the *mbaF* mutant strain (ALE-33) that, although it cannot produce malleobactin, can utilize it under iron-limiting conditions (Table 3). The reason for selecting the *mbaF* mutant was due to the observation of iron-independent transcription of the *fmtA* gene in the *mbaA* mutant strain (data not shown), possibly due to transcription from a promoter of the trimethoprim resistance gene inserted in *mbaA*. Figure 8 shows that *mbaA*, *fmtA*, and *mbaS* genes are regulated by the iron concentration, as was expected from the results described in this work (Fig. 1B, 5, and 6). The presence of malleobactin in the medium did improve growth of the *mbaF* mutant strain under iron-limiting conditions; however, transcription of the genes did not change significantly in the presence of the siderophore, demonstrating that malleobactin does not affect the transcrip-

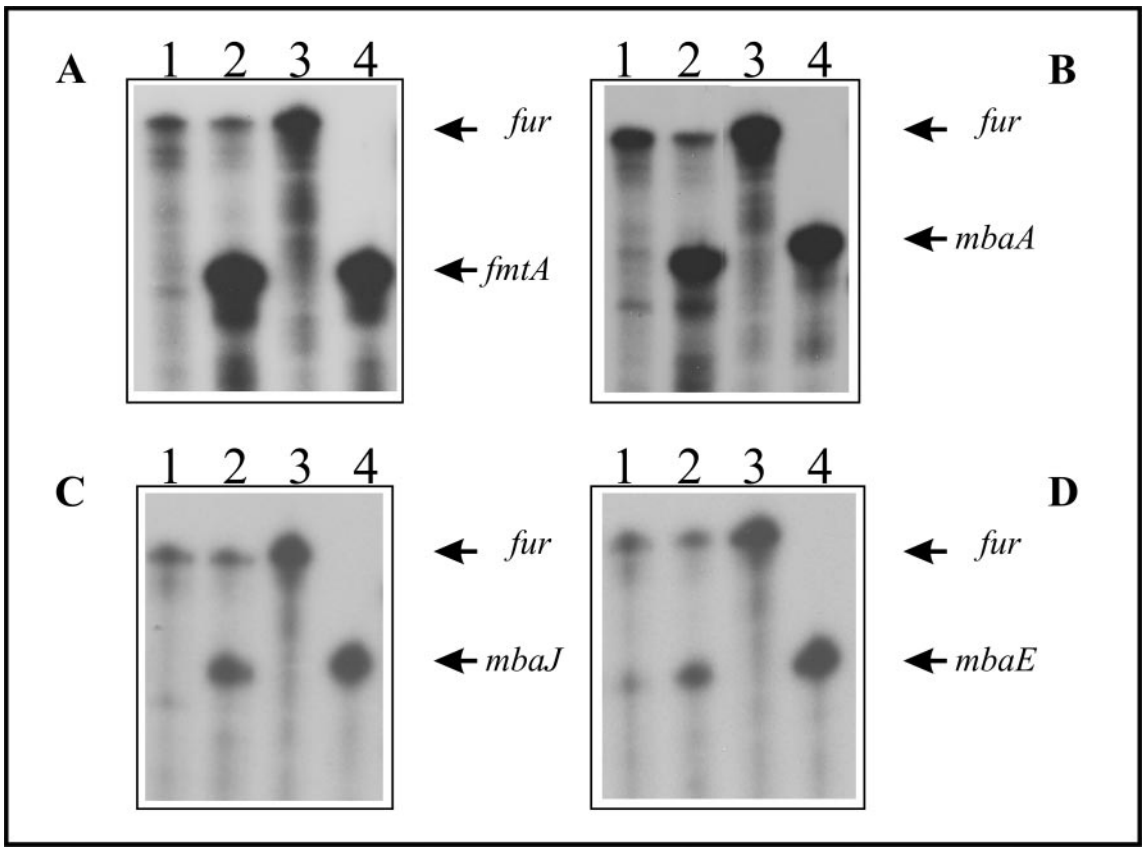


FIG. 7. MbaS controls the transcription of the the *mbaA*, *fmtA*, *mbaJ*, and *mbaE* genes. RNase protection assays were performed with total RNA extracted from the *mbaS* mutant grown in Chelex-treated M9 (lanes 1) and from the *mbaS* mutant complemented with the wild-type *mbaS* gene grown in Chelex-treated M9 plus 1 mM IPTG (lanes 2). (A) *fmtA* gene; (B) *mbaA* gene; (C) *mbaJ* gene; (D) *mbaE* gene. Lanes 3, riboprobe for the *fur* gene without RNase treatment; lanes 4, riboprobe for the *fmtA* (A), *mbaA* (B), *mbaJ* (C), and *mbaE* (D) genes without RNase treatment. The arrows indicate the positions of the protected RNAs. The *fur* gene was used as a control for RNA loading.

tion of the *mbaA*, *fmtA* and *mbaS* genes (Fig. 8, compare lanes 2 and 3 in each panel).

It has been reported that siderophore-inducible outer membrane receptors that interact with the sigma–anti-sigma systems possess an N terminus with an extension, which is absent in those receptors that are not involved in this type of signal transduction (45). Since our results clearly demonstrated that malleobactin was not involved in the transcriptional regulation of *mbaA*, *fmtA*, and *mbaS*, we performed an alignment of the N termini of several outer membrane receptors, including those that possess the extension, and others, such as FepA and FhuA, that do not (45). Our analysis demonstrated that FmtA does not possess this N-terminus extension (data not shown), thereby confirming that it is unlikely that this receptor is involved in the sigma–anti-sigma type of signal transduction.

DISCUSSION

In this work, we characterized the response of the human pathogen *B. pseudomallei* to iron limitation, a situation often encountered by the invading microbe in the host organism and the environment. Our transcriptional analysis indicated that, under iron-limiting conditions, there is induction of a cluster of genes involved in the biosynthesis and transport of the siderophore malleobactin and their regulation. Other ORFs identified in our microarray analysis encode predicted proteins that could function in hemin uptake; since there are no reports on the involvement of this system in the virulence of *B. pseudomallei*, they are of great interest. A cluster of ORFs (BPSS0581 to BPSS0594) harbored in chromosome II showed similarity to the pyochelin biosynthesis and transport gene cluster of *P. aeruginosa* but did not show significant changes in its expression profile under the conditions tested in this work.

Some ORFs, such as those encoding predicted proteins with similarity to the various subunits of the succinate dehydrogenase, showed a strong down-regulation under iron-limiting conditions. Genes with similarity to this cluster reportedly showed comparable repression under iron limitation in other bacteria such as *P. aeruginosa* (66). It is known that transcription of these genes in *P. aeruginosa* is controlled by a pair of small RNAs that are regulated by iron concentration in a Fur-dependent manner (66). Another ORF that was found to be repressed in low iron concentrations encodes a putative aconitase, a gene also controlled by a small RNA in *E. coli* (30). Whether small RNAs are involved in the regulation of the iron homeostasis in *B. pseudomallei* is presently under investigation.

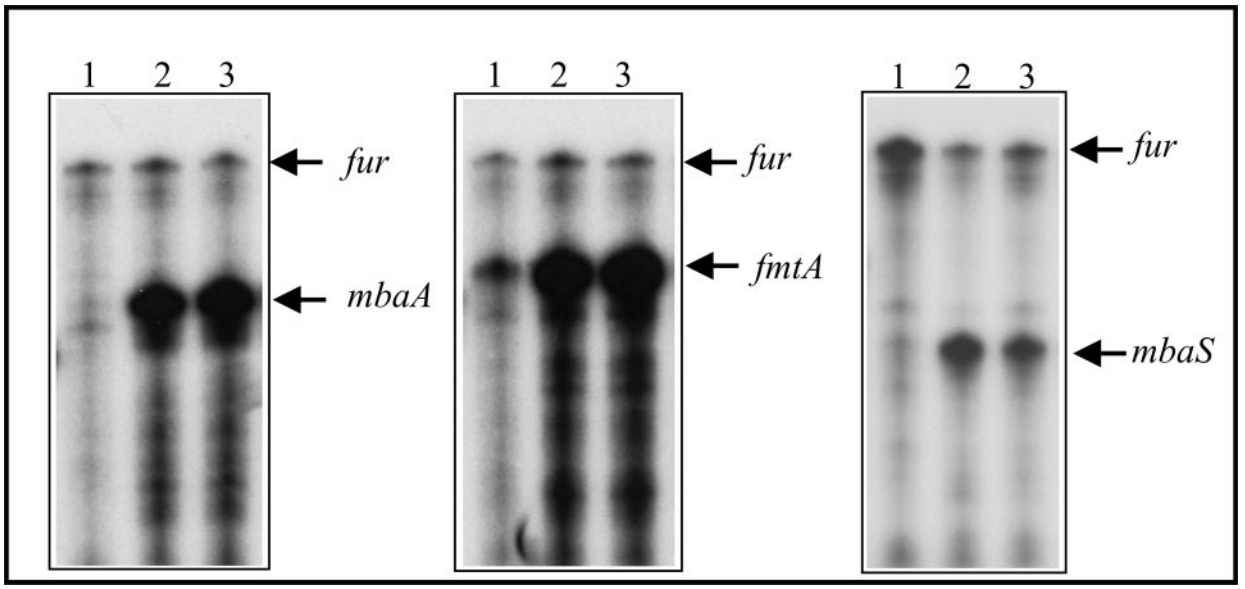


FIG. 8. Transcription of the *mbaA*, *fmtA*, and *mbaS* genes is not controlled by malleobactin. RNase protection assays were performed with total RNA extracted from the *mbaF* mutant strain grown in Chelex-treated M9 plus FAC (lanes 1), Chelex-treated M9 (lanes 2), and Chelex-treated M9 plus malleobactin (lanes 3). The arrows indicate the positions of the protected RNAs. The *fur* gene was used as a control for RNA loading.

In this work, we concentrated on the detailed analysis of a gene cluster present in chromosome II of *B. pseudomallei* that showed similarities to clusters involved in siderophore biosynthesis and uptake in *B. cenocepacia* and *P. aeruginosa*. The genes identified in this cluster showed strong induction under low iron concentrations. One of these genes encodes the ferric malleobactin receptor FmtA, which is also able to transport ornibactin, a siderophore produced by *B. cenocepacia*. Our MS analysis of the purified siderophore malleobactin established the presence of at least three compounds with different molecular masses of 790, 762, and 636 Da. The 790- and 762-Da compounds showed characteristics similar to those of the ornibactins from *B. cenocepacia*, as assessed by MS analysis and bioassays performed with *B. cenocepacia* and *B. pseudomallei*. However, the chemical nature of the compound with an apparent molecular mass of 636 Da remains to be determined. Each of these purified compounds was able to restore growth of the *mbaA* mutant, but not of the *fmtA* mutant, under iron-limiting conditions. Therefore, the compound originally described as malleobactin is a mixture of at least three separable compounds. We also demonstrated that *fmtA* is transcribed together with *mbaJ*, *mbaI*, *mbaA*, and *mbaF* as part of an operon regulated by MbaS. This protein shares sequence similarities with ECF sigma factors operating in iron regulation of various *Pseudomonas* species (59, 60). It was also clear that malleobactin does not induce the expression of *mbaA*, *fmtA*, and *mbaS*. Consistent with this, FmtA does not possess a N-terminal extension like those found in receptors that undergo signal transduction such as FecA in *E. coli* and FpvA in *P. aeruginosa*. These results identify an important difference in regulation between the malleobactin system and those systems mediated by pyoverdin and pseudobactin in *Pseudomonas* species (62). It has been reported that an *orbA* mutant of *B. cenocepacia* cannot produce the siderophore ornibactin (63), suggesting that that there might be involvement of this receptor in signal transduction. However, our sequence alignment analysis indicates that the *B. cenocepacia* OrbA receptor, like FmtA, does not belong to the class of receptors involved in signal transduction. Whether the phenotype observed in the *B. cenocepacia orbA* mutant is due to another type of regulation or condition is still not clear.

We showed that the transcription of *mbaS*, which mediates the transcriptional activation of the malleobactin biosynthesis and transport genes, is repressed under iron-rich conditions. FURTA experiments carried out with *E. coli* suggest that this iron repression observed in *B. pseudomallei* must be Fur dependent. In *P. aeruginosa*, PvdS recognizes the sequence TAA AT-(N_{16})-CGT located in the promoters of genes that are under the transcriptional control of this ECF sigma factor (68). In the *mbaJ* upstream region, we found a sequence that shares similarity with the sequence recognized by PvdS; however, since we did not show that MbaS is able to bind to this promoter, the consensus sequence recognized by MbaS remains to be determined. At this point, we cannot rule out the possibility that this protein is controlling and/or interacting with other factors that can influence the transcription of the *mbaJIAfmt AmbaF* operon. In this vein, the possible involvement of a quorum-sensing system in siderophore production in *B. pseudomallei* was recently described (11). A similar regulation was also described for ornibactin production in *B. cenocepacia* with the quorum-sensing CepR-CepI system (28); however, the mechanisms by which these systems can affect siderophore production in *B. pseudomallei* are currently under investigation.

Furthermore, our results clearly suggest that it is unlikely that the FmtA receptor would transduce any signal with or without malleobactin as the effector. However, we cannot discard the existence of a putative anti-sigma factor in *B. pseudomallei* that could control the activity of MbaS under other environmental conditions. In another bacterium, *Rhizo-*

bium leguminosarum, an ECF sigma factor, RpoI, controls the synthesis of the siderophore vicibactin (9). However, as in the case of MbaS in *B. pseudomallei*, there is no identifiable anti-sigma factor for RpoI; this protein can function in mutants unable to synthesize vicibactin, indicating that this siderophore is not involved in any antagonistic interaction (71). These results are in agreement with those described in the manuscript for the MbaS regulation of malleobactin biosynthesis and transport in *B. pseudomallei*.

It remains to be determined whether MbaS, as it occurs for PvdS in *P. aeruginosa*, plays a role in the pathogenesis of *B. pseudomallei* infections.

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Ankenbauer, R. G., and H. N. Quan.** 1994. FptA, the Fe(III)-pyochelin receptor of *Pseudomonas aeruginosa*: a phenolate siderophore receptor homologous to hydroxamate siderophore receptors. J. Bacteriol. **176:**307–319.
3. **Atkin, C. L., J. B. Neilands, and H. J. Phaff.** 1970. Rhodotorulic acid from species of *Leucosporidium*, *Rhodosporidium*, *Rhodotorula*, *Sporidiobolus*, and *Sporobolomyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. J. Bacteriol. **103:**722–733.
4. **Beare, P. A., R. J. For, L. W. Martin, and I. L. Lamont.** 2003. Siderophore-mediated cell signalling in *Pseudomonas aeruginosa*: divergent pathways regulate virulence factor production and siderophore receptor synthesis. Mol. Microbiol. **47:**195–207.
5. **Bender, C. L., F. Alarcon-Chaidez, and D. C. Gross.** 1999. *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol. Mol. Biol Rev. **63:**266–292.
6. **Bensing, B. A., B. J. Meyer, and G. M. Dunny.** 1996. Sensitive detection of bacterial transcription initiation sites and differentiation from RNA processing sites in the pheromone-induced plasmid transfer system of *Enterococcus faecalis*. Proc. Natl. Acad. Sci. USA **93:**7794–7799.
7. **Bolivar, F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique EcoRI sites for selection of EcoRI generated recombinant DNA molecules. Gene **4:**121–136.
8. **Braun, V., S. Mahren, and M. Ogierman.** 2003. Regulation of the FecI-type ECF sigma factor by transmembrane signalling. Curr. Opin. Microbiol. **6:**173–180.
9. **Carter, R. A., P. S. Worsley, G. Sawers, G. L. Challis, M. J. Dilworth, K. C. Carson, J. A. Lawrence, M. Wexler, A. W. Johnston, and K. H. Yeoman.** 2002. The *vbs* genes that direct synthesis of the siderophore vicibactin in *Rhizobium leguminosarum*: their expression in other genera requires ECF sigma factor RpoI. Mol. Microbiol. **44:**1153–1166.
10. **Challis, G. L., J. Ravel, and C. A. Townsend.** 2000. Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. Chem. Biol. **7:**211–224.
11. **Chan, Y. Y., and K. L. Chua.** 2005. The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence. J. Bacteriol. **187:**4707–4719.
12. **Cheng, A. C., and B. J. Currie.** 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. **18:**383–416.
13. **Cornelis, P., and S. Matthijs.** 2002. Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. Environ. Microbiol. **4:**787–798.
14. **Cox, C. D., and R. Graham.** 1979. Isolation of an iron-binding compound from *Pseudomonas aeruginosa*. J. Bacteriol. **137:**357–364.
15. **Cox, C. D., K. L. Rinehart, Jr., M. L. Moore, and J. C. Cook, Jr.** 1981. Pyochelin: novel structure of an iron-chelating growth promoter for *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **78:**4256–4260.
16. **Cunliffe, H. E., T. R. Merriman, and I. L. Lamont.** 1995. Cloning and characterization of *pvdS*, a gene required for pyoverdine synthesis in *Pseudomonas aeruginosa*: PvdS is probably an alternative sigma factor. J. Bacteriol. **177:**2744–2750.
17. **DeShazer, D., and D. E. Woods.** 1996. Broad-host-range cloning and cassette vectors based on the R388 trimethoprim resistance gene. BioTechniques **20:**762–764.
18. **Escolar, L., J. Pérez-Martin, and V. de Lorenzo.** 1999. Opening the iron box: transcriptional metalloregulation by the Fur protein. J. Bacteriol. **181:**6223–6229.
19. **Essex-Lopresti, A. E., J. A. Boddey, R. Thomas, M. P. Smith, M. G. Hartley, T. Atkins, N. F. Brown, C. H. Tsang, I. R. Peak, J. Hill, I. R. Beacham, and R. W. Titball.** 2005. A type IV pilin, PilA, contributes to adherence of *Burkholderia pseudomallei* and virulence in vivo. Infect Immun. **73:**1260–1264.
20. **Gentleman, R. C., V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. Yang, and J. Zhang.** 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. **5:**R80.
21. **Helmann, J. D.** 2002. The extracytoplasmic function (ECF) sigma factors. Adv. Microb. Physiol. **46:**47–110.
22. **Holden, M. T., R. W. Titball, S. J. Peacock, A. M. Cerdeno-Tarraga, T. Atkins, L. C. Crossman, T. Pitt, C. Churcher, K. Mungall, S. D. Bentley, M. Sebaihia, N. R. Thomson, N. Bason, I. R. Beacham, K. Brooks, K. A. Brown, N. F. Brown, G. L. Challis, I. Cherevach, T. Chillingworth, A. Cronin, B. Crossett, P. Davis, D. DeShazer, T. Feltwell, A. Fraser, Z. Hance, H. Hauser, S. Holroyd, K. Jagels, K. E. Keith, M. Maddison, S. Moule, C. Price, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, M. Simmonds, S. Songsivilai, K. Stevens, S. Tumapa, M. Vesaratchavest, S. Whitehead, C. Yeats, B. G. Barrell, P. C. Oyston, and J. Parkhill.** 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc. Natl. Acad. Sci. USA **101:**14240–14245.
23. **Irizarry, R. A., B. Hobbs, F. Collin, Y. D. Beazer-Barclay, K. J. Antonellis, U. Scherf, and T. P. Speed.** 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics **4:**249–264.
24. **Jones, A. L., T. J. Beveridge, and D. E. Woods.**1996. Intracellular survival of *Burkholderia pseudomallei*. Infect. Immun. **64:**782–790.
25. **Kespichayawattana, W., S. Rattanachetkul, T. Wanun, P. Utaisincharoen, and S. Sirisinha.** 2000. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect. Immun. **68:**5377–5384.
26. **Lamont, I. L., P. A. Beare, U. Ochsner, A. I. Vasil, and M. L. Vasil.** 2002. Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **99:**7072–7077.
27. **Lehoux, D. E., F. Sanschagrin, and R. C. Levesque.** 2000. Genomics of the 35-kb *pvd* locus and analysis of novel *pvdIJK* genes implicated in pyoverdine biosynthesis in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **190:**141–146.
28. **Lewenza, S., and P. A. Sokol.** 2001. Regulation of ornibactin biosynthesis and *N*-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. J. Bacteriol. **183:**2212–2218.
29. **Loprasert, S., R. Sallabhan, W. Whangsuk, and S. Mongkolsuk.** 2000. Characterization and mutagenesis of *fur* gene from *Burkholderia pseudomallei*. Gene **254:**129–137.
30. **Masse, E., and S. Gottesman.** 2002. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **99:**4620–4625.
31. **McMorran, B. J., M. E. Merriman, I. T. Rombel, and I. L. Lamont.** 1996. Characterisation of the *pvdE* gene which is required for pyoverdine synthesis in *Pseudomonas aeruginosa*. Gene **176:**55–59.
32. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Milton, D. L., R. O'Toole, P. Horstedt, and H. Wolf-Watz.** 1996. Flagellin A is essential for the virulence of *Vibrio anguillarum*. J. Bacteriol. **178:**1310–1319.
34. **Morales, V. M., A. Backman, and M. Bagdasarian.** 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97:**39–47.
35. **Mullis, K. B., J. R. Pollack, and J. B. Neilands.** 1971. Structure of schizokinen, an iron-transport compound from *Bacillus megaterium*. Biochemistry **10:**4894–4898.
36. **Munch, R., K. Hiller, H. Barg, D. Heldt, S. Linz, E. Wingender, and D. Jahn.** 2003. PRODORIC: prokaryotic database of gene regulation. Nucleic Acids Res. **31:**266–269.
37. **Munch, R., K. Hiller, A. Grote, M. Scheer, J. Klein, M. Schobert, and D. Jahn.** 2005. Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. Bioinformatics **21:**4187–4189.
38. **Ochsner, U. A., Z. Johnson, I. L. Lamont, H. E. Cunliffe, and M. L. Vasil.** 1996. Exotoxin A production in *Pseudomonas aeruginosa* requires the iron-regulated *pvdS* gene encoding an alternative sigma factor. Mol. Microbiol. **21:**1019–1028.
39. **Ochsner, U. A., P. J. Wilderman, A. I. Vasil, and M. L. Vasil.** 2002. GeneChip expression analysis of the iron starvation response in *Pseudomonas aeruginosa*: identification of novel pyoverdine biosynthesis genes. Mol. Microbiol. **45:**1277–1287.

40. **Penfold, R. J., and J. M. Pemberton.** 1992. An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene **118:**145–146.
41. **Peterson, J. D., L. A. Umayam, T. Dickinson, E. K. Hickey, and O. White.** 2001. The Comprehensive Microbial Resource. Nucleic Acids Res. **29:**123–125.
42. **Ravel, J., and P. Cornelis.** 2003. Genomics of pyoverdine-mediated iron uptake in pseudomonads. Trends Microbiol. **11:**195–200.
43. **Reimmann, C., L. Serino, M. Beyeler, and D. Haas.** 1998. Dihydroaeruginoic acid synthetase and pyochelin synthetase, products of the *pchEF* genes, are induced by extracellular pyochelin in *Pseudomonas aeruginosa*. Microbiology **144:**3135–3148.
44. **Sambrook, B., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
45. **Schalk, I. J., W. W. Yue, and S. K. Buchanan.** 2004. Recognition of iron-free siderophores by TonB-dependent iron transporters. Mol. Microbiol. **54:**14–22.
46. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
47. **Senanayake, S. D., and D. A. Brian.** 1995. Precise large deletions by the PCR-based overlap extension method. Mol. Biotechnol. **4:**13–15.
48. **Serino, L., C. Reimmann, P. Visca, M. Beyeler, V. D. Chiesa, and D. Haas.** 1997. Biosynthesis of pyochelin and dihydroaeruginoic acid requires the iron-regulated *pchDCBA* operon in *Pseudomonas aeruginosa*. J. Bacteriol. **179:**248–257.
49. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**787–796.
50. **Sokol, P. A.** 1986. Production and utilization of pyochelin by clinical isolates of *Pseudomonas cepacia*. J. Clin. Microbiol. **23:**560–562.
51. **Sokol, P. A., P. Darling, S. Lewenza, C. R. Corbett, and C. D. Kooi.** 2000. Identification of a siderophore receptor required for ferric ornibactin uptake in *Burkholderia cepacia*. Infect. Immun. **68:**6554–6560.
52. **Sokol, P. A., P. Darling, D. E. Woods, E. Mahenthiralingam, and C. Kooi.** 1999. Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of *pvdA*, the gene encoding L-ornithine *N*(5)-oxygenase. Infect. Immun. **67:**4443–4455.
53. **Spaink, H. P., J. H. Okker, C. A. Wijffelman, E. Pees, and B. J. J. Lugtenberg.** 1987. Promoters in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1J1. Plant Mol. Biol. **9:**27–39.
54. **Stevens, M. P., and E. E. Galyov.** 2004. Exploitation of host cells by *Burkholderia pseudomallei*. Int. J. Med. Microbiol. **293:**549–555.
55. **Stevens, M. P., A. Haque, T. Atkins, J. Hill, M. W. Wood, A. Easton, M. Nelson, C. Underwood-Fowler, R. W. Titball, G. J. Bancroft, and E. E. Galyov.** 2004. Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei bsa* type III secretion mutant in murine models of melioidosis. Microbiology **150:**2669–2676.
56. **Stevens, M. P., J. M. Stevens, R. L. Jeng, L. A. Taylor, M. W. Wood, P. Hawes, P. Monaghan, M. D. Welch, and E. E. Galyov.** 2005. Identification of a bacterial factor required for actin-based motility of *Burkholderia pseudomallei*. Mol. Microbiol. **56:**40–53.
57. **Stevens, M. P., M. W. Wood, L. A. Taylor, P. Monaghan, P. Hawes, P. W. Jones, T. S. Wallis, and E. E. Galyov.** 2002. An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. Mol. Microbiol. **46:**649–659.
58. **Stojiljkovic, I., A. J. Bäumler, and K. Hantke.** 1994. Fur regulon in gram-negative bacteria. Identification and characterization of new iron-regulated *Escherichia coli* genes by a fur titration assay. J. Mol. Biol. **236:**531–545.
59. **Venturi, V., C. Ottevanger, J. Leong, and P. J. Weisbeek.** 1993. Identification and characterization of a siderophore regulatory gene (*pfrA*) of *Pseudomonas putida* WCS358: homology to the alginate regulatory gene *algQ* of *Pseudomonas aeruginosa*. Mol. Microbiol. **10:**63–73.
60. **Venturi, V., P. Weisbeek, and M. Koster.** 1995. Gene regulation of siderophore-mediated iron acquisition in *Pseudomonas*: not only the Fur repressor. Mol. Microbiol. **17:**603–610.
61. **Visca, P., A. Ciervo, and N. Orsi.** 1994. Cloning and nucleotide sequence of the *pvdA* gene encoding the pyoverdin biosynthetic enzyme L-ornithine N^5-oxygenase in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**1128–1140.
62. **Visca, P., L. Leoni, M. J. Wilson, and I. L. Lamont.** 2002. Iron transport and regulation, cell signalling and genomics: lessons from *Escherichia coli* and *Pseudomonas*. Mol. Microbiol. **45:**1177–1190.
63. **Visser, M. B., S. Majumdar, E. Hani, and P. A. Sokol.** 2004. Importance of the ornibactin and pyochelin siderophore transport systems in *Burkholderia cenocepacia* lung infections. Infect. Immun. **72:**2850–2857.
64. **Warawa, J., and D. E. Woods.** 2005. Type III secretion system cluster 3 is required for maximal virulence of *Burkholderia pseudomallei* in a hamster infection model. FEMS Microbiol. Lett. **242:**101–108.
65. **White, N. J.** 2003. Melioidosis. Lancet **361:**1715–1722.
66. **Wilderman, P. J., N. A. Sowa, D. J. FitzGerald, P. C. FitzGerald, S. Gottesman, U. A. Ochsner, and M. L. Vasil.** 2004. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc. Natl. Acad. Sci. USA **101:**9792–9797.
67. **Wilderman, P. J., A. I. Vasil, Z. Johnson, M. J. Wilson, H. E. Cunliffe, I. L. Lamont, and M. L. Vasil.** 2001. Characterization of an endoprotease (PrpL) encoded by a PvdS-regulated gene in *Pseudomonas aeruginosa*. Infect. Immun. **69:**5385–5394.
68. **Wilson, M. J., B. J. McMorran, and I. L. Lamont.** 2001. Analysis of promoters recognized by PvdS, an extracytoplasmic-function sigma factor protein from *Pseudomonas aeruginosa*. J. Bacteriol. **183:**2151–2155.
69. **Yang, H., C. D. Kooi, and P. A. Sokol.** 1993. Ability of *Pseudomonas pseudomallei* malleobactin to acquire transferrin-bound, lactoferrin-bound, and cell-derived iron. Infect. Immun. **61:**656–662.
70. **Yang, H. M., W. Chaowagul, and P. A. Sokol.** 1991. Siderophore production by *Pseudomonas pseudomallei*. Infect. Immun. **59:**776–780.
71. **Yeoman, K. H., S. Mitelheiser, G. Sawers, and A. W. Johnston.** 2003. The ECF sigma factor RpoI of *R. leguminosarum* initiates transcription of the *vbsGSO* and *vbsADL* siderophore biosynthetic genes in vitro. FEMS Microbiol. Lett. **223:**239–244.