The siderophore-mediated iron acquisition systems of *Acinetobacter baumannii* ATCC 19606 and *Vibrio anguillarum* 775 are structurally and functionally related

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The *Acinetobacter baumannii* type strain, ATCC 19606, secretes acinetobactin, a catechol siderophore highly related to the iron chelator anguibactin produced by the fish pathogen *Vibrio anguillarum* (*Listonella anguillarum*). This paper reports the initial characterization of the genes and gene products involved in the acinetobactin-mediated iron-acquisition process. Insertional mutagenesis resulted in the isolation of several derivatives whose ability to grow in medium containing the iron chelator 2,2′-dipyridyl was affected. One of the insertions disrupted a gene encoding a predicted outer-membrane protein, named BauA, highly similar to FatA, the receptor for ferric anguibactin. Immunological relatedness of BauA with FatA was confirmed by Western blot analysis. Another transposon insertion was mapped to a gene encoding a protein highly similar to FatD, the permease component of the anguibactin transport system. Further DNA sequencing and nucleotide sequence analysis revealed that these *A. baumannii* 19606 genes are part of a polycistronic locus that contains the bauDCEBA ORFs. While the translation products of bauD, -C, -B and -A are highly related to the *V. anguillarum* FatDCBA iron-transport proteins, the product of bauE is related to the ATPase component of Gram-positive ATP-binding cassette (ABC) transport systems. This entire locus is flanked by genes encoding predicted proteins related to AngU and AngN, *V. anguillarum* proteins required for the biosynthesis of anguibactin. These protein similarities, as well as the structural similarity of anguibactin and acinetobactin, suggested that these two siderophores could be utilized by both bacterial strains, a possibility that was confirmed by siderophore utilization bioassays. Taken together, these results demonstrate that these pathogens, which cause serious infections in unrelated hosts, express very similar siderophore-mediated iron-acquisition systems.

### INTRODUCTION

*Acinetobacter baumannii* is a non-motile, Gram-negative rod that causes severe infections in compromised patients. During the last two decades, this opportunistic human pathogen has been connected with a significant and growing number of hospital infections, mainly outbreaks of respiratory infections (Bergogne-Berenzin *et al.*, 1996; Bergogne-Berenzin & Towner, 1996). This bacterium was also found to be associated with a series of fatal cases of community-acquired pneumonia (Anstey *et al.*, 1991). The ability of *A. baumannii* to colonize and invade the human host indicates that it can obtain vital nutrients, such as the essential micronutrient iron, during the infection process. Although iron is abundant, its utilization by this bacterium is normally restricted, as has been described for numerous other human pathogens (Neilands, 1981). It was reported (Yamamoto *et al.*, 1994) that the type strain of *A. baumannii*, ATCC 19606 (Bouvet & Grimont, 1986), produces and secretes acinetobactin, a bacterial siderophore.

**Abbreviations:** ABC, ATP-binding cassette; CAS, Chrome azurol S; DP, 2,2′-dipyridyl; EDDHA, ethylenediamine-di-((o-hydroxyphenyl)acetic acid; HRP, horseradish peroxidase; NTA, nitrilotriacetic acid; TF, human transferrin.

The GenBank/EMBL/DDBJ accession number for the sequence of the *A. baumannii* ATCC 19606 genomic region containing acinetobactin biosynthetic and transport genes is AY571146.
containing catechol and hydroxamate functional groups. This iron chelator was purified from low-iron culture supernatants by adsorption and thin-layer chromatography, and organic solvent extraction at low pH.

Acinetobactin is composed of dihydroxybenzoic acid, threonine and N-hydroxyhistamine. This molecular structure is very closely related to that of anguibactin (Jalal et al., 1989), the pM1-encoded iron chelator secreted by the fish pathogen Vibrio anguillarum ATCC 68554 (Listonella anguillarum) (Actis et al., 1986). The only difference between the two iron chelators is that anguibactin contains a thiazoline ring, derived from the cyclization of a cysteinyl side chain, whereas an oxazoline ring, derived from the cyclization of a threonyl side chain, is found in the acinetobactin molecule (Fig. 1). It is important to note that the chemical properties of acinetobactin distinguish this iron chelator from the catechol siderophore secreted by A. baumannii 8399, a different clinical strain isolated during a nosocomial outbreak of lower respiratory tract infections (Echenique et al., 1992). In addition, HPLC analysis showed that only 33% of a survey of clinical isolates secreted acinetobactin, although all of them produced dihydroxybenzoic acid (Yamamoto et al., 1994). These facts suggest that a single Acinetobacter strain may produce more than one catechol siderophore, in addition to strain-to-strain variation in the type of catechol siderophore produced by different clinical isolates. The latter possibility is supported by our recent report (Dorsey et al., 2003a) showing variations among different A. baumannii clinical isolates in the expression of iron-regulated outer-membrane proteins, which could serve as siderophore receptors, and in the production of histamine, an anguibactin biosynthetic precursor that could play a similar role in the production of acinetobactin in A. baumannii 19606.

In this work we report the identification of an A. baumannii 19606 gene cluster that encodes siderophore transport and biosynthetic proteins, most of which are highly related to those described in the V. anguillarum 775 anguibactin-mediated system (Crosa & Walsh, 2002; Di Lorenzo et al., 2003). Siderophore utilization bioassays showed that V. anguillarum 775 and A. baumannii 19606 can each use either anguibactin or acinetobactin to grow under iron-limiting conditions, demonstrating that these two siderophore-mediated iron-acquisition systems are structurally and functionally related.

**METHODS**

**Bacterial strains and culture conditions.** The bacterial strains and plasmids used in this work are listed in Table 1. The A. baumannii and Escherichia coli strains were grown at 37°C in Luria broth (L) (Miller, 1972) or Luria broth solidified with 1.5% (w/v) agar. Simons citrate agar (Difco) was used as a selection medium to isolate A. baumannii 19606 exconjugants in tri-parental conjugation experiments. M9 minimal medium (Miller, 1972) was used to test for the production of catechol and siderophore compounds by these strains. The V. anguillarum strains were cultured at 30°C either in trypticase soy broth or broth solidified with 1.5% agar, both supplemented with 1% NaCl, or in M9 minimal medium (Crosa, 1980). The iron chelators human transferrin (TF), 2,2’-dipyridyl (DP), ethylenediamine-di-(β-hydroxyphenyl) acetic acid (EDDHA) and nitrilotriacetic acid (NTA) were added to the media to achieve iron-limiting conditions. TF was prepared as a 1 mM stock solution in 100 mM Tris/HCl (pH 7.5), 0.1% NaCl, 50 mM NaHCO₃. Iron-proficient conditions were obtained by adding FeCl₃ dissolved in 0.5 M HCl to a final concentration of 50 μM. The minimal inhibitory concentration of the iron chelators was determined in liquid M9 minimal medium containing increasing concentrations of each iron-chelating compound. Cell growth was determined spectrophotometrically at 600 nm after overnight incubation at 30°C and 37°C for the V. anguillarum and A. baumannii strains, respectively.

**Production and utilization of siderophore compounds, and transferrin binding assays.** The production of extracellular compounds with siderophore activity was investigated using the Chrome azurol S (CAS) reagent (Schwyn &Neilands, 1987). The presence of phenolic extracellular compounds was detected in culture supernatants with the Arnow colorimetric assay (Arnow, 1937). The ability of V. anguillarum strains to use inorganic iron and siderophore compounds was measured from the size of the growth halo around a 7 mm filter disk spotted with different compounds and deposited on M9 minimal medium agar plates containing 25 μM EDDHA (Actis et al., 1986; Walter et al., 1983). Bacterial growth was visually determined after 24 h incubation at 30°C. The ability of the A. baumannii 19606-1 s1 insertion derivative to use inorganic iron, anguibactin or acinetobactin was determined from the size of the growth halo around a 7 mm filter disk spotted with these compounds and deposited on L agar plates containing 120 μM DP and seeded with cells of this insertion mutant. Bacterial growth was visually determined after 24 h incubation at 37°C. Anguibactin and acinetobactin were purified from iron-deficient culture supernatants by adsorption and gel filtration chromatography as described.

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**Fig. 1.** Molecular structure of the siderophores acinetobactin and anguibactin produced by A. baumannii 19606 and V. anguillarum 775, respectively. The structures of acinetobactin and anguibactin were redrawn from the reports published previously by Yamamoto et al. (1994) and Jalal et al. (1989), respectively.
random insertion mutagenesis and rescue cloning. The EZ TN <R6K/ori/KAN-2> Tnp transposome kit was used as indicated by the manufacturer (Epigen). The transposon–transposase complexes were electroporated as described previously (Dorsey et al., 2002) and the insertional derivatives were recovered after a 1 h incubation in SOC (Sambrook & Russell, 2001) and plating on L agar containing 40 μg kanamycin ml−1. Genomic regions containing EZ TN <R6K/ori/KAN-2> insertions were recovered by self-ligation of EcoRI-digested DNA and transformation into E. coli TransforMax EC100D (Dorsey et al., 2002). Both strands of cloned DNA were sequenced with the DYEnamic ET sequence reaction mixture (Amersham), an Applied Biosystems Prism 3100 automated sequencer, and M13 forward and reverse primers (Yanisch-Perron et al., 1985) or custom-designed primers. Sequences were examined and assembled with Sequencher 4.1.2 (Gene Codes Corp.). Nucleotide and amino acid sequences were analysed with DNASTAR (DNASTAR), which is part of the GCG Wisconsin package marketed by Accelrys (http://www.accelrys.com).
II-TOPO (Invitrogen) to generate plasmid pMU420. The basD fragment was excised from pMU420 by EcoRI digestion and subcloned into the EcoRI site of pBCKS 5 to form pMU431. An internal 378 bp fragment, located 645 bp upstream of the 3’ end of the cloned PCR fragment, was deleted by NsiI digestion. The ends were filled by T4 DNA polymerase (New England Biolabs), and a PCR-amplified fragment including the pUC4K aph gene was inserted to form pMU434. To facilitate the allelic exchange of the disrupted basD gene into the A. baumannii chromosome, the entire basD::aph construct was excised from pMU434 with PvuII and inserted at the Smal site of pEX100T to form pMU435.

A tri-parental mating was conducted using A. baumannii 19606 as the recipient, E. coli DH5α cells containing pMU435 as the donor, and E. coli cells containing pRK2073 as a helper. Exconjugants were selected on Simmons citrate agar plates containing 40 μg kanamycin ml −1. Cells growing on these plates were collected and plated on L agar containing 40 μg kanamycin ml −1 and 0·625 % sucrose, to ensure loss of pMU435. Exconjugants able to grow in the presence of sucrose were then tested for growth on L agar containing 120 μg DP ml −1 or 750 μg carbenicillin ml −1. A strain unable to grow on either DP or carbenicillin was named 19606-s1.

Disruption of basD in the A. baumannii 19606-s1 derivative was confirmed by amplifying the internal basD fragment and observing a molecular mass shift consistent with the insertion of the DNA cassette containing aph. The nature of the disruption was further confirmed by Southern blot analysis of the amplicons obtained using total DNA isolated from the parental strain and basD::aph derivative as templates and radiolabelled aph and basD as probes. RT-PCR analysis showed that this genetic manipulation did not impair the transcription of the basC downstream coding region.

Isolation and analysis of whole-cell lysate and membrane proteins. After overnight culturing in L broth or M9 minimal medium under iron-limiting and iron-rich conditions, bacterial cells were used to prepare whole-cell lysates as described before (Dorsey et al., 2003b). Total and outer membranes were isolated from cells by high-speed centrifugation and selective solubilization as previously reported (Actis et al., 1985). Proteins were size-fractionated by electrophoresis on 12·5 % polyacrylamide gels (Actis et al., 1985) and either stained with Fast Stain or blotted to nitrocellulose (Towbin et al., 1979). Protein concentrations were determined as described by Bradford (1976). The production of FatA and BauA was examined by Western blotting with specific polyclonal antisera as probes. RT-PCR analysis showed that this genetic manipulation did not impair the transcription of the basC gene into the A. baumannii chromosome, the entire basD::aph construct was excised from pMU434 with PvuII and inserted at the Smal site of pEX100T to form pMU435.

Biotin labelling of cell surface proteins. Outer-membrane proteins exposed to the extracellular environment were biotinylated with the EZ-Link Sulfo-NHS-Biotinylation kit from Pierce Biotechnology as described previously (Dorsey et al., 2003b). Bacterial cells grown in M9 minimal medium under iron-deficient conditions were washed with PBS and suspended to a concentration of approximately 2·5 × 10 8 cells ml −1. Then 0·5 mg Sulfo-NHS-LC-Biotin was added to 1 ml cells and the mixture was incubated at room temperature for 30 min. The cells were washed with PBS, and total and outer membranes were isolated as described previously (Actis et al., 1985). Biotinylated proteins were detected by chemiluminescence with HRP-labelled avidin (Pierce Biotechnology) and H 2 O 2.

Transcriptional analysis of gene expression. The transcription of the bau genes was tested by RT-PCR analysis using total RNA isolated from bacteria grown under iron-limiting conditions as described before (Wu & Janssen, 1997). The RNA samples were treated with RNase-free DNase I (Roche) and used with an RT-PCR commercial kit (Qiagen) under the conditions suggested by the manufacturer. The basD intergenic regions were amplified as follows: bauD–bauC, primers 209 (5’-AAGCTTATCTGCCTGCG-3’) and 2080 (5’-ACTGGCCTTAAATCCATGCG-3’); bauC–bauE, primers 2005 (5’-CTGGGCGCCTACGGATGC-3’) and 2096 (5’-CTGAAATGTCGCGACATGC-3’); bauE–bauB, primers 2014 (5’-TTAACCAGGC-AAAGATGCCTG-3’) and 2098 (5’-CTCAAGTGTCATATGG-3’); bauD–bauA, primers 2099 (5’-ATTCATGTCAGCTATGGC-3’) and 1696 (5’-GAACATCTATCATTGCTAC-3’). The primers basC.int.F2 (5’-CATTACGGAGTGGCTGCA-3’) and basC.int.R2 (5’-CGATTCACTTGCACGT-3’) were used to test the transcription of basC. The amplicons were analysed by agarose gel electrophoresis and confirmed when necessary by automated DNA sequencing. PCR of total RNA without reverse transcription was used to test DNA contamination of RNA samples.

RESULTS

Iron uptake proficiency and production of iron-regulated siderophore compounds

Although it has already been shown (Yamamoto et al., 1994) that A. baumannii 19606 secretes acinetobactin, a phenolic compound which showed siderophore activity in vitro, the ability of this strain to grow under conditions of iron deficiency has not been thoroughly examined. The determination of the minimal inhibitory concentrations of several iron chelators revealed that this isolate can grow in M9 minimal medium containing up to 1 mM of either EDDHA or NTA, or up to 120 μM DP (data not shown). This strain was also able to grow in liquid M9 minimal medium containing up to 50 μM TF, although no binding of HRP-labelled human transferrin could be detected. Arnow tests demonstrated that the addition of each of these iron chelators induced the secretion of catechol siderophores.

Table 2. Siderophore utilization bioassays

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>V. anguillarum indicator strain (growth halo in mm)*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>775::Tn1-5(pHHC-91)†</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>5</td>
</tr>
<tr>
<td>Anguibactin</td>
<td>13</td>
</tr>
<tr>
<td>Culture supernatant§</td>
<td>5</td>
</tr>
<tr>
<td>Acinetobactin</td>
<td>6</td>
</tr>
</tbody>
</table>

*Bacterial growth around 7 mm filter disks on M9 minimal medium agar plates containing 15 μM EDDHA was determined after 24 h incubation at 30 °C.
†Mutant impaired in the biosynthesis of anguibactin only.
‡Mutant impaired in the biosynthesis and transport of ferric anguibactin.
§Culture supernatant of A. baumannii 19606 incubated in M9 minimal medium under iron-limiting conditions.
compounds into the culture supernatant. Conversely, the addition of 50 μM FeCl₃ reduced the catechol levels below the detection limit of the Arnow colorimetric reaction.

Siderophore utilization assays showed that *V. anguillarum* 775::Tn1-5(pJHC-91), a Tn1 insertion mutant able to utilize ferric-anguibactin complexes but unable to synthesize anguibactin (Walter *et al.*, 1983), grew under iron-limiting conditions around a filter disk containing FeCl₃, purified anguibactin or *A. baumannii* culture supernatant (Table 2). No growth was detected in the case of the negative control strain *V. anguillarum* 775::Tn1-6(pJHC9-8), a deletion mutant that lacks most of the anguibactin-mediated iron-uptake system (Walter *et al.*, 1983). Similar results were obtained when the filter disks were loaded with purified acinetobactin (Table 2). Therefore, *V. anguillarum* can utilize acinetobactin to grow under iron-limiting conditions only when the *V. anguillarum* cells produce the FatDCBA anguibactin-transport proteins encoded by pJM1.

**Detection and characterization of iron-regulated membrane proteins**

SDS-PAGE analysis showed that the iron concentration of the culture medium affected the expression of several *A. baumannii* 19606 genes encoding membrane proteins. Fig. 2 shows that the total membrane fraction of this strain contains several iron-repressed proteins. Further analysis of membrane preparations treated with Sarkosyl showed that four proteins with molecular masses of 72, 74, 78 and 84 kDa, are located in the outer-membrane fraction. Immunoblot analysis of these proteins with anti-FatA serum showed that the 78 kDa iron-regulated protein present in the outer membrane of *A. baumannii* 19606 is immunologically related to the 86 kDa FatA protein (Fig. 3).

The surface exposure of the *A. baumannii* 19606 FatA-like protein was tested by incubating cells cultured under iron-limiting conditions with a water-soluble N-hydroxysuccinimide ester of biotin, which is a membrane-impermeable compound (Wilchek & Bayer, 1988) that we have used previously to examine the location of a potential siderophore receptor present in the *A. baumannii* 8399 strain (Dorsey *et al.*, 2003b). This analysis showed that most of the *A. baumannii* 19606 outer-membrane proteins, including the 78 kDa protein, were labelled with biotin (data not shown). The biotin-labelled 78 kDa protein co-migrated with the single protein detected by immunoblotting using the anti-FatA serum. These results demonstrated that the *A. baumannii* 19606 FatA homologue is not only located in the outer-membrane fraction but also surface exposed.

![Fig. 2. Gel electrophoresis of *A. baumannii* 19606 membrane proteins.](http://mic.sgmjournals.org)

![Fig. 3. Electrophoretic and immunological analysis of outer-membrane proteins.](http://mic.sgmjournals.org)
and capable of interacting with the extracellular milieu. These properties, together with the results obtained using siderophore utilization bioassays, strongly suggested that this protein is the receptor for acinetobactin, and it was therefore named BauA.

Fig. 3 shows that an anti-BauA antiserum recognizes BauA as well as FatA, with the former protein being detected only in the sample obtained from cells cultured under iron-deficient conditions. The stronger signal and the multiple-band pattern produced by the anti-FatA antiserum with the V. anguillarum 775 protein sample is due to the high affinity and titre of this antiserum, which was raised by injecting rabbits with crushed polyacrylamide containing FatA emulsified in Freund’s adjuvant. This antigen preparation resulted in a polyclonal antiserum capable of detecting the native protein as well as its degradation products (Actis et al., 1985). In our hands, the immune response obtained using this antigen preparation is stronger than that obtained with animals injected with soluble antigens only emulsified in Freund’s adjuvant. On the other hand, the lighter signal produced by BauA with anti-FatA most likely reflects the fact that, although related, the predicted amino acid sequence of this protein is only 46.7% identical to that of FatA. Taken together, these results underline the amino acid sequence, subcellular location and surface exposure similarities between these two iron-regulated proteins.

Isolation of iron-acquisition-deficient derivatives

We initiated the genetic and molecular characterization of the factors involved in acinetobactin-mediated iron acquisition by insertional mutagenesis with the EZ::TN <R6K/ori/KAN-2> Tnp transposome kit as described previously (Dorsey et al., 2002). Screening of A. baumannii 19606 Km-resistant derivatives on L agar containing DP resulted in the isolation of several derivatives that were not able to grow in the presence of this synthetic iron chelator. Two of them, named 19606-t6 and 19606-t7, were selected randomly and analysed in more detail. Fig. 4 shows that these two insertional derivatives were unable to grow in M9 minimal medium supplemented with increasing concentrations of DP, although both derivatives showed growth curves in L broth identical to that of the parental strain (data not shown). Furthermore, the Arnow and CAS assays showed that both insertion derivatives and the parental strain produced similar amounts of catechol and siderophore compounds when cultured in M9 minimal medium containing subinhibitory concentrations of DP. All these results suggested that the insertion of EZ::TN <R6K/ori/KAN-2> affected the expression of acinetobactin transport, but not biosynthetic functions. This possibility was confirmed by the ability of the M9 culture supernatants of the 19606-t6 and 19606-t7 derivatives to crossfeed the V. anguillarum 775::Tn155(pHJC-91) anguibactin-transport-proficient reporter strain under iron-limiting conditions. Similar results were obtained when these siderophore utilization assays were repeated with methanol extracts of lyophilized M9 culture supernatants of the 19606-t6 and 19606-t7 insertion mutants.

Identification of genes required for iron acquisition

Rescue cloning and DNA sequencing of the 19606-t6 and 19606-t7 EZ::TN <R6K/ori/KAN-2> insertion derivatives resulted in the identification of an A. baumannii 19606 genomic region containing genes encoding siderophore biosynthetic and transport functions (Fig. 5). The overall G+C content of this fragment (39.4 mol%), as well as that of each of the ORFs located within this region, is consistent with the 39–47% value range reported previously (Bergogne-Berenzin & Towner, 1996).

Siderophore-transport genes. A five-gene locus was identified upon mapping the insertion sites in the 19606-t6 and 19606-t7 derivatives (Fig. 5a). Fig. 5(b) shows that the first ORF encodes a predicted inner-membrane protein that is highly similar to the pJM1-encoded FatD permease protein required for iron acquisition by V. anguillarum via the anguibactin-mediated system (Crosa & Walsh, 2002). The fact that the disruption of this gene in the 19606-t7 derivative abolished acinetobactin utilization but not biosynthesis confirms its role in siderophore utilization. Therefore, this gene was named bauD. The next ORF encodes another predicted inner-membrane protein; this protein shows highest similarity to the V. anguillarum FatC iron-transport protein, and was therefore named bauC.

ORF 3 (Fig. 5b) encodes a potential 256 amino acid protein predicted to be associated with the inner membrane. This protein, which was named BauE, showed the highest similarity to the Pseudomonas putida hypothetical protein PP2592 (Nelson et al., 2002). Interestingly, the P. putida gene encoding this protein is also part of a locus harbouring genes that encode proteins related to siderophore-transport

![Graph showing growth of A. baumannii 19606 and isogenic insertion derivatives under iron-rich and iron-limiting conditions. Cells were cultured in L broth containing inorganic iron or increasing DP concentrations. Cell growth was determined spectrophotometrically at 600 nm after overnight incubation at 37 °C in an orbital shaker.](image-url)
proteins described in other bacteria (Fig. 5c). Further analysis with ScanProsite (http://us.expasy.org/tools/scanprosite/) showed that BauE has a putative P-loop domain found in the ATP-binding cassette of ABC transport proteins, some of which are components of bacterial siderophore-transport systems.

The product of ORF 4 was named BauB since it showed the highest similarity to the FatB anguibactin-transport protein. These two proteins both have the sequence Leu-X-Y-Cys located at the same position in the carboxy-terminal end of the predicted signal peptide. For FatB, this sequence is recognized by the signal peptidase II that cleaves between the Y and Cys residues. The Cys residue serves as the attachment site for a lipid group to FatB and its consequent anchoring to the inner membrane, leaving the active site of this iron-transport protein exposed to the periplasmic space (Actis et al., 1995). The presence of this particular sequence motif together with the overall amino acid sequence similarity between these two proteins is consistent with the possibility that the predicted location and function of BauB are similar to those described for the *V. anguillarum* FatB iron-transport protein (Actis et al., 1995).

The last ORF of this gene cluster (Fig. 5a), which was named BauA, encodes a protein that showed the highest similarity to the *P. putida* PP2590 hypothetical protein (Nelson et al., 2002). However, the second highest BLASTX hit was the FatA protein (Fig. 5b), which is the ferric-anguibactin outer-membrane receptor in *V. anguillarum* (Actis et al., 1988; Tolmasky et al., 1988). The impairment of anguibactin and acinetobactin utilization in the 19606-t6 derivative, which carries an EZ::TN<sup>ΔR6K<sub>ori</sub>/:KAN-2</sup> insertion near the 3<sup>′</sup>-end of the ORF encoding BauA (Fig. 5a), was associated with the absence of an iron-regulated protein...
immunologically related to BauA (Fig. 6b). It is important to note that the disruption of bauD in the derivative 19606-t7 (Fig. 5a) diminished, but did not abolish, the production of BauA (Fig. 6b). This result is consistent with the presence of an alternative promoter element immediately upstream of the 5′ end of bauA. Similar findings were reported for the pJM1 fat locus in V. anguillarum (Crosa, 1997; Waldbeser et al., 1993). The polycistronic nature of the bau locus was confirmed by RT-PCR analysis, which showed that the predicted amplicons were obtained when total RNA and the primers described in Methods were used (data not shown). PCR amplification of the RNA samples with the same primers in the absence of reverse transcriptase produced no amplicons, showing that the total RNA samples tested were not contaminated with chromosomal DNA.

**Siderophore biosynthesis genes.** Next to bauA and transcribed in the opposite direction (Fig. 5a), there is an ORF whose translation product, named BasC, is highly similar to AngU (Fig. 5b). AngU is a putative monooxygenase presumed to be involved, together with AngH, in the biosynthesis of anguibactin (Crosa & Walsh, 2002; Di Lorenzo et al., 2003) by catalysing the production of N-hydroxyhistamine from histidine (Crosa & Walsh, 2002). We have shown previously (Actis et al., 1999) that A. baumannii 19606 produces histamine, an anguibactin biosynthetic precursor (Tolmasky et al., 1995), when cultured in the presence of histidine. These results support the potential role of BasC in the biosynthesis of acinetobactin.

The last ORF of the DNA region sequenced using rescued clones from the 19606-t6 and 19606-t7 insertion derivatives (Fig. 5a) encodes a predicted large protein that showed the highest similarity in amino acid sequence and size to the V. anguillarum AngN protein (Crosa & Walsh, 2002; Di Lorenzo et al., 2003). This protein belongs to a family of non-ribosomal peptide synthetases that include VibF, an enzyme that is required for the biosynthesis of the siderophore vibriobactin in the enteropathogen Vibrio cholerae (Butterton et al., 2000). The role of this A. baumannii 19606 gene, which was named basD, was tested by the generation of an isogenic derivative produced by the deletion of 378 bp and the insertion of a DNA cassette encoding Km resistance within basD. We decided to use this approach since it is more direct than screening the insertion library to isolate such a derivative. In addition, we wanted to test the feasibility of using marker exchange to generate isogenic derivatives affected in particular functions, a genetic approach that, to the best of our knowledge, has not been used with A. baumannii strains. This genetic manipulation, which did not cause a polar effect in the transcription of the downstream gene basC when tested by RT-PCR (data not shown), abolished the ability of the insertion derivative 19606-s1 to grow under iron-limiting conditions. The fact that this derivative tested negative on CAS agar plates suggests that acinetobactin is the only high-affinity siderophore produced by A. baumannii 19606. The growth inhibition imposed by the addition of 120 μM DP was relieved when 19606-s1 cells were fed with either inorganic iron (26 mm growth halo) or purified acinetobactin (18 mm growth halo). Furthermore, these cells grew well around filter disks containing anguibactin (20 mm growth halo). In contrast, the M9 minimal medium culture supernatant of 19606-s1 failed to crossfeed the V. anguillarum anguibactin-transport reporter strains 775::Tn1-5(pJHC9-8) and 775::Tn1-6(pJHC9-8) when tested under the same experimental conditions (data not shown). Methanol extracts of lyophilized A. baumannii 19606-s1 M9 culture supernatants also failed to crossfeed these two V. anguillarum reporter strains, although they were positive with the Arnow test. Taken together, these results demonstrate that the expression of basD is required for the biosynthesis of acinetobactin and the ability of A. baumannii 19606 to grow under iron-limiting conditions.

**Fig. 6.** Production of BauA in the 19606 parental strain and the 19606-t6 and 19606-t7 insertion derivatives. (a) Total cell lysates prepared from equal amounts of cells cultured under iron-rich (Fe) and iron-deficient (DP) conditions were size fractionated by SDS-PAGE and stained with Fast Stain. (b) Samples shown in (a) were blotted to nitrocellulose and probed with anti-BauA serum.

**DISCUSSION**

Although the structure of acinetobactin was elucidated almost 10 years ago (Yamamoto et al., 1994), the genes and gene products involved in its biosynthesis and in the utilization of ferric-acinetobactin complexes remained to be identified and characterized. Based on the structural similarities between acinetobactin and anguibactin, it is possible that A. baumannii 19606 synthesizes proteins related to those involved in anguibactin biosynthesis encoded by genes located in the pJM1 plasmid of V. anguillarum 775 (Actis et al., 1988; Di Lorenzo et al., 2003). Accordingly, we showed previously that this strain...
expresses histidine decarboxylase activity and produces histamine, an anguibactin biosynthetic precursor, when cells are cultured in the presence of 1 % histidine (Actis et al., 1999). In the present work we demonstrated the existence of genes encoding a potential monoxygenase and a non-ribosomal peptide synthetase, with the latter required for acinetobactin biosynthesis. Our previous work (Dorsey et al., 2003a) has also shown that A. baumannii 19606 grown under iron-limiting conditions produces an outer-membrane protein that reacts with anti-FatA serum. This finding supported the hypothesis that this strain might also produce other iron-acquisition components similar to those expressed by the fish pathogen V. anguillarum 775. Indeed, the rescue cloning and DNA sequence analysis data reported here show that the bau gene cluster has the same arrangement as the iron transport-biosynthesis (ITB) operon of V. anguillarum.

The fact that V. anguillarum 775 and A. baumannii 19606 can use either anguibactin or acinetobactin to grow under conditions of iron limitation is in accordance with the molecular and biological findings that these two siderophore-mediated iron-acquisition systems are structurally and functionally related. These similarities are remarkable if one considers that these two siderophore systems are produced by two bacterial pathogens that occupy different ecological niches and cause severe infections in unrelated hosts. In addition, these two gammaproteobacteria are taxonomically different, with V. anguillarum being a member of the order Vibrionales and A. baumannii belonging to the order Pseudomonadales. These two bacterial pathogens are also quite different biologically. For instance, A. baumannii grows well at temperatures up to 45 °C (Bouvet & Grimont, 1986) whereas V. anguillarum grows quite poorly at 37 °C, a temperature at which the plasmid pJM1 is lost, affecting the virulence and ability of this fish pathogen to acquire iron from the environment and the infected host (Crosa et al., 1980).

Although FatA has only 46-7 % identity to BauA, both proteins are recognized by antibodies raised against each polypeptide. In contrast, an anti-FatB serum raised against an amino terminal end peptide (Actis et al., 1995) failed to detect BauB, even though the two proteins have 65-6 % identity. This result is consistent with the utilization of an antigenic peptide that has significant sequence differences with BauB. Nevertheless, BauB has predicted properties similar to those described for FatB, which forecast its role in iron transport as a putative iron-binding periplasmic protein (Actis et al., 1995). It is noteworthy that, when compared with the V. anguillarum fat gene cluster, the bau locus has an extra ORF, bauE, whose translation product has the highest similarity to the P. putida hypothetical protein PP2592. BauE also showed significant similarity with ATP-binding proteins that are part of iron(III)-ABC-transporters described in other bacteria. Among them is the predicted product of the pJM1 ORF40, which is postulated to provide the ATP-binding function missing in the anguibactin-permease system (Di Lorenzo et al., 2003). Interestingly, ORF40 was mapped between anguibactin biosynthetic genes in a pJM1 region that is 35 kb away from the fatDCBA locus (Di Lorenzo et al., 2003).

In silico analysis also revealed the presence of a gene cluster similar to bauDCEBA (Fig. 5c) in the genome of the Gram-negative soil bacterium P. putida KT2440 (Nelson et al., 2002). This finding was unexpected because these two bacterial strains, which were isolated from different ecological niches, are different biologically (Krieg & Holt, 1984) and taxonomically. Although these two gamma-proteobacteria are in the order Pseudomonadales, they are different enough to be classified into two families, with A. baumannii and P. putida belonging to the Moraxellaceae and Pseudomonadaceae, respectively.

The P. putida KT2440 gene cluster includes the PP2590 and PP2592 hypothetical proteins that showed the highest similarity to the A. baumannii 19606 BauA and BauE proteins, respectively. In addition, the length and amino acid composition of the PP2594–PP2590 hypothetical proteins were significantly similar to those of the BauDCEBA proteins. The main difference between these two regions is that the P. putida KT2440 locus includes two additional coding elements, PP2596 and PP2595, whose products were annotated as the permease components of hypothetical ABC transporters with potential ATP-binding activity (Nelson et al., 2002). BLASTX searches showed that these two proteins are related to probable transmembrane ATP-binding proteins that are part of ABC transporters hypothetically involved in drug transport in bacteria such as Mycobacterium bovis (GenBank accession no. NP_855037) and M. tuberculosis (GenBank accession no. CA99981). These proteins are thought to be involved in multidrug resistance by export mechanisms that involve efflux pump proteins, which can play additional transport roles. In the case of E. coli, the activity of EntS, an efflux pump protein encoded by the ybdA gene of the ent operon (Chenault & Earhart, 1991; Shea et al., 1996), is required for the secretion of the enterobactin siderophore (Furrer et al., 2002). Thus, PP2596 and PP2595 could have a similar role in siderophore secretion functions. Analysis of the P. putida KT2440 genome with BLASTX failed to detect gene clusters significantly related to anguibactin biosynthetic genes, suggesting that this bacterium encodes transport but not biosynthetic siderophore functions highly related to those expressed by V. anguillarum and A. baumannii.

Based on the amino acid sequence identity/similarity between BasD and AngN, a protein that is required for the formation of the thiazoline ring present in anguibactin (Crosa & Walsh, 2002), we predict that BasD is involved in the formation of the oxazoline ring present in acinetobactin (Yamamoto et al., 1994). The product of BasC is related to the AngU monoxygenase required, together with the histidine decarboxylase activity of AngH (Tolmasy et al., 1995), for the conversion of histidine into N-hydroxyhistamine, an essential anguibactin biosynthetic
precursor (Crosa & Walsh, 2002). Based on these considerations and the facts that A. baumannii produces histamine (Actis et al., 1999) and that acinetobactin contains an N-hydroxyhistamine moiety, we predict that BasC has an essential function in the biosynthesis of acinetobactin.

GenBank accession no. AB101202 reports the nucleotide sequence of a 32.4 kb genomic region cloned from A. baumannii 19606, the same strain we have used in this work, that includes the genes described in this work. Therefore, we have used the same annotation to avoid confusion. From the information provided by this GenBank reference, which was published (Mihara et al., 2004) during the review of this manuscript, we identified two genes, immediately upstream of the bauDCEBA operon, encoding putative non-ribosomal peptide synthetases related to the AngM and AngR pJM1-encoded proteins. Upstream of these genes is an ORF encoding a putative siderophore-utilization protein found in different bacteria. This ORF is located at what appears to be the 5’ end of this A. baumannii 19606 chromosomal region involved in iron acquisition. On the other side of the region depicted in Fig. 5(a), basD is followed by coding regions whose predicted products are related to AngE and AngB/G, which provide the 2,3-dihydroxybenzoate-AMP ligase and isochorismatase activities, respectively, required for anguibactin biosynthesis (Crosa & Walsh, 2002). Immediately downstream is an angH homologue that encodes the histidine decarboxylase required for the biosynthesis of histamine from histidine. This gene is followed by two genes encoding predicted ABC transport proteins that could play siderophore-secretion functions as proposed for the pJM1 ORF14 and ORF15 coding regions (Di Lorenzo et al., 2003) and the P. putida PP295 and PP2596 ORFs (Nelson et al., 2002). The last three ORFs of this A. baumannii genomic region apparently involved in iron acquisition code for a putative thioesterase, a non-ribosomal peptide synthetase and an isochorismatase that were associated with the products of the pJM1 angT, angD and angC genes, respectively (Di Lorenzo et al., 2003).

This in silico analysis, taken together with our genetic and molecular work, demonstrates that V. anguillarum 775 and A. baumannii 19606 have the capability of expressing highly related proteins involved in the biosynthesis and transport of the respective siderophores, anguibactin and acinetobactin.

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