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Iron acquisition functions expressed by the human pathogen *Acinetobacter baumannii*

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Abstract *Acinetobacter baumannii* is a gram-negative bacterium that causes serious infections in compromised patients. More recently, it has emerged as the causative agent of severe infections in military personnel wounded in Iraq and Afghanistan. This pathogen grows under a wide range of conditions including iron-limiting conditions imposed by natural and synthetic iron chelators. Initial studies using the type strain 19606 showed that the iron proficiency of this pathogen depends on the expression of the acinetobactin-mediated iron acquisition system. More recently, we have observed that hemin but not human hemoglobin serves as an iron source when 19606 isogenic derivatives affected in acinetobactin transport and biosynthesis were cultured under iron-limiting conditions. This finding is in agreement with the observation that the genome of the strain 17978 has a gene cluster coding for putative hemin-acquisition functions, which include genes coding for putative hemin utilization functions and a TonBExbBD energy transducing system. This system restored enterobactin biosynthesis in an *E. coli* ExbBD deficient strain but not when introduced into a TonB mutant. PCR and Southern blot analyses showed that this hemin-utilization gene cluster is also present in the 19606 strain. Analysis of the 17978 genome also showed that this strain harbors genes required for acinetobactin synthesis and transport as well as a gene cluster that could code for additional iron acquisition functions. This hypothesis is in agreement with the fact that the inactivation of the *basD* acinetobactin biosynthetic gene did not affect the growth of *A. baumannii* 17978 cells under iron-chelated conditions. Interestingly, this second iron uptake gene cluster is flanked by perfect inverted repeats and includes transposase genes that are expressed transcriptionally. Also interesting is the observation that this additional cluster could not be detected in the type strain 19606, an observation that suggests some significant differences in the iron uptake capacity between these two *A. baumannii* strains. Transposome mutagenesis of the strain 19606 resulted in the isolation of a derivative unable to grow under iron-chelated conditions. Gene mapping and protein analysis together with complementation assays showed that a protein related to SecA, which is a component of the Sec protein secretion system in a wide range of bacteria, is needed at least for the production of the BauA acinetobactin outer membrane receptor. Furthermore, this derivative was unable to use hemin as an iron source under limiting conditions. Taken together, these results indicate that *A. baumannii* expresses siderophore-mediated and hemin acquisition functions, although different isolates differ in their iron acquisition capacity. Unexpectedly, the ability of this

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pathogen to acquire iron depends on the expression of a SecA protein secretion function, which has not been associated with iron acquisition in bacteria.

Keywords *Acinetobacter baumannii* · Siderophore · Iron · Hemin · Hemoglobin · Acinetobactin · Anguibactin

Introduction

Acinetobacter baumannii is a gram-negative coccobacillus that causes serious infections in compromised human hosts (Bergogne-Berezin and Towner 1996; Bergogne-Berezin 2001; Villegas and Hartstein 2003). This bacterial pathogen has been observed to cause community-acquired pneumonia as well as nosocomial infections and has recently emerged as a problematic infection for troops wounded in the Middle East (Davis et al. 2005; Joly-Guillou 2005; Dijkshoorn et al. 2007). In addition, *A. baumannii* clinical isolates are exhibiting increased resistance to the available antimicrobial chemotherapies, making treatment of serious infections caused by this pathogen a significant challenge in human medicine (Calhoun et al. 2008; Peleg et al. 2008; Perez et al. 2008). Because this organism is capable of forming biofilms on medically relevant materials (Tomaras et al. 2003) and persisting in a variety of environments, including those that are severely desiccated (Houang et al. 1998), this pathogen has proven to be clinically important. Although much is known about antibiotic resistance and the epidemiology of infections caused by *A. baumannii*, the virulence properties and the pathobiology of this important pathogen are poorly understood. Nevertheless, it is evident that this opportunistic pathogen is capable of attaching to and forming biofilms on abiotic surfaces (Tomaras et al. 2003) as well as growing under iron-limiting conditions such as those imposed by the human host (Echenique et al. 1992; Actis et al. 1993; Dorsey et al. 2003, 2004). All these cell properties are known and recognized by their central role in bacterial virulence and the pathogenesis of serious infectious diseases such as those caused by *A. baumannii*. Regarding iron acquisition, initial studies showed that *A. buamannii* clinical isolates secrete iron-regulated catechol and siderophore compounds and produce iron-regulated proteins (Echenique et al. 1992; Actis et al. 1993; Dorsey et al. 2003). Furthermore, the 19606 strain acquires iron via the production of acinetobactin, a catechol–hydroxamate siderophore that is almost identical to the iron chelator anguibactin produced by the fish pathogen *Vibrio anguillarum* (Dorsey et al. 2004; Yamamoto et al. 1994). On the other hand, siderophore utilization assays and detection of histamine production showed that different *A. baumannii* clinical isolates produce different types of catechol siderophores (Dorsey et al. 2003). All these observations suggest that *A. baumannii* is a versatile pathogen that could acquire iron to prosper under iron-limiting conditions, such as those found in the human host, by expressing different iron acquisition systems. However, there are some interesting aspects related to iron acquisition and its role in the virulence of this pathogen that remain uncharacterized.

The acinetobactin-mediated system in the 19606 type strain

The acinetobactin-mediated iron acquisition system was described in the type strain 19606 by us (Dorsey et al. 2004) and Yamamoto et al. (1994). Molecular genetics, bioinformatics and biological assays showed that a 32.4-kbp chromosomal locus containing 18 coding regions harbors the majority of the genes needed for acinetobactin biosynthesis and uptake of iron–acinetobactin complexes. Insertional analysis (Dorsey et al. 2004; Mihara et al. 2004) confirmed this observation by showing that inactivation of *bauA* and *basD*, which code for acinetobactin transport and biosynthesis functions, respectively, affects the ability of isogenic derivatives to grow under iron-restricted conditions imposed by the presence of the synthetic iron chelator 2,2′-dipyridyl (DIP) (Fig. 1). Interestingly, plate and liquid assays clearly showed that the t6 BauA deficient mutant is more sensitive to iron restriction than the s1 BasD deletion/insertion derivative. This phenotype could be due to the production and utilization of acinetobactin intermediates that could provide iron to cells under milder iron chelated conditions because of their lower affinity for iron when compared with that of acinetobactin. Nevertheless, functional analysis of the BauA and BasD mutants proved that the acinetobactin-mediated system is the only siderophore-mediated high-affinity iron acquisition system expressed by the type strain 19606.

Sequence analysis of the 19606 acinetobactin gene cluster showed that it includes two genetic determinants, *barA* and *barB*, which could code for a putative ABC transporter potentially involved in siderophore secretion. Such a hypothesis is supported by the fact that the products of these two genes, which could be regulated by the iron repressor protein Fur, have significant similarity with the *V. anguillarum* pJM1 Orf 14 and Orf 15 predicted cytoplasmic membrane proteins (Di Lorenzo et al. 2003). Random insertion mutagenesis proved that the disruption of Orf 14 results in a severe iron-acquisition deficiency in *V. anguillarum* isogenic derivatives because of the lack of anguibactin production (Tolmasky et al. 1988). Accordingly, interruption of the *barA–barB* coding region by the insertion of a pKnock–Km (Alexeyev 1999) derivative harboring a 1.6-kbp *Pst*I fragment expanding the 3′ end of *barA* and the 5′ end of *barB* resulted in an *A. baumannii* 19606 isogenic derivative with a reduced iron-acquisition phenotype when compared with the parental strain when cultured on solid media or broth under iron chelated conditions (Fig. 2a, b, respectively). These results confirm the role of this ABC transporter in siderophore secretion although the remaining components of the acinetobactin secretion machinery, which appear to be encoded by genes located in a different chromosomal locus or loci, await identification and characterization, a situation similar to that observed with pJM1 even after its complete nucleotide sequence and annotation (Di Lorenzo et al. 2003).

Another significant observation that emerged after the analysis of the 19606 acinetobactin 32.4-kbp gene cluster is the absence of a gene coding for an EntA-like enzymatic activity, which is needed for the production of the acinetobactin and anguibactin precursor dihydroxybenzoic acid. This observation suggests that this strain could have two non-contiguous chromosomal regions involved in acinetobactin production. Such a possibility was confirmed, as it is described in the following section, with the analysis of the iron-uptake functions expressed by the *A. baumannii* strain 17978, the genome of which was the first to be sequenced and annotated for this bacterial species (Smith et al. 2007).

The random insertion mutagenesis of *A. baumannii* 19606 with the EZ::TN<R6Kγ*ori*/KAN-2>transposome from Epicentre resulted in the isolation of an isogenic derivative impaired in its ability to grow on L agar containing 150 μM of DIP (Fig. 3a). Siderophore utilization bioassays and western blotting of membrane proteins with anti-BauA antiserum proved that the iron utilization deficiency of this derivative, named

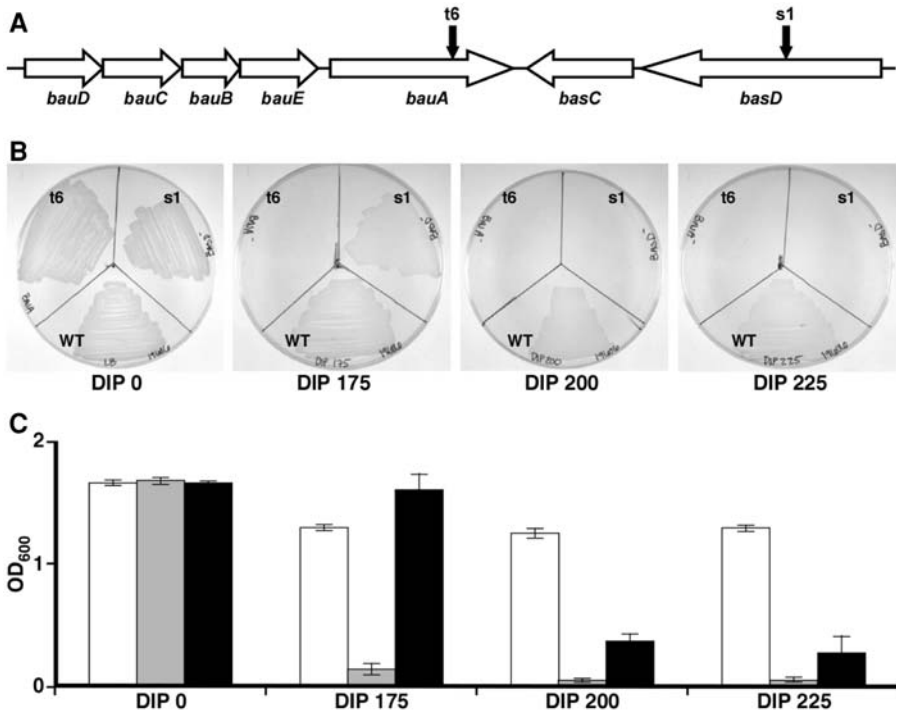

Fig. 1 Iron uptake phenotype of *A. baumannii* affected in acinetobactin biosynthesis and transport functions. **a** Genetic map of the locus containing genes coding for the BauDCEBA acinetobactin transport proteins and the BasC and BasD acinetobactin biosynthesis proteins. The *vertical arrows* locate the transposon insertions t6 and s1 affecting acinetobactin transport and biosynthesis, respectively. Growth of the parental strain (WT, *open bars*) and the t6 (*gray bars*) and s1 (*black bars*) insertion derivatives was tested on L agar (**b**) and L broth (**c**) containing increasing concentrations (μM) of DIP. Cell growth was recorded after overnight incubation at 37°C. *Error bars*, 1 SD

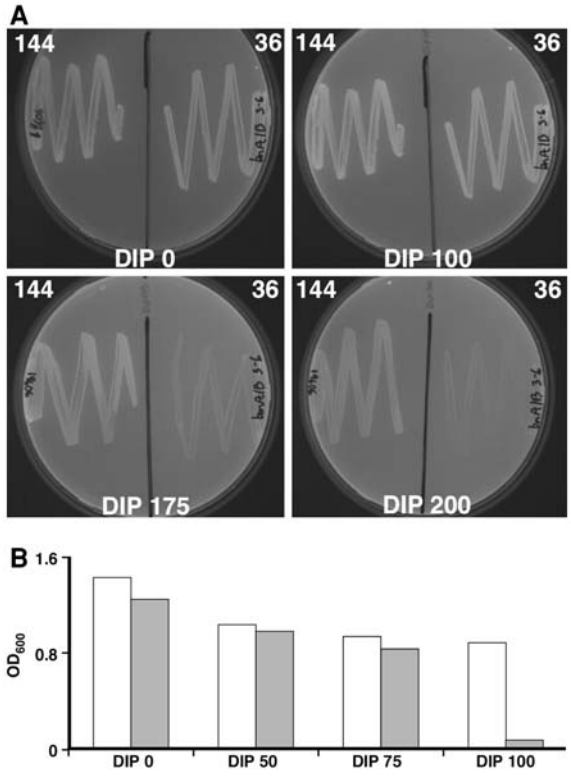

Fig. 2 Iron acquisition phenotype of an *A. baumannii* 19606 isogenic derivative harboring a disruption within the *barA–barB* coding regions. Growth of the iron uptake proficient strain 144 (*open bars*) and the *barA–barB* insertion derivative 36 (*gray bars*) was tested on L agar (**a**) and L broth (**b**) containing increasing concentrations (μM) of DIP. Cell growth was recorded after overnight incubation at 37°C

#2010, was due to the lack of production of the BauA acinetobactin outer membrane receptor (Fig. 3b) but not the production of acinetobactin. Rescue cloning, DNA sequencing and Southern blot hybridization analysis proved that the #2010 derivative harbors a single transposon insertion within the 3′ end of a predicted gene coding for a protein highly related to SecA, which plays a central role in the transport of proteins across the cytoplasmic membrane of a wide range of gram-positive and gram-negative bacteria (Manting and Driessen 2000; Mori and Ito 2001). It is important to note that the growth rate of this derivative is indistinguishable from that displayed by the parental strain under iron-rich conditions. The role of this *A. baumannii* 19606 gene in iron acquisition was confirmed by complementation assays, which proved that the introduction of a plasmid copy of the wild-type allele into the #2010 derivative restored its iron uptake proficiency to wild-type levels (Fig. 3a) as well as the production of the BauA outer membrane protein (Fig. 3b). These results were unexpected because a protein highly related to SecA, which is an important component of the Sec-mediated protein transport pathway, has not been ever reported to be involved in siderophore-mediated iron acquisition processes.

Iron acquisition functions in *A. baumannii* 17978

The genomic sequence of the ATCC 17978 strain, which was isolated from a case of fatal meningitis of a 4-month-old infant, was the first to be reported for *A. baumannii* (Smith et al. 2007) and open new ways to study iron acquisition functions expressed by this human pathogen. However, we tested the iron uptake capacity of this strain before the genomic data was published. Initial culture experiments showed that this strain is capable of growing under iron-chelated conditions imposed by the presence of DIP. Furthermore, the Arnow (Arnow 1937) and CAS (Schwyn and Neilands 1987) colorimetric assays showed that this strain produces catechol and siderophore compounds when cultured under iron-chelated conditions but not in the presence of excess inorganic iron. Additional analyses using PCR and western blotting proved that the 17978 strain harbors the *basD* acinetobactin biosynthesis gene and expresses the BauA acinetobactin outer membrane protein (Fig. 4). These observations are in accordance with the fact that 17978 is capable of crossfeeding the 19606 s1 insertion derivative in which an internal fragment of *basD* was replaced with the kanamycin resistance cassette from the recombinant plasmid pUC4K (Dorsey et al. 2004).

Taken together, all the results described above indicate that the *A. baumannii* 17978 strain harbors genetic determinants needed for the biosynthesis and utilization of acinetobactin. Based on this conclusion, the suicide plasmid pMU435, which was used to generate the *A. baumannii* 19606 s1 isogenic derivative (Dorsey et al. 2004), was conjugated into 17978 to generate the cognate *basD* mutant. However, the iron uptake phenotype of this derivative, which proved to harbor the predicted genetic modification (Fig. 4a), was not different from that of the parental 17978 strain when compared under the same iron-chelated conditions (Fig. 4c). This observation suggested the possibility that

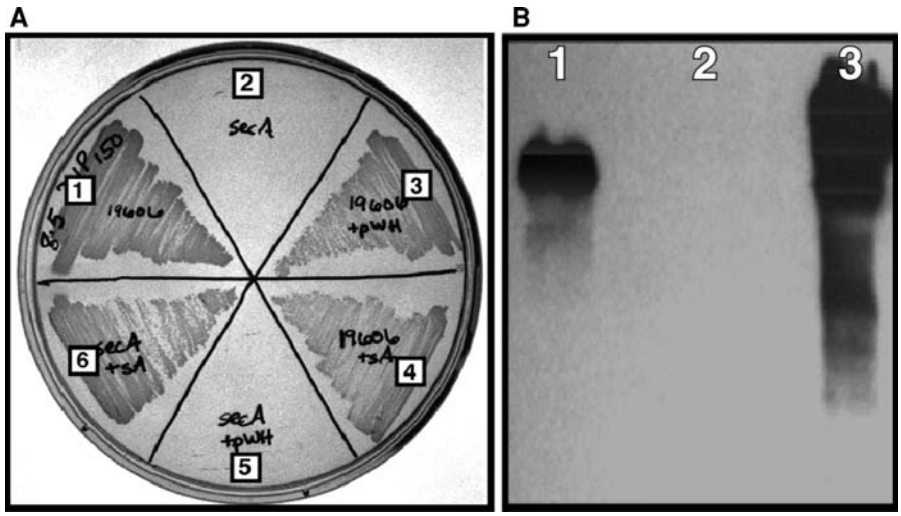

Fig. 3 **a** Iron uptake proficiency of different *A. baumannii* 19606 derivatives plated on L agar containing 150 μM dipyridyl and incubated overnight at 37°C. Sample 1, 19606 parental strain; sample 2, 19606 #2010 insertion derivative; sample 3, 19606 parental strain harboring empty pWH1266 shuttle cloning vector; sample 4, 19606 parental strain harboring pMU472, a pWH1266 derivative containing the wild-type *secA* allele; sample 5, 19606 #2010 insertion derivative harboring empty pWH1266 shuttle cloning vector; and sample 6, 19606 #2010 insertion derivative harboring pMU472. **b** Production of BauA in the parental strain (*lane 1*), the insertion derivative #2010 (*lane 2*) and #2010 harboring pMU472 as determined by immunoblotting of total proteins with anti-BauA polyclonal antibodies

the 17978 could either have two copies of *basD* or express two independent siderophore-mediated iron acquisition systems. Analysis of the genomic sequence of this strain did not support the first possibility, since the genome of 17978 harbors only one predicted copy of *basD*, but provided evidence for the presence of a second iron acquisition locus (Fig. 5), which could explain the results obtained with the 17978 *basD* mutant described above and the fact that this strain tolerates higher amounts of DIP when compared with the type strain 19606. The 26-kbp gene cluster identified in the strain 17978 (Fig. 5) contains 15 genes coding for putative siderophore biosynthesis functions (ORF 1–ORF 11, ORF 14, ORF 15, ORF 18 and ORF 19), one predicted gene (ORF 16) coding for a siderophore outer membrane receptor and two genes (ORF 17 and ORF 20) coding for predicted metal efflux proteins. Interestingly, the predicted translation product of ORF 3 is related to EntA, an observation that supports our hypothesis that the production and utilization of acinetobactin in the strain 19606 involves at least two non-contiguous genetic clusters. It is important to note that almost the entire 26-kbp iron acquisition gene cluster present in *A. baumannii* 17978 is flanked by two 14-base perfect inverted repeats and includes two genes (ORF 12 and 13) coding for putative transposition functions (Fig. 5). RT-PCR of total RNA isolated from 17978 cells grown under iron-chelated conditions proved that these two coding regions are expressed at the transcriptional level. Furthermore, PCR and Southern blot analysis showed that this gene cluster could no be detected in the genome of the type strain 19606. It is also important to mention that DNA hybridization assays failed to detect the 17978 *entA*-like gene in the genome of the type strain 19606, although transformation of *E. coli* AN193 *entA* mutant, which is an enterobactin deficient strain (Cox et al. 1970), with a 19606 cosmid gene library resulted in the isolation of a cosmid clone coding for functions that restored the ability of this *E. coli* mutant to grow under iron-chelated conditions because of the re-establishment of enterobactin biosynthesis.

Taken together, all the results obtained with the strains 19606 and 17978 suggest that these two strains have acquired different siderophore-mediated iron utilization functions from different sources by mechanisms that have involved horizontal gene transfer and transposition. Interestingly, a similar conclusion was drawn after the sequencing and annotation of the *V. anguillarum* pJM1 plasmid and the observation that some of the essential anguibactin biosynthesis functions are encoded by chromosomal genes.

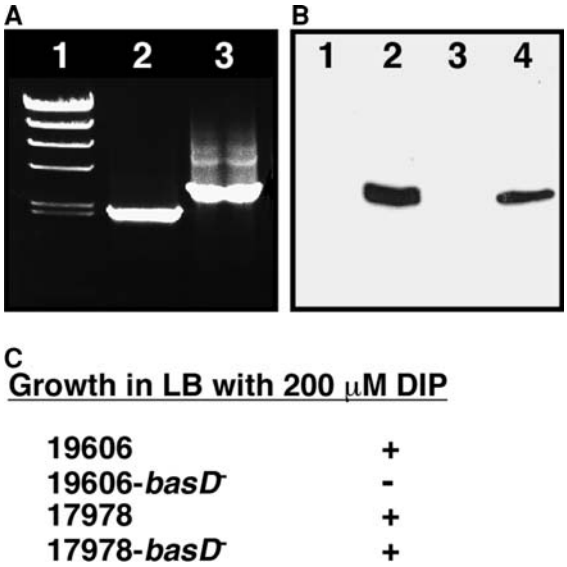

Fig. 4 Effect of *basD* disruption in the iron uptake proficiency of *A. baumannii* 19606 and 17978. **a** Agarose gel electrophoresis confirming the generation of the 17978 *basD*::Km insertion–deletion derivative (*lane 3*) with the pMU435. *Lane 1*, *Hin*dIII-digested λ DNA, *lane 2*, 17978 parental DNA. The amplicons were generated with primers flanking the insertion–deletion site within *basD*. **b** Western blot of total lysates of 19606 (*lanes 1* and *2*) and 17978 (*lanes 3* and *4*) cells cultured in L broth containing either 100 μM $FeCl_3$ (*lanes 1* and *3*) or 100 μM DIP. The blot was probed with anti-BauA polyclonal antibodies and the immunocomplexes were detected by chemiluminescence using HRP-labeled protein A. **c** Growth of *A. baumannii* parental and isogenic derivatives under iron-chelated conditions after overnight incubation at 37°C

A. baumannii hemoglobin and hemin utilization functions

The *A. baumannii* 19606 t6 ($BauA^-$) and s1 ($BasD^-$) mutants were used to test the ability of this pathogen to use human hemoglobin and hemin as iron sources under iron-chelated conditions. The supplementation of L agar or L broth with 50 μM hemin was able to rescue the growth of these mutants in the presence of increasing concentrations of DIP, particularly at 200 μM, to levels similar to those displayed by the parental 19606 strain (panels b and c of Fig. 6). As it was mentioned before, the BauA mutant seems to be more sensitive to iron chelation even in the presence of an alternative iron source such as hemin, which could be used by bacterial pathogens while infecting the human host. In contrast to the effect of hemin, the growth of both mutants was not significantly enhanced by the addition of 50 μM human hemoglobin to either L broth or L agar. It is interesting to note that the *A. baumannii* 19606 derivative #2010, which has a transposon insertion within a gene coding for a SecA related protein and does not transport ferric acinetobactin, is also impaired in its ability to use hemin as an iron source when cultured under iron-chelated conditions.

Based on the aforedescribed experimental observations, we examined the annotation of the *A. baumannii* 17978 genome and found that there is a hypothetical 12-gene locus that could code for functions involved in hemin binding and transport (Fig. 7a). The predicted products of the first four genes, two of which seem to be real *exbD* duplications, are related to the TonBExbBD energy transducing system that is required for hemin utilization in a wide range of bacteria. RT-PCR analysis of total RNA proved that all four coding regions are part of a polycistronic transcript that is present in cells cultured under iron-chelated conditions. The role of these predicted genes was tested by their capacity to restore enterobactin utilization by the *E. coli* RA1051 *exbBD* mutant after PCR amplification and cloning. Figure 7b shows that transformation of *E. coli* RA1051 with pMU673, harboring the parental *exbBD* alleles, restores iron acquisition to level similar to those displayed by the wild-type parental strain W3110. In contrast, transformation of *E. coli*

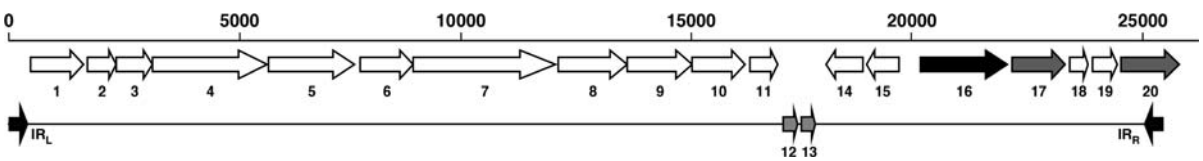

Fig. 5 Genetic organization of an *A. baumannii* 17978 locus harboring genes coding for putative siderophore biosynthesis functions (ORF 1–ORF 11, ORF 14, ORF 15, ORF 18 and ORF 19) as well as functions potentially involved in siderophore recognition (ORF 16) and siderophore secretion (ORF 17 and ORF 20). This locus, which also contains two genes coding for putative transposition functions (ORF 12 and 13), is flanked by two 14-base perfect inverted repeats (IR_L and IR_L, converging *horizontal arrows* in *bottom diagram*)

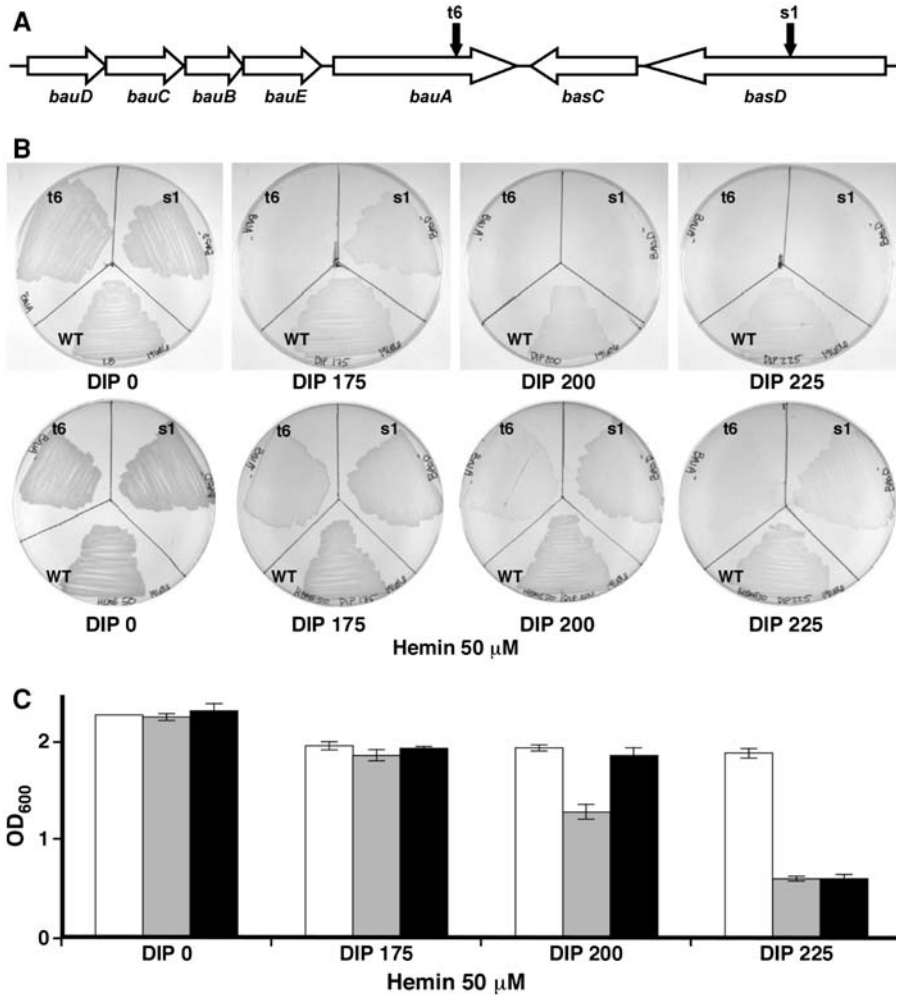

Fig. 6 Iron uptake phenotype of *A. baumannii* affected in acinetobactin biosynthesis and transport functions. **a** Genetic map of the locus coding for the BauDCEBA acinetobactin transport proteins and the BasC and BasD acinetobactin biosynthesis proteins. The *vertical arrows* locate the transposon insertions t6 and s1 affecting acinetobactin transport and biosynthesis, respectively. Growth of the parental strain (WT, *open bars*) and the t6 (*gray bars*) and s1 (*black bars*) insertion derivatives was tested on L agar (**b**) and L broth (**c**) containing increasing concentrations (μM) of DIP in the absence of hemin (**b**, *top row*) or the presence of hemin (**b**, *bottom row* and **c**). Cell growth was recorded after overnight incubation at 37°C. *Error bars*, 1 SD

R1051 with the empty cloning vector failed to restore enterobactin-mediated iron uptake as the DIP concentration in L broth was increased. On the other hand the introduction of the recombinant plasmid pMU672, which harbors the *A. baumannii* 17978 *exbBBDtonB* genes, restored the iron uptake capacity of the RA1051 mutant although at levels lower that those obtained with the parental genes. Interestingly, the iron uptake deficiency of *E. coli* KP1344, which is a W3110 TonB deficient derivative, could be changed to wild-type levels by transformation with the W3110 parental allele but not after transformation with the pMU672 plasmid harboring the *A. baumannii* 17978 *exbBBDtonB* genes. All these results demonstrate that the *A. baumannii* 17978 *exbBD* determinants code for active energy transduction functions needed for hemin and iron utilization. However, the *tonB* component of this gene cluster seems to be dedicated to hemin utilization but not siderophore-mediated iron acquisition, possibilities that are being tested by targeted mutagenesis and additional complementation assays. It is interesting to note that PCR proved that all components of this 17978 locus are also present in the type strain 19606, an observation that validates our experimental data showing the ability of BauA and BasD isogenic derivatives to use hemin to grow under iron limitation.

Effect of gallium and iron in cell growth

It is apparent that antibiotic resistance is a major issue in the treatment of infections caused by *A. baumannii*. Therefore, we need to identify additional essential cellular processes that could be used as new targets to prevent and treat the infections this pathogen causes in humans. One of these targets is

iron utilization because of the central role this metal plays in the physiology of most bacteria, including those that cause diseases in the human host. It has been shown that the addition of Ga(III), a metal that is already being used in human medicine to treat cancer, affects bacterial cell growth, particularly under iron-limiting conditions (Kaneko et al. 2007). This observation offers the possibility of testing this metal as an antimicrobial agent against *A. baumannii*. Therefore, we examined the effect of gallium (III) nitrate using an approach similar to described for the study of *Pseudomonas aeruginosa* (Kaneko et al. 2007). The panel a of Fig. 8 shows that the addition of gallium to 10% trypticase soy broth (TSB) up to a concentration of 50 μM does not have a significant effect on the growth of the *A. baumannii* 19606 parental strain or the BauA and BasD deficient derivatives. However, a significant reduction of bacterial growth was observed when the concentration of gallium was increased in 10% TSB already containing 50 μM DIP (Fig. 8b). This negative effect is independent of the ability of the cells to produce or use acinetobactin. These are encouraging results suggesting that gallium could be used to treat patients infected with *A. baumannii* strains that are highly resistant to a wide range of antibiotics. Unfortunately, almost nothing is known about the mechanisms by which this metal could be internalized and affect functions that are central to cell physiology. Therefore, little can be said about the mechanisms by which bacterial pathogens can develop gallium resistance. Nevertheless, this seems to be a viable alternative that deserves more exploration.

In summary, the data and information presented and discussed in this review show that *A. baumannii* seems to be an adaptable bacterial pathogen that has the ability to use different iron sources and express independent iron acquisition systems that allow it to colonize and prosper in the human host. These conclusions are supported by the genomic information derived from the AYE, SDF (Fournier et al. 2006) (http://www.genoscope.cns.fr/agc/mage/baumannoscope) and ACICU (Iacono et al. 2008) clinical strains showing that the AYE and ACICU are capable of expressing independent siderophore-mediated acquisition systems, with acinetobactin being a common trait, as well as hemin utilization systems. Interestingly, the genome analysis of the SDF strain suggests that its capacity to obtain iron depends on the expression of a hemin acquisition system rather than the expression of biosynthesis and transport functions for a particular siderophore. All these observations demonstrate the plasticity of *A. baumannii* to express different iron acquisition systems, a possibility that we suggested some time ago (Dorsey et al. 2003) after the analysis of outer membrane proteins and the production of siderophores and histamine by different clinical isolates.

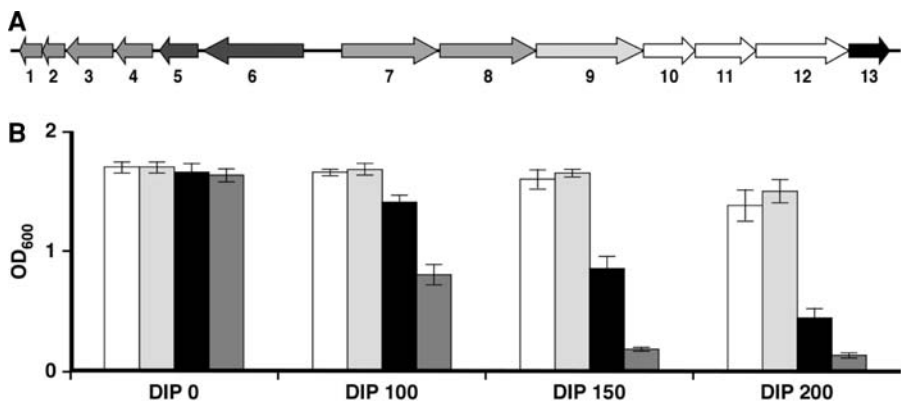

Fig. 7 Genetic map of an *A. baumannii* 17978 locus coding for putative hemin transport and utilization functions. **a** ORF 1–ORF 4 code for ExbBBDTonB energy transducing functions, ORF 5 and ORF 6 code for predicted TonB-dependent receptors, ORF 7 and ORF 8 code for putative hemin receptor proteins, ORF 9 codes for a predicted oligopeptidase, ORFs 10–ORF 12, code for potential dipeptide transport functions, and ORF 13 codes for a hypothetical protein. **b** Genetic complementation of *E. coli* RA1051 (W3110 *exbBD*$^-$) with pMU673 containing the W3110 *exbBD* wild-type allele (*light gray bars*), pMU672 harboring the 17978 *exbBBDtonB* locus (*black bars*) or empty cloning vector (*dark gray bars*). *E. coli* W3110 transformed with empty cloning vector (*open bars*) was used as a positive control. Cell growth was recorded after overnight incubation at 37°C in L broth containing increasing concentrations (μM) of DIP. *Error bars*, 1 SD

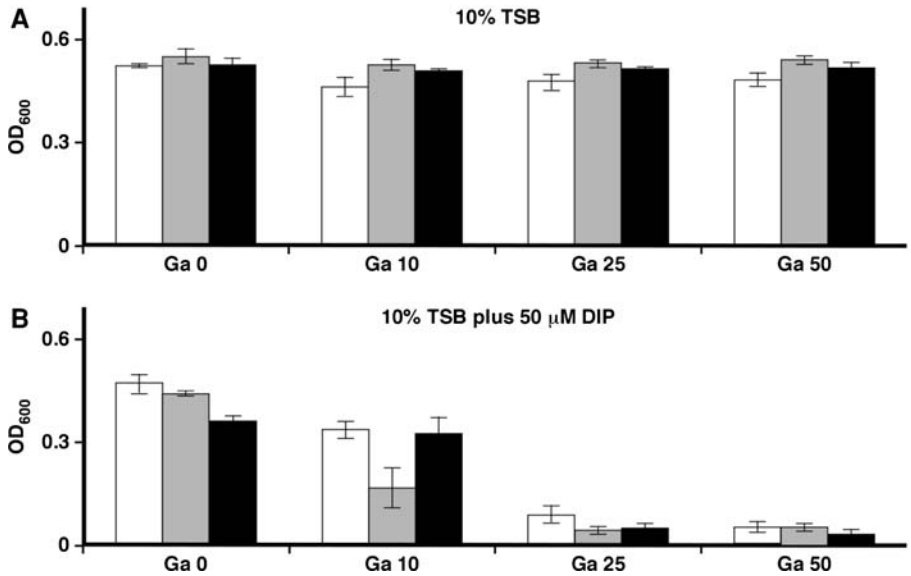

Fig. 8 Effect of gallium nitrate on cell growth. Growth of the parental strain (*open bars*) and the t6 (*gray bars*) and s1 (*black bars*) insertion derivatives was tested in 10% TSB in the absence (**a**) or the presence of DIP (**b**) and increasing concentrations (μM) of gallium nitrate. Cell growth was recorded after overnight incubation at 37°C. *Error bars*, 1 SD

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