The *Acinetobacter baumannii* entA Gene Located Outside the Acinetobactin Cluster Is Critical for Siderophore Production, Iron Acquisition and Virulence

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**Abstract**

*Acinetobacter baumannii* causes severe infections in compromised patients, who present an iron-limited environment that controls bacterial growth. This pathogen has responded to this restriction by expressing high-affinity iron acquisition systems including that mediated by the siderophore acinetobactin. Gene cloning, functional assays and biochemical tests showed that the *A. baumannii* genome contains a single functional copy of an *entA* ortholog. This gene, which is essential for the biosynthesis of the acinetobactin precursor 2,3-dihydroxybenzoic acid (DHBA), locates outside of the acinetobactin gene cluster, which otherwise harbors all genes needed for acinetobactin biosynthesis, export and transport. In *silico* analyses and genetic complementation tests showed that *entA* locates next to an *entB* ortholog, which codes for a putative protein that contains the isochorismatase lyase domain, which is needed for DHBA biosynthesis from isochorismic acid, but lacks the aryl carrier protein domain, which is needed for tethering activated DHBA and completion of siderophore biosynthesis. Thus, *basF*, which locates within the acinetobactin gene cluster, is the only fully functional *entB* ortholog present in ATCC 19606* T*. The differences in amino acid length and sequences between these two EntB orthologs and the differences in the genetic context within which the *entA* and *entB* genes are found in different *A. baumannii* isolates indicate that they were acquired from different sources by horizontal transfer. Interestingly, the AYE strain proved to be a natural *entA* mutant capable of acquiring iron via an uncharacterized siderophore-mediated system, an observation that underlines the ability of different *A. baumannii* isolates to acquire iron using different systems. Finally, experimental infections using *in vivo* and *ex vivo* models demonstrate the role of DHBA and acinetobactin intermediates in the virulence of the ATCC 19606* T* cells, although to a lesser extent when compared to the responses obtained with bacteria producing and using fully matured acinetobactin to acquire iron.

**Introduction**

*Acinetobacter baumannii* is being increasingly recognized as an important pathogen that causes severe infections in hospitalized patients as well as deadly cases of community-acquired pneumonia [1,2,3,4]. More recently, it has been described as the ethiological agent of severe wound infections in military personnel injured in the Middle East [5,6] and cases of necrotizing fasciitis [7]. A serious concern with this pathogen is its remarkable ability to acquire genes and express resistance to a wide range of antibiotics as well as to evade the human defense responses [4]. Among the latter is the capacity of *A. baumannii* to prosper under the iron-limited conditions imposed by the human host’s high-affinity chelators lactoferrin and transferrin [8,9]. Although some progress has been made in recent years, not much is known about the pathobiology of this bacterium and the nature of its virulence factors involved in the serious diseases it causes in humans.

Bacterial pathogens respond to iron limitation imposed by the human host by expressing different high-affinity uptake systems including siderophore-dependent and siderophore-independent systems, as well as systems that remove iron from host compounds, such as hemin, by either direct contact or by producing scavengers known as hemophores [10,11]. In the case of *A. baumannii*, experimental data [12,13,14,15] and *in silico* analyses of fully sequenced and annotated genomes [16,17] show that different *A. baumannii* clinical isolates could express different iron uptake systems. Currently, the best-characterized system is that expressed by the ATCC 19606* T* type strain, which is based on the production and utilization of acinetobactin [14,18,19]. This catechol-hydroxamate siderophore is a non-cyclic derivative of 2,3-dihydroxybenzoic acid (DHBA) linked to threonine and N-hydroxyhistamine [19]. Genetic and functional analyses indicate that the acinetobactin-mediated system is the only high-affinity iron acquisition system expressed by the ATCC 19606* T* type strain [14]. The *bas*, *bau* and *bar* genes needed for the production, transport and secretion of acinetobactin, respectively, are located in a 26.5-kb chromosomal region harboring seven operons [14,18]. However, this locus does not include an *entA* ortholog coding for a 2,3-dihydro-2,3-dihydroxy-benzoate dehydrogenase. This enzyme is involved in the last step of the conversion of chorismate into DHBA, which is essential for the biosynthesis of the catechol moiety of siderophores such as enterobactin [20,21]. This observation indicates that at least two chromosomal regions are involved in the biosynthesis of acinetobactin in the ATCC...
19606T strain; one containing the bas, bau and bar genes and another harboring at least the entA gene. In this report, we present experimental and genomic evidence supporting this hypothesis as well as showing that there are variations not only in nucleotide sequence but also in genetic arrangements among the A. baumannii loci harboring the entA genetic determinant. In addition, we demonstrate that the expression of an active entA gene is needed for the full virulence of the ATCC 19606T strain when tested using A549 human alveolar epithelial cells and Galleria mellonella caterpillars as experimental infection models. We also report the observation that the A. baumannii AYE strain is a natural entA mutant that acquires iron through a siderophore-mediated system that remains to be characterized.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this work are shown in Table S1. Strains were routinely cultured in Luria Bertani (LB) broth or agar [22] at 37°C in the presence of appropriate antibiotics. Iron-rich and iron-limiting conditions were achieved by the addition of FeCl3 dissolved in 0.01 M HCl and 2,2'-dipyridyl (DIP), respectively, to liquid or solid media.

Recombinant DNA techniques

Chromosomal and plasmid DNA were isolated by ultracentrifugation in CsCl density gradients [22,23] or using commercial kits (Qiagen). DNA restriction and Southern blot analyses were conducted using standard protocols and [32P]-dCTP-labelled probes prepared as described before [22,24].

Construction of a gene library and cloning of the entA gene

An A. baumannii ATCC 19606T genomic library was prepared using Escherichia coli LE392 and the cosmid vector pVK100 as described before [13]. Cosmid DNA was isolated en masse from E. coli LE392 clones and used to transform E. coli AN193 by electroporation as described before [13]. Transformants harboring the ATCC 19606T entA gene were selected on LB agar containing 20 µg/ml tetracycline (Tet) and 250 µM DIP. Cosmid DNA was isolated from one of the E. coli AN193 complemented clones, which was named 2631, digested with HindIII and subcloned into pUC118 to generate pMU748 (Fig. 1A). Plasmid DNA was

Figure 1. Genetic organization of A. baumannii chromosomal regions harboring genes coding for siderophore production and utilization. (A) Genetic map of the ATCC 19606T gene cluster containing the entA and entB orthologs. H, HindIII; E, EcoRI. (B) Genetic map of the ATCC 17978 gene cluster that includes the predicted entA and entB orthologs. The horizontal arrows and their direction indicate the location and direction of transcription of predicted genes, respectively. The horizontal lines indicate the chromosomal regions either cloned or used to confirm recombinant derivatives by PCR or Southern blotting. The numbers next to each bar indicate the location of primers used in PCR amplification. The vertical arrows indicate the insertion of the DNA cassette harboring the aph gene, which code for kanamycin resistance, or the EZ-Tn5<KAN-2> transposon (Tn). Numbers under ORFs shown in panel B correspond to those listed in Table 2. Numbers on top of short vertical bars indicate DNA size in kb. doi:10.1371/journal.pone.0036493.g001
isolated from E. coli DH5α recombinant subclones and sequenced with standard automated DNA sequencing methods using M13 forward and reverse [25] and custom-designed primers. Sequences were assembled using Sequencher 4.2 (Gene Codes Corp.). Nucleotide and amino acid sequences were analyzed with DNA STAR, BLAST [26], and the software available through the ExPASy Molecular Biology Server (http://www.expasy.ch).

Construction of an ATCC 19606T entA::aph isogenic derivative

To generate the ATCC 19606T entA::aph insertion mutant, PCR amplified with primers 3504 (5’-CCATCAAGAAGCCTGCTACCACTTTG-3’) and 3505 (5’-ATTCCTGTTCGGTACTGG-3’) (Fig. 1A) and Phusion DNA polymerase (NEB). The amplicon was cloned into the Smal site of the pEX100T and E. coli DH5α transformations were selected on LB agar containing 40 μg/ml kanamycin (Km) and 150 μg/ml ampicillin (Amp). Plasmid DNA (pMU902) was isolated from one of these derivatives and the appropriate cloning was confirmed by automated sequencing using primers 3187 (5’-GGATCCCCAACAAGAACGTCACTT-3’) and 3188 (5’-TTAGCTCACTCATTAACC-3’), which anneal close to the pEX100T Smal site. ATCC 19606T cells were electrorepared with pMU902 as described before [27] recovering the cells in SOC medium [22] for 6 h in a shaking incubator at 37°C. Transformants were selected on LB agar containing 40 μg/ml Km. The generation of the appropriate ATCC 19606T entA::aph derivative was confirmed by PCR using primers 3504 and 3580 (5’-CGATGTTCGGATACCACTTTG-3’) and 3188 (5’-TTAGCTCACTCATTAACC-3’), which anneal close to the pEX100T Smal site. The ATCC 19606T genomic region encompassing the predicted entA and mbt genes with primers 3606 (5’-GAAGTGAACGACGATTGCG-5’) and 3607 (5’-CGCAATGTTCTCATGTT-3’) was obtained with Phusion DNA polymerase using primers 3504 (5’-CCAACAAGAACGTCACTT-3’) and 3632 (5’-GGATCCCCAACAAGAACGTCACTT-3’), both of which included BamHI restriction sites.

Production and utilization of catechol and siderophore compounds and expression of EntA and EntB activity by cloned genes

A. baumannii cells were cultured in a chemically defined medium containing sodium succinate as a carbon source [19]. Production of extracellular compounds with siderophore activity was investigated with the Chroma Azurol S (CAS) reagent [28]. The presence of catechol compounds in cell-free succinate culture supernatants [19] was detected with the Arnow test [29]. Briefly, 1 vol of reagent A (0.5 N HCl), reagent B (10% Na nitrite, Na molybdate), and reagent C (1 N NaOH) were successively added to 1 vol of culture supernatant cleared by centrifugation at 16,000 × g. The reaction was measured by determining OD510 after 10 min incubation at room temperature. DHBa (Sigma-Aldrich) was used as a standard in chemical and biological assays. Production of DHBa was biologically examined with cross-feeding assays using the Salmonella typhimurium emb-1 enterobactin mutant as previously described [15]. Minimal inhibitory concentrations (MICs) of DIP, which were repeated at least three times in duplicate each time, were determined using M9 minimal medium [30] containing increasing concentrations of DIP. OD600 was used to monitor cell growth after overnight incubation at 37°C. Expression of EntA and EntB activity of cloned DNA was tested by transforming the E. coli AN193 and E. coli AN192 mutants with pMU923, which was obtained by PCR amplification and cloning of the ATCC 19606T genomic region encompassing the predicted entA and mbt genes with primers 3606 (5’-GAAGTGAACGACGATTGCG-5’) and 3607 (5’-CGCAATGTTCTCATGTT-3’) (Fig. 1A). The same set of primers were used to PCR clone the cognate chromosomal region from the ATCC clinical isolate to generate the derivative pMU960. Primers 3206 (5’-CGCAAGGACATCG-TAAAGG-5’) and 3211 (5’-TCTGGACAGCATACACCG-3’) were used to PCR amplify and clone the ATCC 19797 entA and mbt orthologs (pMU804) (Fig. 1B).

The genetic nature of the entB deficient phenotype of E. coli AN192 was confirmed by automated DNA sequencing of amplicons obtained using the primers 3783 (5’-CGGTGAA-CAGGGTATTGCG-3’) and 3785 (5’-CAGGTAACGATTGCGTGCAG-3’) and chromosomal DNA obtained form this mutant and the parental strain AB1515 [31] as templates. PCR sequencing reactions were done using primers 3783, 3785, 3784 (5’-CCITTTGAACGCGAAGTCG-3’), 3786 (5’-CGATATCACCATGACCTT), 3792 (5’-CAGATTGCGTTGATGACC-3’), and 3793 (5’-CTCCAGGCGGAGACGATTG-3’). Production of DHBa and acinetobactin was examined by HPLC analysis with an Agilent 1100 LC instrument using succinate culture supernatants filtered with 0.45 μm cellulose acetate filter units (Spin-x centrifuge filter units, Costar, Cambridge, MA). Supernatants were fractionated with a Vydac C-8, 5 μm, 250 mm×4.6 mm reversed-phase column (Grace Davison Discovery Sciences, Deerfield, IL). Water and acinetotilin containing 0.13% and 0.1% trifluoroacetic acid, respectively, were used as mobile phases. The gradient was as follows: 17% acinetotilin for 5 min and then from 17% to 30% within 30 min, and thereafter held for 15 min. Detection was at 317 nm with a flow rate of 0.5 ml/min.

A549 infection assays

A549 human alveolar epithelial cells [32], which were provided by Dr. E. Lafontaine (College of Veterinary Medicine, University of Georgia, USA) were cultured and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum at 37°C in the presence of 5% CO2 as previously described [33]. A549 monolayers maintained in modified Hank’s balanced salt solution (mHBSS, same as HBSS but without glucose) for 24 h at 37°C in 5% CO2 without infection remained viable as determined by trypan blue exclusion assays. 24-well tissue culture plates were seeded with approximately 10⁶ epithelial cells per well and then incubated for 16 h. Bacterial cells were grown 24 hr in LB broth at 37°C with shaking at 200 rpm, collected by centrifugation at 15,000 rpm for 10 min, washed, resuspended, and diluted in mHBSS. The A549 monolayers were singly infected with 10⁶ cells of the ATCC 19606T parental strain, the s1 (basD) or 3069 (entA) isogenic derivatives. Inocula were estimated spectrophotometrically at OD₆₃₀ and confirmed by plate count. Infected monolayers were incubated 24 h in mHBSS at 37°C in 5% CO2. The tissue culture supernatants were collected, the A549 monolayers were lysed with sterile distilled H₂O and lysates were added to the cognate tissue culture supernatants. Bacteria were collected from the resulting suspensions by centrifugation, resuspended in 1 ml sterile distilled H₂O, serially diluted and then plated on nutrient agar. After overnight incubation at 37°C, the colony forming units (CFUs) were counted and CFU/ml values for each sample were calculated and recorded. Counts were compared using the Student’s t-test; P values<0.05 were considered significant. Experiments were done four times in triplicate using fresh biological samples each time. To determine bacterial relative...
fitness, the recovered CFUs were divided by the CFUs of the inoculum used to infect monolayers.

**G. mellonella** killing assays

Bacteria grown in LB broth were collected by centrifugation and suspended in phosphate-buffered saline solution (PBS). The number of bacteria was estimated spectrophotometrically at OD_{600} and diluted in PBS to appropriate concentrations. All bacterial inocula were confirmed by plating serial dilutions on LB agar and determining colony counts after overnight incubation at 37°C. Ten freshly-received final-instar *G. mellonella* larvae (Grubco, Fairfield, OH) weighing 250–350 mg were randomly selected and used in killing assays as described previously [34]. Briefly, the hemocoele at the last left proleg was injected with 5–10^5 bacteria containing 1 x 10^5 bacteria ±0.25 log of each tested strain using a syringe pump (New Era Pump Systems, Inc., Wantagh, NY) with a 26 G 1/2 needle. Each test series included control groups of non-injected larvae or larvae injected with sterile PBS or PBS containing 100 μM FeCl₃. The test groups included larvae infected with the parental strain ATCC 19606T, the s1 basD mutant or the 3069 insertion derivative, which were injected in the absence or presence of 100 μM FeCl₃. Injected larvae were incubated at 37°C in darkness, assessing death at 24-h intervals over six days. Larvae were considered dead and removed if they did not respond to probing. Results were not considered if more than two deaths occurred in the control groups. Experiments were repeated three times using 10 larvae per experimental group and the survival curves were plotted using the Kaplan-Meier method [35]. P values<0.05 were considered statistically significant for the log-rank test of survival curves (SAS Institute Inc., Cary, NC).

**Results and Discussion**

The acinetobactin locus in different *A. baumannii* strains

Previous reports described a 26.5-kb *A. baumannii* ATCC 19606T chromosomal gene cluster involved in acinetobactin production and utilization [14,18]. Table 1 shows that the same *bas-bau-bar* 18-gene cluster, recently referred to as the acinetobactin gene cluster [17], is also found in *A. baumannii* AYE [36]. In contrast, the cognate clusters in strains AB0057 and ACICU include the additional putative genes AB57_2807 and AB57_2818 [37], and ACICU_02575 and ACICU_02586 [38], respectively. Furthermore, the *A. baumannii* AB307-294 *bas-bau-bar* gene cluster has three additional predicted genes; ABBFA_001054, ABBFA_001053, and ABBFA_001065 [37]. It is not clear whether these additional coding units are the result of sequencing and/or annotation artifacts and their potential role in siderophore production and utilization remains to be tested considering that most of them (AB57_2807, AB57_2818, ACICU_02575, ACICU_02586, ABBFA_001054 and ABBFA_001065) code for polypeptides containing 43 to 51 amino acid residues. Similarly, the annotation of the ATCC 17978 *bas-bau-bar* gene cluster [39] encompasses 21 rather than 18 predicted genes because of potential nucleotide sequencing errors (Table 1). The products of coding regions AIS_2382 and AIS_2383 are highly related to the BasD predicted protein, while the products of the AIS_2376-AIS_2378 coding regions highly match that of the *basA* gene originally described in the ATCC 19606T strain [18]. Unfortunately, these errors were also included in a recent report analyzing potential iron acquisition functions expressed by *A. baumannii* using bioinformatics [17].

In spite of all these differences, a common feature of the *bas-bau-bar* gene cluster present in all these strains is the absence of an *entA* gene coding for a 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase needed for the biosynthesis of DHBA, which is a precursor needed for the production of catechol siderophores, such as enterobactin [21]. This observation indicates that a second locus needed for the production of catechol siderophores, such as enterobactin, has been lost or is not present in these strains. Our initial finding that the genome of the *A. baumannii* ATCC 17978 strain has an additional gene cluster (Table 2) potentially involved in siderophore biosynthesis and utilization [40]. The coding region AIS_2579 of this cluster, which was identified as cluster 2 [17], was annotated as a putative *entA* ortholog [39]. However, initial BLAST searches using AIS_2579 as a query did not identify *entA* orthologs as top matches in any of the fully sequenced and annotated *A. baumannii* genomes and the ATCC 19606T partial genomic data (GenBank accession number NZ_ACQB00000001) deposited in GenBank by the Broad Institute as part of the Human Microbiome Project (http://www.broadinstitute.org/). Furthermore, attempts to identify the ATCC 19606T *entA* gene by PCR amplification or Southern blotting, using ATCC 17978 genomic information to design appropriate primers and probes, failed to produce positive results (data not shown). Taken together, all these observations indicate that there are variations in the nucleotide sequence and chromosomal arrangements among the *entA* genes present in different *A. baumannii* strains.

### Table 1. Components of the *bas-bau-bar* gene cluster in different *A. baumannii* genomes.

| Strain   | Annotated coding regions | Additional coding regions | Accession # |
|----------|--------------------------|---------------------------|-------------|
| 19606T   | BAC87896-BAC87913        | NA                        | AB101202    |
| AYE      | ABAYE1087-ABAYE1104      | None                      | NC_010410   |
| AB0057   | AB57_2802-AB57_2822      | 2807/2818                 | NC_011586.1 |
| ACICU    | ACICU_02570-ACICU_02589 | 02575/02586               | NC_010611.1 |
| AB307-294| ABBFA_001050-ABBFA_001070| 001054-001053/001065      | NC_011595.1 |
| 17978    | A15_2372-A15_2392        | 2382-2383/2376-2378*      | NC_009085.1 |

*Additional coding regions compared to the ATCC 19606* bas-bau-bar gene cluster. Numbers should be preceded by the cognate letters and underscore symbols.

*Not applicable.

**entA**-related coding sequences.

**barA**-related coding sequences.

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*Plasmid pLOR2372 was constructed by inserting a 2.3-kb fragment containing *entA* from *A. baumannii* strain AYE, which encodes a putative siderophore synthetase (accession number AB101202), into the *E. coli* plasmid pBAD24 and subsequently transforming it into *E. coli* AN193. The plasmid was isolated and sequenced. The *entA* gene of *A. baumannii* was amplified using primers designed from the sequences of the *entA* gene and cloned into the *E. coli* plasmid pBAD24. After transformation into *E. coli* AN193, the plasmid was isolated and sequenced. The *entA* gene was then transferred to *A. baumannii* strain AB307-294 by conjugation.

**Cloning and testing of the ATCC 19606T** *entA* gene

Since the in silico approach failed to locate the ATCC 19606T *entA* gene, a functional complementation approach was applied using *E. coli* AN193, an *entA* mutant that does not produce DHBA.**
enterobactin because of its inability to make the DHBA precursor. Transformation of this mutant with plasmid DNA isolated en masse from an ATCC 19606^T genomic library made in E. coli LE392, using the cloning cosmid vector pVK100, resulted in the isolation of the AN193-2631 derivative capable of growing in the presence of significantly higher DIP concentrations when compared with AN193 transformed with empty cloning vector. Transformation of E. coli AN193 with plasmid pMU711 isolated from the AN193-2631 derivative confirmed the ability of this cosmid clone to restore the iron uptake capacity of E. coli AN193 (data not shown). Restriction analysis of pMU711 digested with HindIII showed that it has an insert larger than 20 kb (data not shown). Subcloning into HindIII-digested pUC118 and nucleotide sequencing resulted in it having an insert larger than 20 kb (data not shown). Subcloning into

### Table 3. Components of the gene cluster containing the entA-entB genes in different A. baumannii genomes.

| Strain   | Annotated coding regions | Additional coding regions | Accession # |
|----------|--------------------------|---------------------------|-------------|
| 19606^T | 00624.1-00618.1^b         | NA^c                      | NZ_GG704572 |
| AB0057  | AB57_1980-AB57_1985       | None                      | NC_00586.1  |
| AB307-294 | ABBFA_001744-ABBFA_001739 | None                      | NC_00585.1  |
| ACICU   | ACICU_01787-ACICU_01792   | None                      | NC_00566.1  |
| AYE     | AABAY1887-AABAY1894       | AABAY1890                 | NC_00440    |

^aAdditional coding regions compared to the ATCC 19606^T gene cluster shown in Fig. 1A.
^bNumbers should be preceded by the annotation HMPREF0010_, according to the information presented in the A. baumannii Broad Institute genome site.
^cNot applicable.

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Figure 2. Iron acquisition phenotype of E. coli enterobactin deficient mutants. (A) Growth of the E. coli AN193 entA mutant harboring no plasmid or transformed with pMU925, pMU858, pMU968, pMU804 or pMU807 in LB broth in the absence or the presence of 250 μM DIP. (B) Growth of the E. coli AN192 entB mutant harboring no plasmid or transformed with pMU925 or pMU964 in LB broth in the absence or the presence of 250 μM DIP. The plasmids pMU925, pMU968 and pMU804 harbor the entA/entB orthologs cloned from ATCC 19606T, AYE, and ATCC 17978, respectively. Plasmid pMU858 harbors an insertionally inactivated ATCC 19606T entA derivative. Plasmid pMU964 harbors the ATCC 19606T basF gene.

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Figure 3. Iron acquisition phenotype of A. baumannii derivatives and their capacity to produce catechol, DHBA and acinetobactin. (A) The ATCC 19606T parental strain (19606), the s1 BasD acinetobactin synthesis mutant (s1) and the 3069 entA isogenic derivative harboring no plasmid (3069) or transformed with empty pWH1266 vector (3069.E) or pMU951 harboring (3069.C) were cultured in M9 minimal medium in the absence or the presence of increasing concentrations of DIP. pMU951 is a pWH1266 derivative harboring the ATCC 19606T entA allele. (B) Arrow’s reaction of succinate culture supernatants obtained from the strains shown in panel A. (C) Crossfeeding of the S. typhimurium entB-7 enterobactin deficient derivative by FeCl₃, distilled water or succinate culture supernatants from the ATCC 19606T parental strain (19606) or the 3069 entA isogenic derivative harboring either no plasmid (3069) or pMU951 (3069.C) under iron-chelated conditions. (D) HPLC profiles of sterile succinate medium (medium) or culture supernatants from the ATCC 19606T parental strain (19606) or the 3069 entA mutant (3069). Succinate culture supernatants cleared by high-speed centrifugation and filtration trough 0.45 μm cartridges were spiked with either DHBA (3069+DHBA) or purified acinetobactin (3069+Ab) immediately before HPLC analysis. The elution time for DHBA and acinetobactin are indicated on top of the cognate peaks.

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the ATCC 19606<sup>T</sup> 3069 mutant, they could be detected in the ATCC 19606<sup>T</sup> 3069 sample only when it was spiked with either pure DHBA or purified acinetobactin before HPLC analysis (Fig. 3D). These observations showed that the two peaks detected in ATCC 19606<sup>T</sup> culture supernatants indeed correspond to DHBA and mature acinetobactin. Interestingly, the chromatograms shown in Fig. 3D and Fig. 4 indicate that ATCC 19606<sup>T</sup> cells produce and secrete a significant amount of DHBA in addition to fully matured acinetobactin. A similar analysis of s1 succinate culture supernatants showed the presence of DHBA but not fully matured acinetobactin (data not shown), a result that is in accordance with the colorimetric data shown in Fig. 3B.

Finally, the role of the ATCC 19606<sup>T</sup> entA ortholog was confirmed with genetic complementation assays, which proved that electroporation of pMU951, a derivative of the shuttle vector pWH1266 harboring the entA wild-type allele expressed under the promoter controlling the expression of tetracycline resistance, was enough to restore the parental iron utilization phenotype in the ATCC 19606<sup>T</sup> 3069.C derivative (Fig. 3A) as well as its capacity to produce DHBA (Fig. 3, panels B and C). This complementation was not observed with the ATCC 19606<sup>T</sup> 3069.E derivative harboring the empty cloning vector pWH1266. Although not shown, there were no significant growth differences among the parental strain, the mutants and the complemented strains used in these experiments when they were cultured in LB broth without any selection pressure. Furthermore, the plasmid pMU951 was stably maintained in the ATCC 19606<sup>T</sup> 3069.C strain as an independent replicon without detectable rearrangements.

Taken together, all these results strongly indicate that the entA ortholog shown in Fig. 1A is essential for the production of DHBA, which is used by A. baumannii ATCC 19606<sup>T</sup> cells as a key precursor for acinetobactin biosynthesis. The location of the entA gene outside the main locus involved in the biosynthesis, transport and secretion of acinetobactin resembles the arrangement of genes needed for the biosynthesis of anguibactin in the fish pathogen V. anguillarum 775 [41]. The pJM1 plasmid present in this strain contains most of the genes coding for anguibactin biosynthesis and transport functions, including an angA ortholog that is inactive because of a frameshift mutation [42]. Further analysis showed that the 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase activity needed for anguibactin biosynthesis is indeed encoded by the chromosomal ortholog vabA, which is located within a 11-gene cluster that contains all the genetic determinants needed for the production and utilization of vanchrobactin [43]. Although this system is fully active in V. anguillarum RV22, an O2 serotype isolate that lacks pJM1, vanchrobactin is not produced in the serotype O1 V. anguillarum 775 (pJM1) strain because of an RS1 transposon insertion within vabF. Furthermore, in silico analysis of the predicted product of vabA and angA loci, after virtual correction of the frameshift present in the later gene, showed that they are only 32% identical [41]. These observations and the fact that the angA pJM1 plasmid copy is near transposable elements led to the hypothesis that transposition events resulted in the acquisition by horizontal transfer of two genes potentially coding for the same function that may have evolved independently [41]. A mechanism similar to this one could also explain the location of the ATCC 19606<sup>T</sup> entA gene outside the acinetobactin cluster [17], a possibility that is supported by the recent observation that A. baumannii has an underappreciated capacity to rearrange its genome by swapping, acquiring or deleting genes coding for a wide range of functions, including those involved in iron acquisition [44].

Our results also provide strong support to our previous report that acinetobactin is the only high-affinity siderophore produced by this strain [14], when cultured under iron-chelated laboratory conditions, although it contains genetic determinants that could code for additional iron acquisition functions as deduced from recent comparative genomic analyses [16,17]. Considering these reports and the experimental data presented here, it is possible to speculate that the additional iron-acquisition related genes present in A. baumannii ATCC 19606<sup>T</sup> are either not expressed or do not code for complete functional iron acquisition systems and represent remnants of DNA fragments acquired from other sources by horizontal gene transfer. Such outcomes, which are currently being explored, could be due to a situation similar to that reported for the fish pathogen V. anguillarum, where a vanchrobactin-producing ancestor acquired the pJM1 plasmid coding for the production and utilization of anguibactin [43]. Since anguibactin is potentially a better iron chelator than vanchrobactin, evolution and adaptation processes favored the emergence of V. anguillarum O1 serotype strains harboring the pJM1 plasmid and producing only anguibactin because of mutations in the vanchrobactin coding genes, but capable of using both anguibactin and vanchrobactin as it is the case with the 775 (pJM1) strain [45].
Analysis of the ATCC 19606T chromosomal region harboring entA and entB orthologs

Sequence analysis of flanking DNA regions showed that a modE-modK-modB-modC gene cluster, which could code for an uncharacterized molybdenum transport system, locates upstream of ATCC 19606T entB gene (Fig. 1A). Downstream, entC is separated by a 72-nt intergenic region from a predicted gene coding for an isochorismatase (EntB) ortholog, which is followed by a 524-nt intergenic region preceding a fur iron-dependent regulatory gene. The nucleotide sequence and genetic arrangement is the same as that reported for this strain by the Broad Institute Human Microbiome Project. Table 3 shows that the same gene cluster is also found in the genome of A. baumannii AB0057, AB307-294 and ACICU.

The predicted product of the ATCC 19606T entB gene depicted in Fig. 1A showed significant amino acid sequence similarity and the same length when compared with the products of the cognate AB0057 (AB57_1954), AB307-294 (ABBF0_001740), ACICU (ACICU_01791), and AYE (ABAYE1889) genes. The product of the ATCC 19606T entB ortholog is also related to that of basF, which is located within the acinetobactin gene cluster originally described in this strain [18]. The product of basF is a 299-amino acid protein that contains the N-terminal isochorismatase lyase (ICL) domain, which is needed for DHBA biosynthesis from isochorismic acid, and the C-terminal aryl carboxylate (ArCP) protein domain, which is needed for tethering activated DHBA and chain elongation in the biosynthesis of siderophores such as enterobactin [46] and anguibactin [47]. In contrast, in silico analysis showed that the entB ortholog shown in Fig. 1A codes for a 213-amino acid residue protein that includes the N-terminal ICL domain but lacks the C-terminal ArCP protein domain. Based on this predicted protein structure, it is possible to speculate that this shorter EntB ortholog should code for the isochorismatase lyase activity. This possibility was examined by testing the iron-uptake proficiency of the E. coli AN192 entB mutant harboring either no plasmid or the derivatives AN192-3171 and AN192-3170 transformed with pMU925, which harbors the entA and entB genes shown in Fig. 1A, or pMU964, which harbors a copy of the basF gene present in the acinetobactin cluster, respectively. Fig. 2B shows that AN192-3171 (pMU925) and AN192-3170 (pMU964) grew significantly better (P<0.0001 and P=0.0013, respectively) than the non-transformed AN192 strain when cultured in LB broth containing 250 μM DIP. This response indicates that both ATCC 19606T entB orthologs complement the E. coli AN193 entB mutant, allowing it to produce enterobactin and grow under iron-limited conditions. The complementation by the BasF ortholog is straightforward since this protein contains the ICL and ArCP protein domains needed for enterobactin biosynthesis. On the other hand, the complementation by the shorter ATCC 19606T EntB ortholog can be explained by the previous observation that the mutation in E. coli AN192 affects only the functionality of the ICL domain of the protein produced by this derivative that was obtained by chemical mutagenesis [31]. This functional observation is further supported by our DNA sequencing data showing that the AN192 entB ortholog has two point mutations when compared with the parental AB1515 allele. One is a silent mutation that resulted in a G-to-A base transition at position 801 of the gene, which maps within the ArCP protein domain and produced no change in amino acid sequence. In contrast, the other mutation, which locates at position 593 and is also a G-to-A base transition, produced a Gly-to-Asp amino acid change at position 198 that maps within the ICL domain. This amino acid change, which is one residue away from Arg196 that is predicted to play a role in the interaction of the ICL domains in the functional EntB dimer protein [48], could be responsible for the lack of isochorismatase lyase in the AN192 mutant. Taken together, these results indicate that basF, which locates within the acinetobactin gene cluster, is the only fully functional entB ortholog present in the genome of the A. baumannii strains fully sequenced and annotated.

Considering the different genetic context in which basF and the entB ortholog shown in Fig. 1A are found in different A. baumannii genomes and the differences in length (289 vs. 213 amino acid residues) and amino acid composition (52.4% similarity and 40.1% identity to one another) of their predicted products, it is possible to speculate that these two genes were transferred from unrelated sources by at least two independent horizontal mechanisms, one of which may have been driven by the need of acquiring the entB trait required for acinetobactin biosynthesis. These observations are in agreement with the recently described capacity of A. baumannii to rearrange its genome content [44], as proposed above for the presence of the entA ortholog in a genomic region outside the acinetobactin cluster.

Analysis of the A. baumannii AYE entA ortholog

The comparative genomic analysis of the ATCC 19606T entA ortholog with other A. baumannii sequenced genomes showed that the clinical isolate AYE also harbors the gene cluster shown in Fig. 1A, which was annotated as ABAYE1887-ABAYE1894 [36]. However, this cluster contains an additional 210-nucleotide gene (ABAYE1890) (Table 3), which was not included in any of the other fully sequenced A. baumannii genomes. ABAYE1890 codes for a hypothetical 69-amino acid protein and overlaps by 35 nucleotides with ABAYE1889, which codes for a predicted 229-amino acid protein. Both genes were recently recognized by comparative genomic analysis as components of the siderophore biosynthesis gene cluster 5 [17]. However, it is important to note that the predicted product of ABAYE1889 is 26 amino acids shorter than the putative ACICU, AB0057 and AB307-0294 EntA orthologs. This observation suggests that the A. baumannii AYE ABAYE1889 entA gene may not code for a functional product needed for the biosynthesis of DHBA and acinetobactin. This possibility was supported by the HPLC analysis of AYE succinate culture supernatants, which showed an elution profile that does not include peaks corresponding to DHBA and acinetobactin that were detected in the ATCC 19606T culture supernatant (Fig. 4). Interestingly, AYE succinate culture supernatants promoted the growth of S. typhimurium entB-7 in the presence of DIP in spite of the fact that it does not produce DHBA (data not shown). This observation indicates that A. baumannii AYE produces an uncharacterized non-catechol-based siderophore(s) capable of promoting growth under iron-limited conditions.

The failure of the ABAYE1889-ABAYE1890 genetic region to code for EntA activity was further confirmed by the fact that AN193-3179, a derivative transformed with pMU968 harboring the 2.8-kb AYE chromosomal region encompassing these predicted genes, grew as poorly as the non-complemented AN193 mutant when cultured in the presence of 250 μM DIP (Fig. 2A). All these results indicate that the AYE isolate is a natural A. baumannii entA mutant that does not make acinetobactin because of the lack of DHBA production. HPLC analysis of AYE culture supernatants of cells grown in succinate medium supplemented with 100 μM DHBA confirmed this possibility since a peak with a retention time corresponding to acinetobactin could be detected only under this experimental condition (Fig. 4).

Cloning, DNA sequencing and bioinformatic analysis of the 2.8-kb AYE chromosomal region encompassing the ABAYE1889 and ABAYE1890 genes confirmed the original genome sequence.
but can be used by ATCC 19606 T cells via acinetobactin-uncharacterized siderophore, which is different from acinetobactin supporting the ability of the ATCC 19606T strain to use pathogens.
xenosiderophores produced by related and unrelated bacterial vanchrobactin to acquire iron under chelated conditions [43]. but uses this siderophore as well as externally provided to that of mutant incapable of producing acinetobactin, although this isolate tests positive with the CAS reagent. This situation could be similar to that of V. anguillarum 775 (pJM1) that only produces anguibactin but uses this siderophore as well as externally provided vanchrobactin to acquire iron under chelated conditions [43]. This is due to the inactivation of the vskF vanchrobactin chromosomal gene and the possibility that anguibactin is an iron chelator stronger than vanchrobactin, a condition that does not justify the production of two siderophores. Accordingly, the production and utilization of the AYE uncharacterized siderophore, which may have a higher affinity for iron than acinetobactin, could be mediated by genes located in cluster 1 identified by in silico genomic analysis [17] that remain to be characterized genetically and functionally. Furthermore, preliminary siderophore utilization bioassays showed that AYE cell-free succinate culture supernatants crossfeed the ATCC 19606\(^2\) s1 (basD) and 3069 (entA) acinetobactin production deficient mutants as well as the t6 (bauF) acinetobactin uptake mutant. These findings indicate that A. baumannii AYE acquires iron via an uncharacterized siderophore, which is different from acinetobactin but can be used by ATCC 19606\(^3\) cells via acinetobactin-independent mechanisms. All these results provide evidence supporting the ability of the ATCC 19606\(^3\) strain to use xenosiderophores produced by related and unrelated bacterial pathogens.

**Analysis of the ATCC 17978 chromosomal region harboring the entA ortholog**

The ATCC 17978 A1S_2562-A1S_2581 cluster (Table 2), cluster 2 according to Eijkenkamp et al. [17], includes the A1S_2579 gene coding for a putative EntA ortholog [39]. The role of this gene in DHBA production was confirmed by the observation that the AN193-2943 transformant harboring pMU804 (Fig. 1B) displays an iron-restricted response similar to that detected with AN193-3172 harboring pMU925, a response that was not detected with AN193-2944 harboring pMU807 (Fig. 2A). The latter plasmid is a derivative of pMU804 with a transposon insertion within the annotated entA (A1S_2579) coding region (Fig. 1B).

Detailed analysis of the nucleotide sequence and annotation of the A1S_2562-A1S_2581 cluster (GenBank accession number NC_009085.1) showed that because of potential DNA sequencing errors, this cluster is most likely composed of 18 predicted coding regions rather than the 20 genes originally reported [39]. Fig. 1B and Table 2 show that A1S_2563 and A1S_2564 could be a single genetic unit coding for a predicted siderophore interacting protein that belongs to the ferredoxin reductase protein family. Our DNA sequencing data, which confirmed the DNA sequence originally reported [39], and in silico analysis showed that there are two potential coding regions between A1S_2568 and A1S_2570 (Fig. 1B and Table 1), with one of them annotated as the A1S_2569 coding region and the other omitted in the original report. Our analysis showed that the predicted products of these two genes are truncated transposases found in a wide range of bacterial genomes including members of the *Acinetobacter* genus. We also observed that A1S_2573 and A1S_2574 most likely correspond to a single genetic unit coding for a predicted 2,3-dihydroxybenzoate-AMP ligase (EntE) (Fig. 1B and Table 1), which is needed for the activation of DHBA and further biosynthesis of DHBA-containing siderophores such as enterobactin and anguibactin [10]. Nucleotide and amino acid comparative analyses also showed that the products of A1S_2573/A1S_2574, A1S_2580 and A1S_2581 are significantly related to that of the *basE*, *basF* and *baeF* genes, respectively. The products of these three genes, which work together with EntA, are needed for the biosynthesis and activation of DHBA using chorismate as a precursor. Taken together, all these observations indicate that there is a potential redundancy in the *A. baumannii* ATCC 17978 functions needed for the biosynthesis and utilization of DHBA as a siderophore precursor. In contrast, and as it was observed with the other *A. baumannii* genomes, the ATCC 17978 entA ortholog, which is represented by the coding region A1S_2579 (Fig. 1B and Table 1), is a unique coding region that locates outside the *bau-bau-bar* gene cluster. Furthermore, the genetic arrangement and content of this ATCC 17978 gene cluster containing the entA ortholog is different from that shown in Fig. 1A and described in the genome of other *A. baumannii* strains. This observation resulted in the classification of this cluster as cluster 2 [17], which at the time that report was published was found only in the ATCC 17978 genome. However, genomic data recently uploaded into the BaumannScope web site [https://www.genoscope.cns.fr/ugc/microscope/about/collabprojects.php?P_id=8] indicate that the same gene cluster is also present in the genome of the *A. baumannii* strains 6013113 (GenBank accession number ACYR02000000.2) and 6013150 (GenBank accession number ACYQ0000000.2).

Interestingly, the ATCC 17978 A1S_2562-A1S_2581 gene cluster has the same genetic content and organization as that of the *A. baylyi* ADP1 ACIAD2761-ACIAD2776 gene cluster (GenBank accession number NC_005966.1) with the exception of the presence of the transposase coding regions. A gene potentially coding for a transposase fragment is located outside the *A. baylyi* ADP1 ACIAD2761-ACIAD2776 gene cluster, downstream of ACIAD2761 [49]. In contrast, the ATCC 17978 cluster 2 includes two putative genes coding for transposase-related proteins located between the A1S_2568 and A1S_2570 annotated coding regions (Fig. 1B and Table 1), one of which was not included in the original report [39]. Furthermore, our previous analysis showed that cluster 2 includes perfect inverted repeats located at the ends of the cluster [40]. Taken together, these observations suggest that this particular cluster was mobilized by horizontal gene transfer among environmental and clinical *Acinetobacter* strains, which must acquire iron in different free-iron restricted ecological niches.

**Role of the ATCC 19606\(^{T}\) entA gene in virulence**

The role of the *entA* gene in the virulence of *A. baumannii* ATCC 19606\(^{T}\) was tested using A549 human alveolar epithelial cells and *G. mellonella* caterpillars as *ex vivo* and *in vivo* experimental models. The tissue culture assays showed that the number of ATCC 19606\(^{T}\) bacteria recovered after 24 h incubation was significantly greater than the s1 (*P* = 0.0048) and 3069 (*P* = 0.000045) derivatives (Fig. 5A), affected in acinetobactin biosynthesis at intermediate (*bauD*) and early (*entA*) biosynthetic stages, respectively. It was also noted that the persistence of 3069 was significantly lower than that of the s1 mutant (*P* = 0.0024) (Fig. 5A). It is important to mention that incubation of *A. baumannii* ATCC
controls.

s1 or 3069 iron-deficient isogenic derivatives in the absence (panel B) or the presence (panel C) of 100 μM 

Moths death was determined daily for six days. Caterpillars injected with comparable volumes of PBS or PBS plus 100 μM Fe₃Cl were used as negative controls.

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Figure 5. Virulence of A. baumannii ATCC 19606⁷ parental and isogenic derivatives. (A) Persistence of bacteria in the presence of A549 monolayers. Bacteria (10⁷) were added to 95% confluent monolayer maintained in mHBSS. The resulting CFUs were quantified after 24 h incubation at 37°C in 5% CO₂. (B and C) G. mellonella killing assays. Caterpillars were infected with 1×10⁶ bacteria of the ATCC 19606⁷ parental strain (19606), or the s1 or 3069 iron-deficient isogenic derivatives in the absence (panel B) or the presence (panel C) of 100 μM Fe₃Cl and incubated at 37°C in darkness. Moth death was determined daily for six days. Caterpillars injected with comparable volumes of PBS or PBS plus 100 μM Fe₃Cl were used as negative controls.

19606⁷ in mHBSS at 37°C for 24 h did not result in significant bacterial growth.

Infection of G. mellonella larvae showed that more than 40% of them died six days after injected with the parental ATCC 19606⁷ strain, a value that is significantly different (P=0.0014) from that obtained with animals injected with sterile PBS (Fig. 5B). Infection of caterpillars with the 3069 entA mutant showed that the killing rate of this derivative is not statistically different from that of the PBS negative control (P=0.6705) but significantly different from the killing rate of the parental strain (P=0.0005). On the other hand, the s1 derivative is significantly more virulent than the 3069 mutant (P=0.043) and almost significantly different from the parental strain (P=0.0820). The killing rates of the s1 and 3069 mutants were corrected to values statistically indistinguishable from the parental strain when the inocula used to infect the larvae were supplemented with 100 μM Fe₃Cl (Fig. 5C). This response shows that the virulence defect of the s1 and 3069 isogenic mutants is due to their inability to acquire iron when injected into G. mellonella.

Taken together, these results demonstrate that inactivation of entA produces a more drastic reduction in the capacity of A. baumannii ATCC 19606⁷ cells to persist in the presence of A549 cells, which represent a target affected during the respiratory infections this pathogen causes in humans, or infect and kill G. mellonella larvae, an invertebrate host capable of mounting a complex innate immune response similar to that of vertebrate animals [50], when compared to the response obtained with the basD mutant. This response could be due to the fact that the 3069 derivative does not produce DHBA and acinetobactin intermediates, which are secreted by the s1 mutant and could have some functions in iron acquisition and virulence as it was shown with Brucella abortus [51,52]. Thus, these acinetobactin precursors together with DHBA could bind iron, although less efficiently than acinetobactin, and provide s1 cells with enough iron to persist and cause host injury, although to a much reduced extent when compared with the response obtained with ATCC 19606⁷ cells.

Conclusions

The work described in this report shows that a single A. baumannii entA functional ortholog, which is essential for the biosynthesis of the acinetobactin precursor DHBA, is located outside the acinetobactin gene cluster, which otherwise codes for all biosynthesis, secretion and transport functions related to iron acquisition mediated by this high-affinity siderophore. Although the same genetic arrangement is found in all fully sequenced and annotated A. baumannii genomes, the genetic context within which the entA ortholog is found varies among different clinical isolates. In one group of strains this gene is next to genes coding for putative molybdenum transport, as in ATCC 19606⁷. In another group, entA is located within a large gene cluster, which could code for an alternative uncharacterized siderophore-mediated system, as in ATCC 17978. Interestingly, this cluster also includes genes coding for potential DNA mobility functions and is flanked by perfect inverted repeats, a feature that may explain its transfer by lateral processes. Nevertheless, in all cases examined the entA ortholog is always next to an entB ortholog, which codes for a protein that is able to catalyze the production of DHBA from isochorismic acid but cannot promote the completion of the acinetobactin biosynthesis process because of the lack of the C-terminal aryl carrier (ArCP) protein domain. This is in contrast to the presence of basF within the acinetobactin cluster that codes for a fully functional EntB ortholog. All these findings indicate that all genetic components needed for iron acquisition via the acinetobactin-mediated system involved horizontal transfer as well as complex chromosomal rearrangement processes. All these observations are in agreement with the recent observation that A. baumannii has the capacity to acquire, lose and/or shuffle genes, a number of which could code for virulence factors involved in the pathogenesis of the infections this bacterium causes in humans [44]. Our study also shows that the presence of all genetic determinants needed for the biosynthesis and utilization of acinetobactin does not warrant its active expression and may not reflect a virulence advantage when compared to other strains; the AYE clinical isolate is a natural entA mutant incapable of producing DHBA and acinetobactin. However, AYE is an iron-uptake proficient strain that seems to acquire this essential metal due to the expression of a siderophore-mediated system that remains to be functionally characterized. This finding reinforces our initial observation that different A. baumannii clinical isolates can express different iron acquisition systems [12]. Finally, we show that acinetobactin intermediates and DHBA, which are produced in addition to acinetobactin in the ATCC 19606⁷ strain, play a role in the virulence of A. baumannii when tested using "ex vivo..."
and in vivo infection experimental models, although to a lesser extent when compared to the role of the fully matured acinetobactin-mediated system.

Supporting Information

Table S1  Bacterial strains and plasmids used in this work. (DOC)

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Author Contributions

Conceived and designed the experiments: WFP BAA LAA. Performed the experiments: WFP BAA. Analyzed the data: WFP BAA LAA. Contributed reagents/materials/analysis tools: WFP BAA LAA. Wrote the paper: WFP BAA LAA.

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