Identification and characterization of a cluster of genes involved in biosynthesis and transport of acinetoferrin, a siderophore produced by *Acinetobacter haemolyticus* ATCC 17906T

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*Acinetobacter haemolyticus* ATCC 17906T is known to produce the siderophore acinetoferrin under iron-limiting conditions. Here, we show that an operon consisting of eight consecutive genes, named *acbABCD* and *actBCAD*, participates in the biosynthesis and transport of acinetoferrin, respectively. Transcription of the operon was found to be iron-regulated by a putative Fur box located in the promoter region of the first gene, *acbA*. Homology searches suggest that *acbABCD* and *actA* encode enzyme proteins involved in acinetoferrin biosynthesis and an outer-membrane receptor for ferric acinetoferrin, respectively. Mutants defective in *acbA* and *actA* were unable to produce acinetoferrin or to express the ferric acinetoferrin receptor under iron-limiting conditions. These abilities were rescued by complementation of the mutants with native *acbA* and *actA* genes. Secondary structure analysis predicted that the products of *actC* and *actD* may be inner-membrane proteins with 12 membrane-spanning helices that belong to the major facilitator superfamily proteins. *ActC* showed homology to *Sinorhizobium meliloti* RhtX, which has been characterized as an inner-membrane importer for ferric rhizobactin 1021 structurally similar to acinetoferrin. Compared to the parental ATCC 17906T strain, the *actD* mutant displayed about a 35% reduction in secretion of acinetoferrin, which was restored by complementation with *actD*, suggesting that *ActD* acts as an exporter of the siderophore. Finally, the *actB* product was significantly similar to hypothetical proteins in certain bacteria, in which genes encoding ActBCA homologues are arranged in the same order as in *A. haemolyticus* ATCC 17906T. However, the function of *ActB* remains to be clarified.

INTRODUCTION

Iron is a requisite nutrient for the growth and proliferation of the vast majority of microbes. However, iron bioavailability is often limited due to the insolubility of iron in aerobic environments at neutral-to-alkaline pH or to sequestration of iron by high-affinity iron-binding proteins such as lactoferrin and transferrin within the host to avoid microbial infections through non-specific mechanisms (Ratledge & Dover, 2000; Bullen et al., 2005; Miethke & Marahiel, 2007). Thus, to overcome this iron restriction in the competitive local environment, many microbes have evolved diverse strategies, one of the most prominent being the biosynthesis of low-molecular-mass chelators, called siderophores (Neilands, 1981; Hider & Kong, 2010). They are secreted into the local extracellular milieu, where they bind ferric iron with high specificity. In Gram-negative bacteria, iron-loaded siderophores (ferric siderophores) are transported back into the cell cytoplasm across the outer and inner membranes by iron-repressible outer-membrane protein (IROMP) receptors and ATP-binding cassette

**Abbreviations:** DAP, 1,3-Diaminopropane; DPD, 2,2′-dipyridyl; Fur, ferric uptake regulator; FURTA, Fur titration assay; IROMP, iron-repressible outer-membrane protein; MFS, major facilitator superfamily; OMP, outer-membrane protein.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AB661448.

A supplementary figure and a supplementary table are available with the online version of this paper.
transport systems or major facilitator superfamily (MFS) transporters, respectively. (Braun et al., 1998; Andrews et al., 2003; Cuiv et al., 2004). The receptors display relatively tight specificity for their cognate siderophores. Translocation of a ferric siderophore through the receptor into the periplasmic space is dependent on the energy-transducing TonB system, comprising two cytoplasmic membrane proteins, ExbB and ExbD, in addition to TonB, which traverses the periplasmic space (Letain & Postle, 1997). The receptors commonly possess a conserved sequence, the TonB box, which interacts with TonB to gain the energy necessary to transport the ferric siderophore into the periplasm. Most siderophores can be classified into three major groups, i.e. catecholate, hydroxamate and α-hydroxycarboxylate (Hider & Kong, 2010). The ability to synthesize and utilize siderophores has been shown to be one of the virulence determinants in numerous bacterial pathogens, since the siderophores can facilitate the acquisition of iron from iron-withholding proteins, such as transferrin and lactoferrin in animal hosts (Schaible & Kaufmann, 2004; Bullen et al., 2005; Weinberg, 2009). On the other hand, high intracellular concentrations of iron may damage bacteria through the formation of undesired reactive oxygen species. In Gram-negative bacteria, maintenance of iron homeostasis is mainly regulated by the ferric uptake regulator (Fur), which functions as a transcriptional regulator (Bagg & Neilands, 1987; Escolar et al., 1999). When the intracellular iron concentration is increased, Fur dimersizes with Fe$^{2+}$ as a cofactor and binds to a 19 bp consensus sequence, termed the Fur box (de Lorenzo et al., 1987; Calderwood & Mekalanos, 1988), present in the promoters of genes generally involved in iron acquisition

Acinetobacter haemolyticus has emerged as an environmental bacterium and an opportunistic, multidrug-resistant, intrahospital human pathogen, which causes septicaemia, pneumonia, meningitis, skin and wound infections, and urinary tract infection (Bergogne-Bérézin & Towner, 1996). Recently, a comparative study on the virulence potential of Acinetobacter species has suggested that A. haemolyticus and Acinetobacter baumannii are likely to be the most hazardous species with regard to growth ability in the mammalian environment, toxicity and intracellular infectivity (Tayabali et al., 2012).

We have previously reported that, when grown under conditions of iron depletion, A. haemolyticus ATCC 17906$^T$ produces a citrate-based dihydroxamate siderophore, acinetoferrin (Fig. 1), which is characterized by 1,3-diaminopropane (DAP) moieties and monounsaturated acyl appendages (Okujo et al., 1994). In this regard, acinetoferrin is very similar in chemical structure to rhizobactin 1021 (Fig. 1) produced by Sinorhizobium meliloti (Persmark et al., 1993), in which the relevant biosynthesis gene cluster has been identified and characterized (Lynch et al., 2001). This study was undertaken to obtain insight into the genetic basis of the acinetoferrin-mediated iron uptake system of A. haemolyticus ATCC 17906$^T$. The Fur titration assay (FURTA) (Stojilkovic et al. 1994) was used to identify a cluster of Fur-regulated genes involved in acinetoferrin biosynthesis and transport. We describe the identification and characterization of an operon consisting of eight ORFs, whose functions were elucidated by homology search and mutation-complementation analysis.

**METHODS**

**Bacterial strains, plasmids, oligonucleotides and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Sequences of all oligonucleotides used are presented in Table S1. A. haemolyticus ATCC 17906$^T$ and its mutants and Escherichia coli were routinely grown with shaking at 30 and 37 °C, respectively, in Luria–Bertani (LB) medium or on LB agar plates (1.5 % agar) containing 0.5 % NaCl. To impose iron limitation, the LB medium was supplemented with the iron chelator 2,2′-dipyridyl (DPP) at 150 μM. Media with and without DPP were named +Fe medium and +Fe medium, respectively. As necessary, appropriate antibiotics were added to the growth media at the following concentrations: ampicillin (50 μg ml$^{-1}$), apramycin (50 μg ml$^{-1}$) and tetracycline (10 μg ml$^{-1}$).

**FURTA.** The FURTA was performed as described elsewhere (Stojilkovic et al. 1994). A. haemolyticus ATCC 17906$^T$ chromosomal DNA fragments (0.5–1 kb) partially digested with Sau3AI were ligated into the BamHI site of pBluescript II KS(+) and the resulting plasmids were transformed into E. coli H1717. Transformants were incubated at 37 °C for 15–24 h on MacConkey lactose agar plates (Difco) containing 0.1 mM FeCl$_3$ and ampicillin (50 μg ml$^{-1}$). Red colonies (Lac $^+$) denote a FURTA-positive phenotype and indicate binding of the Fur–Fe$^{2+}$ complex to the promoter region transformed in the indicator strain. Plasmid DNA was isolated, and nucleotide sequences of the inserts were determined.

**Growth assays.** A. haemolyticus ATCC 17906$^T$ and its mutant strains were grown overnight in LB medium, and aliquots of the preculture were inoculated into 5 ml fresh LB medium at OD$_{600}$ 0.005 and

![Fig. 1. Chemical structures of acinetoferrin (a) and rhizobactin 1021 (b).](http://mic.sgmjournals.org)
shaken at 70 r.p.m. The OD_{600} was measured with an Advantec TV5062CA biophotorecorder (Advantec Toyo).

**DNA manipulation, nucleotide sequencing, and protein sequence analysis.** Standard DNA manipulations were carried out as described by Sambrook et al. (1989). Chromosomal DNA and plasmid DNA were extracted using a Wizard Genomic DNA Purification kit (Promega) and a High Pure Plasmid Isolation kit (Roche Diagnostics, respectively). Restriction enzymes were purchased from Roche Diagnostics. A Ligation-Convenience kit (Wako (Roche Diagnostics), respectively. Restriction enzymes were purified from agarose gels using a MagExtractor-PCR & Gel Clean up DNA fragment purification kit (Promega) and a High Pure Plasmid Isolation kit (Roche Diagnostics). DNA fragments required minimal errors. Oligonucleotide primers (Table S1) were designed according to the determined sequence of A. haemolyticus ATCC 17906. Protein homology searches and alignments were carried out using the BLAST program on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1997). Transmembrane helices in ActC and ActD were predicted using the HMMTOP 2.0 server (http://www.enzim.hu/hmmtop/html/adv_submit.html) (Tusnády & Simon, 2001).

**RNA isolation and RT-PCR.** A. haemolyticus ATCC 17906 was grown in LB broth to an OD_{600} of 0.3. The culture was split into two aliquots; one was left untreated to prepare +Fe cells, and the other was supplemented with DPT at 150 μM to prepare –Fe cells. Both aliquots were further incubated until an OD_{600} of 0.5 was reached. Total RNA was extracted from each cell pellet using an RNase-free DNase mini kit (Qiagen) and then treated, according to the manufacturer’s instructions, with RNase-free DNase I (Ambion) to exclude possible contamination with traces of chromosomal DNA. RT-PCR was carried out with a ReverTra Dash RNA PCR kit (Toyobo), according to the manufacturer’s directions. For first-strand cDNA synthesis, 1 μg pretreated total RNA was incubated in a total volume of 20 μL at 42°C for 1 h with the primer AIRT-R complementary to the internal sequence of actD. Subsequent PCR was performed with 1 μL of reverse transcriptase reaction mixture using the primer pairs designed for each gene to be tested. PCR conditions were as follows: after an initial denaturation of 2 min at 94°C, DNA was amplified for 30 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. Total RNA without treatment with the M-MLV reverse transcriptase was used as a negative control reaction for PCR to confirm the lack of genomic DNA contamination. 16S rRNA was used as an established endogenous internal control. The

<table>
<thead>
<tr>
<th>Table 1. Bacterial strains and plasmids used in this study</th>
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<td><strong>A. haemolyticus strains</strong></td>
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<tr>
<td>ATCC 17906&lt;sup&gt;T&lt;/sup&gt;</td>
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<tr>
<td>TF-achA</td>
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<tr>
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<td>TF-actB</td>
</tr>
<tr>
<td>TF-actC</td>
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<td>TF-actD</td>
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<tr>
<td><strong>E. coli strains</strong></td>
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<tr>
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<tr>
<td>H1717</td>
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<tr>
<td><strong>Plasmids</strong></td>
</tr>
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</tr>
<tr>
<td>pRK415</td>
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<tr>
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</tr>
<tr>
<td>pBC-actf1</td>
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<tr>
<td>pRK415-actC</td>
</tr>
<tr>
<td>pRK415-actD</td>
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primers 16S-F and 16S-R were designed according to the nucleotide sequence of *A. haemolyticus* ATCC 17906T 16S rRNA (accession no. Z93437). RT-PCR products were detected in a 1.5% agarose gel stained with ethidium bromide and visualized in a Gel Doc XR (Bio-Rad).

**Outer-membrane protein (OMP) analysis.** Cells of ATCC 17906T and mutant strains grown for 12 h in +Fe and −Fe media were harvested by centrifugation. The OMP-rich fractions were prepared and analysed by SDS-PAGE as previously described (Yamamoto et al., 1995a). Protein concentrations were determined by the method of Markwell et al. (1978). The developed gel was stained with Coomassie brilliant blue R-250 followed by visualizing with a Gel Doc XR system (Bio-Rad). The IROMPs were electroblotted onto a pre-wetted PVDF membrane (ProBlott; Applied Biosystems) with a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad) for determination of their N-terminal amino acid sequences by automated Edman degradation method with a Procise model 491 protein sequencer (Applied Biosystems).

**Construction of deletion/insertion mutant and complementing strains.** Mutant strains of *A. haemolyticus* ATCC 17906T, i.e. TF-acbA, TF-actA, TF-actB, TF-actC and TF-actD, were constructed by gene replacement as described by Aranda et al. (2010), and each mutant carried a partially deleted gene with a non-polar insertion cassette conferring apramycin resistance (*aacC4*). Briefly, the upstream and downstream regions (approx. 0.5 kb each) of the target gene were amplified from genomic DNA of the ATCC 17906T strain using primer pairs (AF and AR, and BF and BR; Table S1) for construction of amplicons A and B, respectively, of each target gene. On the other hand, the apramycin cassette was amplified using primer pairs appropriately designed to link amplicons A and B to the 5’ and 3’ sites of the *aacC4* cassette derived from vector pJG1011 (Gomez & Bishai, 2000) as a template. The AR and BF primers contained an extension of about 20 nt complementary to the acc-F and acc-R primers, respectively. The three PCR products purified were mixed and subjected to PCR-driven overlap extension ( Heckman & Pease, 2007) with the AF and BR primers and a KOD-Plus-DNA polymerase (Toyobo). The purified PCR product containing the target gene inactivated by the *aacC4* cassette was introduced into the ATCC 17906T strain by electroporation according to the procedure of Leathy et al. (1994). Recombinant mutants, whose wild-type genes were replaced by allelic exchange via double-crossover recombination, were selected on LB agar plates containing apramycin. Candidate clones were examined by PCR with the AF and BR primers to verify gene replacement (data not shown). The resulting mutants were named TF-acbA, -actA, -actB, -actC and -actD (Table 1), and grown in the presence of apramycin. To provide the five above-mentioned mutated genes with *in trans* complementation, the plasmids pRK415-acbA, pRK415-actA, pRK415-actB, pRK415-actC and pRK415-actD were constructed by PCR using *A. haemolyticus* ATCC 17906T genomic DNA with the comp-F and comp-R primer sets (Table S1), all of which contained the corresponding entire genes. These plasmids were introduced into the respective mutant strains by electroporation (Leathy et al., 1994). Empty strains containing only pRK415 were also constructed. Mutants containing pRK415 were grown in the presence

<table>
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<th>ORF (aa)</th>
<th>Homologous protein (aa)</th>
<th>Micro-organism</th>
<th>Identity/similarity (%) (aa overlap)</th>
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<td>acbB (493)</td>
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of tetracycline and apramycin. All final constructs were sequenced to confirm that the sequences were correct.

**HPLC analysis of acinetoferrin.** Production of acinetoferrin was examined by HPLC with a Hitachi LaChrom Elite HPLC system equipped with an Inertsil C8-3 reversed-phase column (150 × 4.6 mm, 5 μm; GL Sciences). ATCC 17906<sup>T</sup> and its mutant strains were grown to OD<sub>600</sub> 0.4 in 50 ml of a chemically defined Tris-buffered succinate medium (pH 7.4) containing 0.1 μM FeCl<sub>3</sub> (Okujo et al., 1994), and culture supernatants filtered with 0.22 μm pore-size cellulose acetate filters were adjusted to pH 2.0 with solid citric acid. Acinetoferrin in the culture supernatants (50 ml) was adsorbed on an Amberlite XAD-7 column (120 × 10 mm ID, 20–60 mesh; Sigma-Aldrich), washed with 50 ml distilled water, and desorbed with 50 ml methanol. The methanol eluate was evaporated to dryness. The residue was resuspended in 1 ml methanol and a 10 μl aliquot was injected into the HPLC system for analysis. Forty-five per cent acetonitrile in 0.1 % trifluoroacetic acid and 54 % acetonitrile in 0.1 % trifluoroacetic acid were used as mobile phases. The gradient was as follows: 45 % acetonitrile for 5 min and then from 45 % to 54 % acetonitrile within 30 min, and held at 54 % acetonitrile for 10 min. Detection was at 220 nm with a flow rate of 0.5 ml min<sup>−1</sup>.

**RESULTS**

**Isolation of the DNA region encoding acinetoferrin biosynthesis and transport genes**

The FURTA system (Stojiljkovic et al., 1994) was successfully used to isolate Fur box-containing gene fragments from the <i>A. haemolyticus</i> ATCC 17906<sup>T</sup> chromosome. Out of the positive clones, one clone named pFURTA-Af (Table 1, Fig. S1) was found to contain a fragment, part of which (915 bp) was predicted to encode a product that was 73 % identical to...

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**Fig. 2.** Nucleotide sequences in the intergenic regions of the acinetoferrin biosynthesis and transport genes in <i>A. haemolyticus</i> ATCC 17906<sup>T</sup> and the partial deduced amino acid sequence of ActA. Acinetoferrin cluster genes were also identified as belonging to an iron-regulated operon by RT-PCR. (a) The putative –10 and –35 promoter elements, start codons (in bold type) and stop codons are presented. The putative Fur box sequence is underlined with nucleotide matches with the 19 bp <i>E. coli</i> consensus Fur box sequence. The terminator signal is indicated by opposing arrows, and the amino acid sequence deduced from actA, which is compatible with the N-terminal sequence determined for the ActA protein, is indicated by double underlines. Numbers correspond to sequence positions in GenBank accession number AB661448. (b) Lanes contain RT-PCR amplicons amplified from total RNA isolated from –Fe and +Fe cells of <i>A. haemolyticus</i> ATCC 17906<sup>T</sup>. The sizes of the amplicons derived from the representative genes are as follows: acbA, 334 bp; actA, 368 bp; and actD, 300 bp. As an internal control, a 225 bp fragment of the 16S rRNA gene was included. Lanes +RT and –RT represent RT-PCR products with and without reverse transcriptase, respectively; lane M, molecular size standard.
A1S_1647, one of the biosynthetic enzymes for a predicted hydroxamate siderophore produced by *A. baumannii* strains (Antunes et al., 2011; Eijkelkamp et al., 2011), and was 27% identical to RhbC, one of the biosynthetic enzymes for rhizobactin 1021 produced by *S. meliloti* (Lynch et al., 2001). As expected, the potential Fur box, which matched 15 of 19 nt of the consensus Fur box (de Lorenzo et al., 1987; Calderwood & Mekalanos, 1988), was detected in the promoter region. Then, based on these findings, the DNA sequence of the 13.1 kb region was determined, identifying eight ORFs (Fig. S1a). Cloning and sequencing of this region were performed using four plasmids (pBC-acf1, pBC-acf2, pRK-acf3 and pRK-acf4) with overlapping DNA fragments (Fig. S1b) which had been isolated by colony hybridization with DIG-labelled probes (Fig. S1c).

**Predicted protein sequences**

The deduced amino acid sequences of eight ORFs shared significant homology to known or predicted siderophore biosynthetic enzymes and transporters in other bacteria (Table 2). The presence of ORFs that encode proteins homologous to putative ω-amino acid monooxygenases, acetyltransferases and TonB-dependent siderophore receptors implied a cluster associated with biosynthesis and transport of the hydroxamate siderophore, acinetoferrin. Protein products of two genes, *orf9* and *orf10*, not linked to the preceding gene, were homologous to a putative IS4 family transposase ORF1 and hypothetical protein, respectively, in *A. baumannii* strains, suggesting that they were not involved in acinetoferrin-mediated iron acquisition. On the basis of potential functions inferred from homology, the biosynthetic and transport genes were named *acbABCD* (*acb* stands for acinetoferrin biosynthesis) and *actBCAD* (*act* stands for acinetoferrin transport; Fig. 2a). The predicted amino acid sequences of AcbABCD shared 34–41% identity with those of the *S. meliloti* rhizobactin 1021 RhbCDEF (Lynch et al., 2001), in keeping with the structural similarities between the siderophores produced by these species (Fig. 1). However, the

![Fig. 3. HPLC profiles of acinetoferrin secreted by the wild-type *A. haemolyticus* ATCC 17906T strain and deletion mutants into culture supernatants. The growth and HPLC conditions are described in Methods. A 10 μl aliquot of the acinetoferrin extract (1 ml) was injected into the HPLC instrument. The peak with a retention time of 19.5 min was collected and identified as acinetoferrin by MS analysis.](http://mic.sgmjournals.org)
gene order in *A. haemolyticus* was not the same as that in *S. meliloti*. Moreover, unlike in *S. meliloti*, the *A. haemolyticus* acinetoferrin cluster did not contain genes involved in DAP biosynthesis. Interestingly, *A. haemolyticus* AcbABD proteins (excluding AcbC) were highly similar (49–77% identity, 63–87% similarity) to the corresponding enzyme proteins in eight fully sequenced *A. baumannii* genomes, e.g. A1S_1647, _1648, and _1657 in *A. baumannii* ATCC 17978 (Eijkelkamp *et al.*, 2011), whereas the arrangement of these genes in *A. baumannii* strains was quite different from that in the ATCC 17906T strain. An AcbC homologue was not found in any of the whole-genome-sequenced *A. baumannii* strains.

The product of *actA* showed a significant similarity with various TonB-dependent outer-membrane receptors for ferric siderophores, including RhtA for ferric rhizobactin 1021 in *S. meliloti* (Lynch *et al.*, 2001). The amino acid sequence in the N-terminal region deduced from *actA* was identical to the first 10 N-terminal amino acids determined for the 79.3 kDa IROMP (see below). The product of *actB* showed homology to hypothetical proteins whose functions have not been ascertained for any species. The product of *actC* showed 61% identity to bacterial MFS proteins (Table 2). It was noteworthy that ActC also showed significant homology to the *S. meliloti* RhtX, an inner-membrane importer for ferric rhizobactin 1021 subsequent to outer-membrane translocation (Cuı´v *et al.*, 2004). HMMTOP analysis (Tusnády & Simon, 2001) placed ActC within the group of inner-membrane proteins with 12 transmembrane domains belonging to the MFS. ActD, the last gene product, showed homology to MFS efflux permeases and was divided into an inner-membrane protein with 12 transmembrane domains by HMMTOP analysis. Its subcellular location and similarity to MFS proteins in eight fully sequenced *A. baumannii* genomes, e.g. A1S_1647, _1648, and _1657 in *A. baumannii* ATCC 17978 (Eijkelkamp *et al.*, 2011), whereas the arrangement of these genes in *A. baumannii* strains was quite different from that in the ATCC 17906T strain. An AcbC homologue was not found in any of the whole-genome-sequenced *A. baumannii* strains.

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proteins suggested that ActD could be involved in acinetoferrin secretion. Finally, although there were no proven functions for actB homologues in the three bacterial species cited, we note that all of them were located upstream of putative MFS importer genes followed by putative ferric siderophore outer-membrane receptor genes in the same order as actB in the ATCC 17906\textsuperscript{T} strain.

The \textit{acbABCD} and \textit{actBCAD} genes form an iron-regulated operon

The eight genes were located consecutively and oriented in the same direction with a single set of putative promoter elements in front of \textit{acbA} and with a single rho-independent transcription terminator sequence just downstream of the stop codon of \textit{actD}, suggesting that the eight genes are transcriptionally linked (Fig. 2a). This transcriptional organization was also suggested by the overlaps that occurred at the gene junctions as well as the presence of relatively short intervening sequences without any promoter elements. Then, in order to confirm that this gene cluster was organized as an iron-regulated operon, RT-PCR analysis was performed for \textit{acbA}, \textit{actA} and \textit{actD} with three different primer combinations (Table S1) that amplified internal fragments of the corresponding genes. The primer (AFRT-R) complementary to the internal sequence of the last gene, \textit{actD}, was used for the first-strand cDNA synthesis. Each of the reactions yielded an amplicon of the expected size without interference from DNA contamination, only when total RNA from cells grown under iron-limiting conditions was used (Fig. 2b). In addition, the level of 16S rRNA expression as a control was not affected by the iron conditions in the medium. In particular, the results of PCR for \textit{acbA} and \textit{actD}, the first and last genes within the eight-gene cluster, demonstrated that these genes were co-transcribed from the promoter upstream of \textit{acbA} as a single polycistronic message under iron-limiting conditions. The presence of a potential Fur box in the promoter region of \textit{acbA} implied that Fur was responsible for this iron-regulated transcription.

Phenotypic analysis of the \textit{acbA} mutant

In order to characterize the acinetoferrin biosynthesis cluster, the first gene, \textit{acbA}, was mutated using gene replacement with insertion (Aranda \textit{et al.}, 2010). No discernible growth defect was observed for the \textit{acbA} mutant strain when grown in +Fe medium (data not shown). As expected, \textit{A. haemolyticus} ATCC 17906\textsuperscript{T} produced acinetoferrin when grown in the –Fe medium alone, but the TF-\textit{acbA} mutant defective in \textit{acbA} did not produce acinetoferrin under the same conditions (Fig. 3). In contrast, the complementing strain, TF-\textit{acbA}/pRK415-\textit{acbA}, produced acinetoferrin at a level similar to that of the wild-type parental strain, indicating that the \textit{acbA} gene was one of the determinants essential for acinetoferrin biosynthesis. Nevertheless, the \textit{acbA} mutant could grow to a modest extent with a reduced growth rate in the –Fe medium but could not reach the same final OD\textsubscript{600} as the wild-type strain (Fig. 4a). The culture supernatant of the \textit{acbA} mutant exhibited a catecholate siderophore titre similar to that of the parental strain, as determined by the Arnow colorimetric assay (Payne, 1994). It was also positive in the Chrome azurul S liquid assay, which can monitor siderophore production (Schwyn & Neilands, 1987). These results were consistent with the report that the ATCC 17906\textsuperscript{T} strain also produces a small amount of the catecholate siderophore acinetobactin, which is also produced by \textit{A. baumannii} (Yamamoto \textit{et al.}, 1994; Wuest \textit{et al.}, 2009). Therefore, the modest growth observed for the \textit{acbA} mutant may be attributable to acinetobactin. These results suggested that under iron-limiting conditions, acinetobactin could partially replace acinetoferrin in supplying iron to \textit{A. haemolyticus} cells. However, the possibility was not ruled out that the ATCC 17906\textsuperscript{T} strain can acquire iron, although to a lesser extent, via an uncharacterized siderophore, which is different from both acinetobactin and acinetoferrin but capable of promoting growth of this strain under iron-limiting conditions. On the other hand, the complementing strain, TF-\textit{acbA}/pRK415-\textit{acbA}, had a reduced growth rate but eventually reached almost the same final OD\textsubscript{600} as the parental strain.
The strain (TF-abcA/pRK415) containing an empty plasmid also showed a more reduced growth rate than the complementing strain. However, a highly reduced growth rate was observed for TF-abcA containing pRK415 or pRK415-abcA when it was grown in –Fe medium supplemented with both tetracycline and apramycin, although the reason for this is currently unknown. The growth assay at least indicated that abcA participates in acinetoferrin production and that acinetoferrin was the preferred siderophore of this strain under the growth conditions used in this study. Taken together, these data indicate that A. haemolyticus ATCC 17906T produced a second siderophore, acinetobactin, but in an amount insufficient to grow at a level similar to the parental strain. Additionally, the abcA mutant showed normal expression of the ActA receptor protein, the seventh gene product (data not shown), confirming that the gene replacement mutation in abcA caused no polar effect on the downstream genes.

Phenotypic analysis of the actA mutant

A. haemolyticus ATCC 17906T was grown in +Fe and –Fe media, and the profiles of total outer-membrane proteins

**Fig. 8.** Amino acid sequence alignment of A. haemolyticus ATCC 17906T ActC with the three proteins showing the highest homology and S. mellioti RhtX. The numbers refer to amino acid positions in the unprocessed protein. Amino acids identical in the five proteins are marked by asterisks, and conserved amino acids are denoted by colons. The accession numbers of the aligned proteins are as follows: *Pectobacterium carotovorum* PC1-3088 (NC012917), *P. mendocina* NK-01 MDS_4916 (CP002620), *Stenotrophomonas maltophilia* R551-3 Sma1_0074 (NC011071) and *S. mellioti* 1021 RhtX (AE006469). The four conserved amino acid residues that are predicted to be located in a cytoplasmic loop of a novel family of MFS permeases (Cuiv et al., 2004) are boxed.
(OMPs) were then compared by SDS-PAGE (Fig. 5). As a result, at least five IROMP bands were observed, only when the ATCC 17906T strain was grown in –Fe medium (Fig. 5, lanes 1 and 3). The first 10 N-terminal amino acid sequence of the 79.3 kDa IROMP (the predicted molecular mass of the mature ActA was 79 249 kDa) was identical to that deduced from actA (Fig. 2a). This also indicated that the 23 amino acid residues were removed from the ActA pre-protein as a signal peptide. The actA mutant TF-actA was constructed to determine loss of expression of the corresponding protein. As expected, the 79.3 kDa IROMP band disappeared in this mutant (Fig. 5, lanes 2 and 4), and the corresponding band was again detected in the complementing strain, TF-actA/pRK415-actA, although it was not detected in the mutant containing the empty plasmid (Fig. 5, lanes 5 and 6). Next, we determined the effect of defective actA on growth in the –Fe medium. TF-actA showed an approximate 50% reduction in growth compared with the parental strain (Fig. 6), as observed for the acbA mutant (Fig. 4). In contrast, the actA mutant complemented with the actA gene outgrew the parental strain when incubated for 24 h under iron-limiting conditions (Fig. 6). Altogether, these data indicated that ActA could act as the outer-membrane receptor for ferric acinetoferrin.

**Phenotypic analysis of the actD mutant**

The inactivation of actD (TF-actD) resulted in an approximate 35% reduction in the ability to secrete acinetoferrin compared with the parental strain, whereas the introduction of pRK415-actD into the actD mutant (TF-actD/pRK415-actD) regained the strain’s original ability to secrete acinetoferrin (Fig. 3). In addition, TF-actD/pRK415-actD outgrew the actD mutant (Fig. 6). These results suggested that ActD may function as an inner-membrane exporter for acinetoferrin.

**Phenotypic analysis of the actB and actC mutants**

When TF-actB, TF-actC and their complementing strains (TF-actB/pK415-actB and TF-actC/pRK415-actC) were cultured in –Fe medium, no significant differences in the levels of growth after 24 h were observed (Fig. 6), and they were able to grow at levels similar to the parental strain (Fig. 4). Thus, mutational analysis failed to confirm the functions of actB and actC in ferric acinetoferrin transport. In particular, it was assumed that the additional production of acinetobactin could hamper the assessment of the function of ActC as an inner-membrane importer for ferric acinetoferrin.

**DISCUSSION**

The Fur box-containing DNA fragment isolated from A. haemolyticus ATCC 17906T by the FURTA method facilitated cloning and sequencing of neighbouring ORFs of related functions. Homology and mutational analyses revealed a cluster of eight ORFs encoding biosynthetic enzymes and transport components for acinetoferrin. We demonstrated that these eight ORFs were regulated by iron and were co-transcribed as a single polycistronic message. The results reported here indicate that the biosynthesis of acinetoferrin in A. haemolyticus ATCC 17906T likely proceeds as shown in Fig. 7. This represents another example of the siderophore biosynthesis pathways independent of nonribosomal peptide synthetases (Oves-Costales et al., 2009).

It has been reported that neighbouring genes encoding RhbA (L-2,4-diaminobutyrate:2-ketoglutarate 4-amino-transferase) and RhbB (L-2,4-diaminobutyrate decarboxylase), which are required for DAP production, are present as the first and second ORFs in the iron-regulated rhizobactin 1021 biosynthesis operon of S. meliloti (Lynch et al., 2001). Although such genes were not present in the acinetoferrin biosynthesis operon, significant activities of these two enzymes were found in the ATCC 17906T strain even when grown in +Fe medium (Yamamoto et al., 1995b; Ikai & Yamamoto, 1997). Moreover, there was a gene located at the same direction as acbA immediately upstream of acbA, whose protein product was homologous to hypothetical proteins of A. baumannii strains not related to DAP production. These data support the possibility that genes involved in DAP production may be located in another chromosomal region of the strain and that the transcription of these genes may not be iron-regulated. Consistent with this, it has recently been reported that A. baumannii, belonging to the same genus as A. haemolyticus, produces DAP, which is responsible for its surface-associated motility and virulence, even under iron-replete conditions (Skiebe et al., 2012). Besides rhizobactin 1021 (Persmark et al., 1993) and acinetoferrin, schizokinen (Mullis et al., 1971) and synechobactin (Ito & Butler, 2005) are known as hydroxamate siderophores characteristically containing DAP moieties for structural assembly. The results in this study would be useful to elucidate the genetic determinants involved in biosynthesis and transport of these siderophores.

To date, three different siderophore biosynthesis gene clusters have been described in A. baumannii (Antunes et al., 2011; Eijkelkamp et al., 2011). One gene cluster that is common to all the whole-genome-sequenced A. baumannii strains is proposed to function in biosynthesis of a hydroxamate siderophore of unknown structure. The acba, acbb and acbd orthologues in the acinetoferrin biosynthesis cluster were found to be present in the hydroxamate siderophore cluster of A. baumannii. However, the genetic arrangement differs considerably between the two species, and A. baumannii has no acbc orthologue. These findings suggested that the predicted hydroxamate siderophore in A. baumannii may be structurally related to acinetoferrin.
The proteins *Pseudomonas aeruginosa* FptX (Cuiv et al., 2004) and *Legionella pneumophila* LbtC (Chatfield et al., 2012), which show homology to *S. meliloti* RhtX, a member of a novel family of permeases (Cuiv et al., 2004), have also been reported to be encoded in the gene clusters that are involved in siderophore-mediated iron acquisition systems. The actC gene encoding the protein homologous to RhtX is located between actB and actA in the acinetoferrin cluster (Fig. 2a), and amino acid sequence alignment of these ActC homologues with RhtX revealed that a motif of four amino acids, QDI(V/I)/A, was conserved (Fig. 8), which is predicted to be located in a cytoplasmic loop in RhtX (Cuiv et al., 2004). These observations suggested that ActC may also be a member of a novel family of permeases and function in part as an importer of ferric acinetoferrin. Moreover, it is of interest that all of the genes encoding proteins with the highest similarity to ActC that are cited in Table 2 are preceded by genes encoding ActB homologues of unknown function. *Pseudomonas mendocina* has also been reported to produce a hydroxamate siderophore of unknown structure (Awaya & Dubois, 2008). The function of ActB in *A. haemolyticus* ATCC 17906T, however, remains obscure.

It is of great interest to elucidate how siderophores newly synthesized under iron-limiting conditions are secreted into the extracellular milieu, since intracellular accumulation of iron-free siderophores may be toxic due to deprivation of the cellular components of essential iron cofactors. MFS efflux pumps with 12 transmembrane segments in several bacteria, e.g. in *E. coli* (EntS) (Furrer et al., 2002), *Bordetella* species (AlcS) (Brickman & Armstrong, 2005), *Legionella pneumophila* (lbtB) (Allard et al., 2006) and *Vibrio parahaemolyticus* (PvsC) (Tanabe et al., 2006), have been identified as siderophore-specific exporters. This work indicated that *A. haemolyticus* ATCC 17906T also secretes acinetoferrin with the help of ActD. However, it should be considered that the actD mutant was still able to secrete acinetoferrin in response to iron limitation, although to a lesser extent than the wild-type parental strain (Fig. 3). This may be explained by compensation by another efflux pump in this species to avoid accumulation of acinetoferrin at burdensome levels, similar to *P. aeruginosa*, in which the siderophore pyoverdine is also exported by another known multidrug efflux pump (Poole et al., 1993).

Recently, we identified the gene cluster involved in biosynthesis and transport of acinetobactin in *A. haemolyticus* ATCC 17906T (GenBank accession no. AB621369), whose gene order is very similar to that in the characterized acinetobactin gene cluster of *A. baumannii* ATCC 19606T (Mihara et al., 2004). However, genes encoding an integrase catalytic subunit and a transposase IS3/IS911 family protein intervene in the 3’-terminal region of a thioesterase gene, thereby resulting in the absence of the C-terminal 24 amino acid residues. Deletion of angT, encoding a thioesterase which is involved in anguibactin biosynthesis in *Vibrio anguillarum*, has been reported to lead to a 17-fold decrease in its production (Wertheimer et al., 1999). Since anguibactin is structurally similar to acinetobactin, it was assumed that *A. haemolyticus* ATCC 17906T may also produce acinetobactin even in small amounts. For this reason, we attempted to generate a double mutant both in *acbA* and in the gene involved in acinetobactin biosynthesis. However, in spite of extensive efforts, such a mutant could not be obtained. Construction of a double mutant with regard to two different siderophore biosynthesis clusters may be necessary to clarify the advantages of possession of independent siderophore biosynthesis pathways in *A. haemolyticus*.

In conclusion, we have identified the *A. haemolyticus* ATCC 17906T gene cluster spanning ~13 kb which participates in acinetoferrin biosynthesis and transport. This cluster includes eight ORFs which are all co-transcribed from the promotor located in front of *acbA*. The existence of the iron acquisition system mediated by acinetoferrin would give *A. haemolyticus* the opportunity to utilize different iron sources, ultimately enhancing its ability to colonize and prosper in its natural habitat and human host. Further studies will be focused on identification and characterization of genes encoding the inner membrane active transport system for ferric acinetoferrin in connection with the function of ActC.

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