Response to Bile Salts in Clinical Strains of Acinetobacter baumannii Lacking the AdeABC Efflux Pump: Virulence Associated with Quorum Sensing

Maria López1,2†, Lucia Blasco1†, Eva Gato1,2, Astrid Perez1,2, Laura Fernández-Garcia1,2, Luis Martinez-Martínez3,4, Felipe Fernández-Cuenca5,6, Jesús Rodríguez-Bañó5,6, Alvaro Pascual5,6, German Bou1,2, and María Tomás1,2* on behalf of the GEIH-GEMARA (SEIMC)

Introduction: Acinetobacter baumannii is an opportunistic nosocomial pathogen associated with multiple infections. This pathogen usually colonizes (first stage of microbial infection) host tissues that are in contact with the external environment. As one of the sites of entry in human hosts is the gastrointestinal tract, the pathogen must be capable of tolerating bile salts. However, studies analyzing the molecular characteristics involved in the response to bile salts in clinical strains of A. baumannii are scarce.

Material and Methods: Microbiological and transcriptional studies (arrays and RT-PCR) in the response to bile salts were carried out in isogenic (A.baumannii ΔadeB ATCC 17978 and A. baumannii ΔadeL ATCC 17978) and clinical strains from clone ST79/PFGE-HUI-1 which is characterized by lacking the AdeABC efflux pump and by overexpression the AdeFGH efflux pump.

Results and Discussion: In presence of bile salts, in addition to the glutamate/aspartate transporter were found overexpressed in A. baumannii ΔadeB ATCC 17978, the virulence factors (surface motility, biofilm, and Type VI Secretion System) which are associated with activation of the Quorum Sensing system. Overexpression of these factors was confirmed in clinical strains of clone ST79/PFGE-HUI-1.

Conclusions: This the first study about the adaptive response to bile salts investigating the molecular and microbiological characteristics in response to bile salts of an isogenic model of A. baumannii ATCC 17978 and clinical isolates of A. baumannii (clinical strains
of ST79/PFGE-HUI-1) lacking the main RND efflux pump (AdeABC). Clinical isolates of A. baumannii lacking the AdeABC efflux pump (clone ST79/PFGE-HUI-1) displayed a new clinical profile (increased invasiveness) possibly associated with the response to stress conditions (such as the presence of bile salts).

Keywords: Acinetobacter baumannii, bile salts, quorum sensing, type VI secretion

INTRODUCTION

Acinetobacter baumannii is an important pathogen that is known to be a major agent in healthcare-associated and nosocomial infections (Antunes et al., 2013). The pathogen is increasingly involved in hospital outbreaks of infection, particularly in Intensive Care Units (ICUs) (del Mar Tomas et al., 2005). In most of these outbreaks, various inanimate objects in the hospital environment have been identified as the principal source of infection (Sherertz and Sullivan, 1985; Cefai et al., 1990). However, the genus Acinetobacter is known to be a normal inhabitant of human skin. Some researchers have therefore postulated that, in the context of an outbreak of A. baumannii infection, humans may be transient skin carriers, thus facilitating cross-contamination and also representing a potential source of hospital spread of the infection (Mulin et al., 1995). The digestive tract of ICU patients is an important reservoir of multiresistant A. baumannii in hospital settings (Corbella et al., 1996). The types of surveillance samples most frequently analyzed in patients include sputum and tracheostomy exudate, wounds, armpit/groin, and rectal smears. In a Spanish study involving the detection of A. baumannii in different surveillance samples from ICU patients, the microorganism was identified in 75% of axillary-pharyngeal samples and in 77% of rectal swabs; the pathogen was identified in 90% of patients following analysis of a combination of axillary/rectal/pharyngeal specimens and in 96% of patients following analysis of a combination of pharyngeal-rectal samples (Rodriguez-Baño et al., 2004).

Several factors affect the ability of the microorganism to persist in high numbers in the gut, both as a commensal and as an opportunistic pathogen. One such factor is tolerance to bile salts (Pumbwe et al., 2007). Bile salts (i.e., salts of bile acids) are formed from secondary bile acids (bile acids conjugated to amino acids) which attach to a sodium or potassium ion to form a salt. Bile acids are retained in the gallbladder as bile salts and are secreted into the intestine (Malik, 2016). Two mechanisms of tolerance to bile salts have been identified in bacteria to date: RND efflux pumps (Lin et al., 2003; Pumbwe et al., 2008) and glutamate transporters (Kraestel et al., 2010).

Three types of RND efflux pumps have been described in clinical strains of A. baumannii: AdeABC (expression of which is controlled by AdeRS); AdeIJK (in which the regulatory gene is adeN); and AdeFGH (in which adeL is the negative regulatory gene). The AdeFGH pump and in particular the AdeABC pump play a major role in acquired resistance (Coyne et al., 2010; He et al., 2015), whereas the AdeIJK pump is responsible for intrinsic resistance (Coyne et al., 2011). Moreover, overexpression of AdeABC and AdeFGH efflux pumps has been associated with increased biofilm production (He et al., 2015; Yoon et al., 2015; Richmond et al., 2016). However, neither these RND efflux pumps nor glutamate transporters have been associated with tolerance to bile salts in A. baumannii strains.

Finally, several studies have investigated how the virulence factors associated with the Quorum Sensing (QS) system can be regulated differently throughout the intestine by bile salts and, therefore, by the different commensal bacteria present (Zheng et al., 2010; Bachmann et al., 2015). The QS system enables bacterial populations to live and proliferate in an environment (sometimes hostile) with effective intercellular communication. However, this has not yet been investigated in strains of A. baumannii.

In this study, we carried out microbiological and transcriptional studies to investigate the response to bile salts in clinical strains of A. baumannii (clone ST79/PFGE-HUI-1) lacking the AdeABC efflux pump, as well as in isogenic mutant strains of A. baumannii ATCC 17978.

MATERIALS AND METHODS

Strains, Susceptibility Testing, and Growth with Bile Salts

Isogenic and Clinical Strains

A. baumannii ATCC 17978 was used as a reference strain. This strain was used to produce two stable mutants with the pMo130 plasmid; following the instructions of Hamad and colleagues (Hamad et al., 2009), A. baumannii ΔadeB ATCC 17978 and A. baumannii ΔadeL ATCC 17978 mutants were obtained. The mutants were confirmed by sequencing analysis and RT-PCR assays (Rumbo et al., 2013). The primers used are listed in Table S1 (Supplementary Material).

The Ab421 GEIH-2010 strain and other 10 clinical strains of A. baumannii, all belonging to clone ST79/PFGE-HUI-1 and identified during the second multicenter Spanish study of this pathogen (GEIH-REIPI-2010-Ab project), were included in the present study. These isolates were characterized in a previous study (Rumbo et al., 2013). Species identification was confirmed by detection of the blaOXA51 gene, and the adeA, adeB, adeC, adeR, and adeS genes were not detected (Rumbo et al., 2013).

To confirm the absence of the AdeABC system and regulatory genes, we applied Next Generation Sequencing (NGS) to a representative clinical strain (Ab421 GEIH-2010) of clone ST79/PFGE-HUI-1. The genome of this strain was recently published in the Genome Announcements (Lopez et al., 2016). Moreover, the LysR-type regulator protein (AdeL) upstream of the AdeF protein (AdeFGH) contained a new mutation that introduced an amino acid substitution (Met7→Stop) (Lopez et al., 2016).
The antibiotic susceptibility profile (isogenic and Ab421 GEIH-2010 strain) was determined by microdilution, according to CLSI recommendations (CLSI, 2015). The MICs were determined in the presence of bile salts (cholic acid sodium salt 50% and deoxycholic acid sodium salt 50%, Sigma Aldrich, Germany) and Phe-Arg β-naphthylamide dihydrochloride (PAbetaN), a commonly assumed RND efflux pump inhibitor (Pannek et al., 2006). The bile salts were used at a concentration 0.5% as the physiological concentration in the human intestine ranges between 0.1 and 1.3% (Pumbwe et al., 2007).

**Quantitative Assays**

Biofilm formation was quantified following the procedure described by Álvarez-Fraga et al. (2016). The strains were grown on Luria Broth for 18 h at 37°C and used to inoculate 5 mL of LB broth. Cultures were grown at 37°C with shaking. Overnight cultures were pelleted, washed and resuspended in 5 mL of modified LB-LN in presence and absence of 0.5% bile salts. A 1:100 dilution of each strain was incubated at 37°C for 48 h under static conditions. Growth of the culture was measured at OD_{600} to estimate total cell biomass. Biofilm formation was quantified by staining with crystal violet and solubilized with ethanol-acetone. The OD_{580}/OD_{600} ratio was used to normalize the amount of biofilm formed to the total cell content of each sample tested, to overcome variations due to differences in bacterial growth under several experimental conditions. Eight independent replicates were performed. A student’s t-test was performed to evaluate the statistical significance of the observed differences between the strains considered.

**Gene Expression**

Gene expression studies were carried out by microarray and RT-PCR analysis. In both types of analysis, RNA was isolated using hot phenol extraction and subjected to DNase I treatment (Invitrogen). The RNA was then cleaned on an RNeasy column (Qiagen) following the manufacturer’s mini cleanup protocol (Hamner et al., 2013) to obtain Dnase-treated RNA from late log-phase cultures in LB-LN (OD = 0.4–0.6) in the absence and presence of 0.5% bile salts in static conditions and tigecycline (0.5 mg/L). The RNA samples were quantified in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). The quality and integrity of the samples were determined in an Agilent 2100 Bioanalyzer with RNA 6000 Nano reagents and RNA Nano Chips (Agilent Technologies), and only samples with an RNA integrity number (RIN) >8 were included. Analysis of controls without reverse transcriptase confirmed the absence of contaminating DNA in the samples.

The A. baumannii strain ATCC 17978 and mutants A. baumannii ΔadeL ATCC 17978 and A. baumannii ΔadeB ATCC 17978 were included in the microarrays (Bioarray Diagnostico Geneticco, Alicante, Spain). The analysis was conducted using eArray (Agilent) (Aranda et al., 2013). Labeling was carried out by two-color microarray-based prokaryote analysis by Fair Play III labeling, version 1.3 (Agilent). Four independent RNA extractions per condition (biological replicates) were used in each experiment. Statistical analysis was carried out with the Bioconductor software package RankProd for the R computing environment. A gene was considered induced when the ratio of the treated to the untreated preparation was ≥1.5 and the P-value was < 0.05.

For RT-PCR assays, we used the Ab421 GEIH-2010 and other clinical isolates (Ab427, Ab428, Ab435, and Ab436) of ST79/PFGE-HUI-1 clone and A. baumannii ΔadeL ATCC 17978 strains. The following were analyzed by RT-PCR: (i) expression...
of the adeG gene (AdeFGH efflux pump) in the strains cultured in the presence of bile salts (0.5%) and tigecycline (0.5 mg/L), and (ii) expression of genes determined by microarray analysis (A1S_1490, A1S_0115, A1S_1295, and A1S_1510). The studies were carried out with the Lightcycler 480 RNA MasterHydrolisis Probe (Roche, Germany). The UPL Taqman Probes (Universal Probe Library-Roche, Germany) and primers used are listed in Table S1. The concentrations of the samples were adjusted to efficiencies of 90–110% (50 ng of RNA), and all experiments were performed in triplicate from three RNA extractions. For each strain, the expression of all genes was normalized relative to that of the rpoB gene. The normalized expression of each gene of interest was then calibrated relative to its expression by strains cultured in the absence of the bile salts or tigecycline in A. baumannii ATCC 17978, which were assigned a value of 1.0 (Mean Relative Expression, RE) (Aranda et al., 2013). Overexpression of the gene was defined by RE values of ≥ 1.5 (Tomas et al., 2010).

RESULTS
Antimicrobial Susceptibility in A. baumannii ATCC 17978 Isogenic Model and Ab421 GEIH-2010 (Table 1)
Resistance of the A. baumannii strains to several antimicrobials increased in the presence of 0.5% bile salts, although not significantly, which may indicate that expression of the AdeFGH efflux pump (RND type) is not modulated by bile salts. The MICs of tobramycin, tigecycline, norfloxacin, ciprofloxacin, gentamicin, tetracycline, and netilmicin were lowered in A. baumannii strain ΔadeL ATCC 17978 in the presence of the RND-efflux pump inhibitor Phe-Arg β-naphthylamide dihydrochloride (PAbetaN). These results confirm the overexpression of the AdeFGH efflux pump in relation to antibiotic resistance. Moreover, the levels of expression of the adeG gene (AdeFGH) revealed by RT-PCR explained the results of the MIC assays. The relative expression (RE) of the adeG gene in A. baumannii ΔadeL ATCC 17978 strains cultured in the presence of 0.5% bile salts was 0.5 (i.e., it was not overexpressed relative to expression in the absence of bile salts). However, the level of expression of adeG was 2.53 times higher in the presence than in the absence of tigecycline (0.5 mg/L).

In clinical strain Ab421 GEIH-2010 (belonging to clone ST79/PFGE-HUI-1), the MICs of tobramycin, tigecycline, norfloxacin, ciprofloxacin, gentamicin, tetracycline, and netilmicin decreased (by 2–4 times) in the presence of PAbetaN. The RE was also 2.71 times higher in the presence than in the absence of tigecycline (0.5 mg/L) in this isolate.

Virulence Phenotype: Surface Motility and Biofilm (A. baumannii ATCC 17978 Isogenic Model and Ab421 GEIH-2010)
In relation to surface motility studies in Normal LB (Figure 1), the A. baumannii ATCC and A. baumannii ATCC 17978ΔadeL strains did not grow, while A. baumannii ΔadeB ATCC 17978 and Ab421 GEIH-2010 both grew in the presence of 0.5 and 1% bile salts. The surface motility of isogenic strains (A. baumannii ATCC 17978 and mutants) was also higher in the presence of bile salts (0.5%) in this modified medium-Low Salt (LB-LS). However, the greatest increase in the motility of strain Ab421 GEIH-2010 cultured in presence of bile salts (0.5%) was observed in modified LB-Low Nutrients (LB-LN). Therefore, this concentration of bile salts (0.5%) and the modified medium LB-LN were used for all further experiments in this study.

In the biofilm assays, the presence of bile salts (0.5% in LB-LN) increased the capacity of biofilm production in Scanning Electron Microscopy (SEM) studies (Figure 2) and quantitative assays (Student’s t-test, P < 0.05) in all strains considered in this study (Figure 3). However, different phases of biofilm formation were observed in the biofilm matrix in Ab421 GEIH-2010 and microcolonies (slime layer) in A. baumannii ΔadeB ATCC 17978 cells (Figure 2: SEM analysis). Moreover, these isolates showed the highest capacity for biofilm production in quantitative assays (Figure 3).

Gene Expression in Relation to Bile Salts in the A. baumannii ATCC 17978 Isogenic Model: Microarray Analysis
Microarray analysis yielded the following results (GEO database GSE85264).
A) Comparison of A. baumannii ATCC 17978 Isogenic Strains Cultured in the Presence of 0.5% Bile Salts (LB-LN)
Gene expression in the presence of bile salts (0.5%) in isogenic A. baumannii strains is summarized in Table 2. In the first experiment comparing A. baumannii ATCC 17978ΔadeL and the A. baumannii wild type strain, only one gene was overexpressed (lysR regulator family) in the mutant strain. This may be related to tolerance to bile salts in this isolate, although this was not confirmed. However, comparison of A. baumannii ATCC 17978ΔadeB and the A. baumannii wild type strain showed that seven genes were overexpressed in mutant strains in relation to acid tolerance (glutamate/aspartate transporter), gene mobility (transposases) and surface motility/biofilm formation (csuA/B). Finally, 25 genes associated with acid tolerance (glutamate/aspartate transporters), quorum sensing (acyl-CoA dehydrogenase, acyl-CoA synthase/AMP-acid ligases II, amino acid adenylation, acyl carrier protein, and RND superfamily transporter), iron/sulfur metabolism (ring hydroxylation dioxygenase Rieske [2Fe-2S] and aromatic-ring-hydroxylation dioxygenating dioxygenase β subunit), gene mobility (transposases), T6SS/Type VI Secretion System (vipA, hcp-1, putative signal peptide, putative membrane, and vipB), and motility/biofilm formation (csuA/B and fimbrial protein) were revealed by comparing A. baumannii ATCC 17978ΔadeB and A. baumannii ATCC 17978ΔadeL.
B) Comparison of Each Isolate Cultured in the Absence and Presence of Bile Salts (LB-LN)
Use of arrays to investigate gene expression in A. baumannii ATCC and mutant strains (A. baumannii ΔadeB ATCC 17978 and A. baumannii ΔadeL ATCC 17978) cultured in the absence and presence of bile salts (0.5%) revealed similar
TABLE 1 | MICs of different antimicrobial agents against clinical and isogenic strains of *A. baumannii* in the presence or absence of bile salts (0.5%) or in the presence of Phe-Arg β-naphthylamide dihydrochloride (PAbetaN, 100 µg/mL).

| Strain                     | Antimicrobial | MIC (mg/L) | MIC (mg/L-Bile Salts 0.5%) | MIC (mg/L-PAbetaN 100 µg/mL) |
|----------------------------|---------------|------------|-----------------------------|-------------------------------|
| *A. baumannii* ATCC 17978  | Tobramycin    | 0.5        | 0.5                         | 0.5                           |
|                            | Sulfamethoxazole | 4,864        | 4,864                       | 4,864                        |
|                            | Tigecycline   | 1          | 2                           |                               |
|                            | Norfloxacin   | 4          | 2                           |                               |
|                            | Ciprofloxacin | 0.5        | 0.25                        |                               |
|                            | Gentamicin    | 1          | 1                           |                               |
|                            | Tetracycline  | 4          | 1                           |                               |
|                            | Netilmicin    | 1          | 1                           |                               |
| *A. baumannii* ATCC 17978 ΔadeB | Tobramycin    | 0.25       | 1                           | 0.5                           |
|                            | Sulfamethoxazole | 4,864        | 4,864                       | 4,864                        |
|                            | Tigecycline   | 1          | 1                           |                               |
|                            | Norfloxacin   | 4          | 4                           |                               |
|                            | Ciprofloxacin | 0.5        | 0.5                         | 0.125                         |
|                            | Gentamicin    | 0.5        | 2                           | 0.5                           |
|                            | Tetracycline  | 4          | 4                           |                               |
|                            | Netilmicin    | 1          | 1                           |                               |
| *A. baumannii* ATCC 17978 Δadel | Tobramycin    | 1          | 1                           | 0.25                          |
|                            | Sulfamethoxazole | 4,864        | 4,864                       | 4,864                        |
|                            | Tigecycline   | 1          | 0.5                         | 0.25                          |
|                            | Norfloxacin   | 8          | 8                           | 0.5                           |
|                            | Ciprofloxacin | 0.5        | 2                           | 0.25                          |
|                            | Gentamicin    | 1          | 4                           | 0.5                           |
|                            | Tetracycline  | 4          | 4                           |                               |
|                            | Netilmicin    | 2          | 1                           | 0.5                           |
| Ab421 GEIH-2010            | Tobramycin    | 64         | 64                          | 8                             |
|                            | Sulfamethoxazole | 9,728       | 9,728                       | 4,864                        |
|                            | Tigecycline   | 16         | 16                          | 4                             |
|                            | Norfloxacin   | 1,024      | 2,048                       | 512                           |
|                            | Ciprofloxacin | 512        | 512                         | 512                           |
|                            | Gentamicin    | 512        | 512                         | 128                           |
|                            | Tetracycline  | 16         | 32                          | 8                             |
|                            | Netilmicin    | 256        | 256                         | 8                             |

Gene expression in all three isolates. Genes involved in the following processes were expressed under stress conditions: (i) Ferric iron binding (ii) Oxidoreductase/Transferase (iii) Isomerase/Fumarylacetoacetate, (iv) Response to toxic substances, and (v) DNA metabolism (Table 3).

Both results (A and B) of the microarray analysis are consistent with findings of motility studies in modified LB-LN (Figure 1).

**Growth Curves on Presence of Bile Salts (0.5% in LB-LN) in Clinical Strains of the ST79/PFGE-HUI-1 Clone**

To analyze the results obtained with isogenic models and Ab421 GEIH-2010, we used five clinical strains of *A. baumannii* belonging to the clone ST79/PFGE-HUI-1 (including the Ab421 GEIH-2010).

Interestingly, in the first 12 h, the *A. baumannii* clinical strains from PFGE-HUI-1 clone showed a greater growth than the strain *A. baumannii* ATCC 17978. However, after this time, the clinical strains had a stagnation (from 12 to 24 h). After 24 h, *A. baumannii* clinical strains from PFGE-HUI-1 and the *A. baumannii* ATCC 17978 strain reached to an OD₆₀₀ of 0.5 and 0.3 (the bacterial growth was inoculated on LB plates). This may indicate the development of biofilm formation in the clinical strains from PFGE-HUI-1 clone not being possible the study of growth by optical density (Figure 4).

**Gene Expression in Relation to Bile Salts in Clinical Strains of ST79/PFGE-HUI-1 Clone as Revealed by RT-PCR**

Gene overexpression detected by microarray analysis was confirmed by RT-PCR in clinical strains of clone
ST79/PFGE-HUI-1 (in addition to the Ab421 GEIH-2010). The levels of overexpression (RE) of A1S_1490 (Acid tolerance), A1S_0115 (Quorum Sensing), A1S_1295 (T6SS), and A1S_1510 (Surface motility/Biofilm formation) were statistically significantly higher in clinical isolates of the ST79/PFGE-HUI-1 clone cultured in the presence of bile salts (0.5%) than in the same clone cultured in LB-LN (Table 4). These results obtained with the clinical strains of the PFGE-HUI clone confirmed the overexpression of genes involved in the response to bile salts in the isogenic model of A. baumannii ATCC 17978. Moreover, all strains of this ST79/PFGE-HUI-1 clone (17 isolates) were considered to be the cause of infection in all six patients. Interestingly, five of the six patients had bacteraemia (primary or secondary).

**DISCUSSION**

The RND type of multidrug efflux pumps play several roles in bacterial pathogens: (i) provision of resistance to antimicrobial and antiseptic compounds, including those naturally present in mucosa; (ii) regulation of virulence factors via involvement in quorum-sensing regulation; (iii) detoxification of intracellular metabolites; and finally, (iv) mediation of cell homeostasis and intercellular signal trafficking (Beceiro et al., 2013).

In A. baumannii, the AdeABC RND-pump is the main efflux system involved in antimicrobial resistance. However, 25–30% of clinical strains of A. baumannii do not possess the AdeABC efflux pump (Chu et al., 2006; Lin et al., 2009).

In the GEIH-REIPI-2010 Ab Project (a multicentre study in which 45 hospitals participated), we studied clinical strain Ab421 GEIH-2010, which lacks the AdeABC efflux pump as well as regulatory genes and also overexpresses the AdeFGH system. This clinical strain belongs to clone ST79/PFGE-HUI-1 (seventeen isolates) (Rumbo et al., 2013). The mechanisms associated to the adaptive response to bile salts were analyzed in mutant strains of A. baumannii ATCC 17978 by considering an isogenic model (A. baumannii ΔadeB ATCC 17978 and A. baumannii ΔadeL ATCC 17978). Interestingly, the Ab421 GEIH-2010 strain displayed a higher capacity for surface motility and biofilm formation when cultured in the presence of bile salts than when cultured in the absence of these.

The mechanisms associated to the response to bile salts (essential for survival in the gastrointestinal tract) are not well-known in A. baumannii. In a study of the profile of protein overexpression in response to monovalent cations (200 mM of NaCl), Hood et al. observed glutamate/aspartate transport (Hood et al., 2010). Moreover, glutamate transport (glnQHMP operon) has also been implicated with acid tolerance in Streptococcus mutans (Krasel et al., 2010). Our results with A. baumannii ΔadeB ATCC 17978 (microarray analysis) and clinical strains of ST79/PFGE-HUI-1, including strain Ab421 GEIH-2010 (RT-PCR), revealed the involvement of this
FIGURE 2 | SEM analysis of A. baumannii cells cultured in the absence (A,C,E,G) and presence of 0.5% bile salts (B,D,F,H). (A,B) A. baumannii ATCC 17978; (C,D) A. baumannii ΔadeB ATCC 17978; (E,F) A. baumannii ΔadeL ATCC 17978; (G,H) Ab421 GEIH-2010. (Scale bars: 20 μm). It is observed in presence of bile salts, the state of adhesion in A. baumannii ATCC 17978 (B), slime layer-micro colonies (previous state of biofilm formation) in A. baumannii ΔadeB ATCC 17978 (D), proliferation in A. baumannii ΔadeL ATCC 17978 (F) and finally, biofilm formation in Ab421 GEIH-2010 (H). The arrows indicate the most advanced stages of biofilm development.
transporter and associated proteins in tolerance to physiological concentrations of bile salts (Prouty et al., 2004; Sánchez et al., 2005).

Virulence factors associated with activation of the QS system may be regulated differently throughout the intestine depending on the bile salts present, the level of tolerance and the presence of different commensal bacteria (Bachmann et al., 2015). We observed overexpression of the QS genes (A1S_0112 to A1S_0116) in strains lacking the AdeABC efflux pump (A. baumannii ΔadeB ATCC 17978 and clinical strains of ST79/PFGE-HUI-1) cultured in the presence of bile salts. In a study using transcriptomic analysis, Clemmer et al. confirmed that this cluster of genes was induced by the 3-OH C12-HSL molecule (a signal in the QS network). Interestingly, the A1S_0116 gene encodes a RND superfamily transporter which may be involved in efflux of the molecules from the QS system (Clemmer et al., 2011). Moreover, virulence factors modulated by the QS system were overexpressed, i.e., surface motility, biofilm formation (A1S_2218 and A1S_1510) (Clemmer et al., 2011; Rumbo-Feal et al., 2013) and the Type VI Secretion System (T6SS) (A1S_1292 to A1S_1296) (Sana et al., 2012).

Zheng et al., investigated the role of the QS system in Vibrio cholerae in modulating the expression of virulence factors such as T6SS (Zheng et al., 2010). The authors established that a high density of bacteria is critical for expression of the T6SS pandemic V. cholerae C6706. The T6SS may be a potent mediator of survival of the pathogen or commensals in multi-bacterial environments, biofilms and in polymicrobial

| Gene name | Protein Description | Fold | Function |
|-----------|--------------------|------|----------|
| Acinetobacter baumannii ATCC 17978ΔadeL vs. Acinetobacter baumannii ATCC 17978 | A1S_2303 | LysR regulator family | 2.50 | Regulatory |
| Acinetobacter baumannii ATCC 17978ΔadeB vs. Acinetobacter baumannii ATCC 17978 | A1S_1490 | Glutamate/Aspartate transporter | 1.50 | Acid tolerance |
| | A1S_0658 | Transposase (ISAba1) | 2.57 | Mobility of genes |
| | A1S_0657 | Transposase (ISAba2) | 2.21 | |
| | A1S_2218 | CsuA/B | 1.76 | Surface motility/biofilm |
| | A1S_1071 | Hypothetical protein | 1.88 | – |
| | A1S_2652 | Hypothetical protein | 1.88 | |
| | A1S_3020 | Hypothetical protein | 1.84 | |
| Acinetobacter baumannii ATCC 17978ΔadeB vs. Acinetobacter baumannii ATCC 17978ΔadeL | A1S_1493 | Glutamate/aspartate transport protein | 1.60 | Acid tolerance |
| | A1S_1490 | Glutamate/aspartate transport protein | 1.59 | |
| | A1S_1492 | Glutamate/aspartate transport protein | 1.58 | |
| | A1S_0113 | Acyl-CoA dehydrogenase | 1.73 | Quorum sensing |
| | A1S_0112 | Acyl-CoA synthetase/AMP-acid ligases II | 1.65 | |
| | A1S_0115 | Amino acid adenylation | 1.52 | |
| | A1S_0114 | Acyl carrier protein | 1.50 | |
| | A1S_0116 | RND superfamily transporter | 1.50 | |
| | A1S_1860 | Ring hydroxylating dioxygenase Rieske (2Fe-2S) | 1.54 | Iron/Sulfur metabolism |
| | A1S_1859 | Aromatic-ring-hydroxylating dioxygenase β-subunit² | 1.50 | |
| | A1S_0658 | Transposase (ISAba1) | 2.74 | Mobility of genes |
| | A1S_0657 | Transposase (ISAba2) | 2.41 | |
| | A1S_1294 | Type VI secretion system-associated protein (VpA) | 2.05 | T6SS |
| | A1S_1296 | Type VI secretion system-associated protein (Hcp-1) | 1.79 | |
| | A1S_1292 | Putative signal peptide | 1.64 | |
| | A1S_1295 | Type VI secretion system-associated protein (Putative Membrane protein) | 1.62 | |
| | A1S_1293 | Type VI secretion system-associated protein (VpB) | 1.99 | |
| | A1S_2218 | CsuA/B | 1.57 | Surface motility/biofilm |
| | A1S_1510 | Fimbrial protein (type I) | 1.55 | |
| | A1S_1865 | Glu-tRNA amidotransferase | 1.64 | Transferase activity |
| | A1S_1466 | Glutaminase-aspirginase | 1.58 | |
| | A1S_1071 | Hypothetical protein | 1.92 | – |
| | A1S_2652 | Hypothetical protein | 1.91 | |
| | A1S_3020 | Hypothetical protein | 1.80 | |
TABLE 3 | Microarray analysis of the expression of genes isolated from *Acinetobacter baumannii* isogenic strains cultured in the presence (0.5%) and absence of bile salts.

| Gene name          | Protein Description                   | Fold | Function                   |
|--------------------|---------------------------------------|------|----------------------------|
| *Acinetobacter baumannii* ATCC 17978 vs. *Acinetobacter baumannii* ATCC 17978 (0.5 % Bile Salts) |
| A1S_3175           | Bacterioferritin                       | 5.21 | Feric iron binding         |
| A1S_0800           | Bacterioferritin                       | 2.71 |                            |
| A1S_1860           | Ring hydroxylation dioxygenase Rieske (2Fe-2S) protein | 2.27 | Iron/Sulfur metabolism     |
| A1S_1859           | Aromatic-ring-hydroxylation dioxygenase beta subunit | 2.21 |                             |
| A1S_2102           | Aldehyde dehydrogenase 1              | 3.57 | Oxidoreductase activity    |
| A1S_1864           | Acyl-CoA dehydrogenase-like protein    | 2.34 |                             |
| A1S_1858           | Short-chain dehydrogenase/reductase SDR | 2.17 |                             |
| A1S_1865           | Glu-TRNA amidotransferase              | 2.66 | Transferase activity       |
| A1S_3415           | Malonylcoacetate isomerase             | 2.5  | Isomerase activity         |
| A1S_1857           | Vanillate C-demethylase oxidoreductase | 2.31 | Catalytic activity         |
| A1S_3414           | Fumarylcoacetase                      | 2.14 | Fumarylcoacetase activity  |
| A1S_2809           | Bacteriolytic lipoprotein entericidin B | 2.03 | Response to toxic substance |
| A1S_1228           | Cold shock protein                     | 2    | DNA binding                |
| A1S_1924           | Cytochrome d terminal oxidase polypeptide subunit I | 2.34 | Component of membrane     |
| *Acinetobacter baumannii* ATCC 17978adeB vs. *Acinetobacter baumannii* ATCC 17978adeB (0.5% Bile Salts) |
| A1S_3175           | Bacterioferritin                       | 4.17 | Feric iron binding         |
| A1S_0800           | Bacterioferritin                       | 3.64 |                            |
| A1S_1860           | Ring hydroxylation dioxygenase Rieske (2Fe-2S) protein | 2.79 | Iron/Sulfur Metabolism     |
| A1S_1859           | Aromatic-ring-hydroxylation dioxygenase beta subunit | 2.73 |                             |
| A1S_1861           | Benzoate dioxygenase large subunit     | 2.43 | Oxidoreductase activity    |
| A1S_2102           | Aldehyde dehydrogenase 1              | 2.39 |                             |
| A1S_1864           | Acyl-CoA dehydrogenase-like protein    | 2.79 |                             |
| A1S_1858           | Short-chain dehydrogenase/reductase SDR | 2.53 |                             |
| A1S_1075           | D-amino-acid dehydrogenase            | 2.62 |                             |
| A1S_1856           | P-hydroxyphenylacetate hydroxylase C1/reductase component | 2.01 |                             |
| A1S_1865           | Glu-TRNA amidotransferase              | 2.97 | Transferase activity       |
| A1S_3415           | Malonylcoacetate isomerase             | 2.59 | Isomerase activity         |
| A1S_1857           | Vanillate C-demethylase oxidoreductase | 2.47 | Catalytic activity         |
| A1S_3414           | Fumarylcoacetase                      | 2.12 |                             |
| A1S_0804           | Trehalose-6-phosphate phosphatase      | 3.66 | Metal ion binding          |
| A1S_1498           | TetR family transcriptional regulator  | 2.28 | Transcriptional regulator  |
| A1S_1687           | Transcriptional regulator              | 3.34 |                             |

(Continued)
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FIGURE 4 | Curves showing growth of the A. baumannii isolates belonging to clone ST79/PFGE-HUI-1 in the presence of bile salts (0.5%) in LB-LN. A. baumannii ATCC 17978 was included as a control.

TABLE 4 | Expression of genes involved in Acid tolerance, Quorum sensing, T6SS, and surface motility/biofilm mechanisms in clinical strains (clone ST79/PFGE-HUI) cultured in the presence of bile salts (0.5%), determined by RT-PCR.

| A. baumannii GEIH-2010 (PFGE-HUI-1 clone) | Acid tolerance (A1S_1490 gene<sup>a</sup>) | Quorum sensing (A1S_0115 gene<sup>a</sup>) | T6SS (A1S_1295 gene<sup>a</sup>) | Surface motility/biofilm (A1S_1510 gene<sup>a</sup>) | Bacteraemia |
|------------------------------------------|----------------------------------------|----------------------------------------|---------------------------------|----------------------------------------|-------------|
| Ab421                                    | 4.16                                    | 4.28                                    | 3.38                            | 3.83                                    | Yes         |
| Ab427                                    | 2.42                                    | 1.68                                    | 2.28                            | 2.42                                    | Yes         |
| Ab428                                    | 2.60                                    | 1.87                                    | 2.36                            | 2.41                                    | Yes         |
| Ab435                                    | 9.00                                    | 9.78                                    | 7.83                            | 8.39                                    | Yes         |
| Ab436                                    | 4.72                                    | 2.80                                    | 3.27                            | 4.31                                    | Yes         |

| Control*                                 | Acid tolerance (A1S_1490 gene<sup>a</sup>) | Quorum sensing (A1S_0115 gene<sup>a</sup>) | T6SS (A1S_1295 gene<sup>a</sup>) | Surface motility/biofilm (A1S_1510 gene<sup>a</sup>) | Bacteraemia |
|------------------------------------------|----------------------------------------|----------------------------------------|---------------------------------|----------------------------------------|-------------|
| A. baumannii ATCC 17978                 | 1.23                                    | 1.03                                    | 1.10                            | 1.05                                    | –           |

*A. baumannii ATCC 17978 was included as a control. The same grown in the absence of bile salts were used as references strains (RE = 1).<sup>a</sup>Genome A. baumannii ATCC 17978.

Gram-negative bacteria to kill competitors. The A. baumannii clinical strains carry T6SS repressors (TetR regulators) in plasmids harboring resistance genes that prevent expression of these T6SS systems (Weber et al., 2015, 2016). When Multi-Drug-Resistant (MDR) strains of A. baumannii are not exposed to antibiotics, such as in the inanimate hospital environment or in untreated polymicrobial infections, there is an increased likelihood of encountering competitors that will activate bacterial T6SS systems (Weber et al., 2015). In Spanish hospitals, ST79/PFGE-HUI-1 was the only carbapenem-susceptible clone that did not possess a plasmid carrying OXA 24 β-lactamase or the AbKAB Toxin-Antitoxin system (Rumbo et al., 2013; Mosqueda et al., 2014). In the present study, resistance to antimicrobials (aminoglycosides, quinolones and glycin) in Ab421 GEIH-2010 (belonging to ST79/PFGE-HUI-1) and A. baumannii ΔadeL ATCC 17978 was associated with overexpression of the AdeFGH pump.

Finally, although only a small number of cases were considered (out to seven), all patients from whom strains of A. baumannii clone ST79/PFGE-HUI-1 were isolated had infections and five out of patients developed bacteraemia (72%). This contrasts with usual observations for A. baumannii, as about half of colonized patients do not usually have infections due to the pathogen (Cisneros and Rodríguez-Baño, 2002; Villar et al., 2014), and the prevalence of bacteraemia in infected patients is usually lower than 10% (Thom et al., 2010). Thom and collaborators found that in 86% of patients from ICUs who had the gastrointestinal tract colonized by A. baumannii clinical strains, they had bacteraemia through genetically similar strains (Thom et al., 2010). This implies
that those clinical isolates of *A. baumannii* that present a higher capacity to survive gastrointestinal conditions (including bile salts tolerance) through biofilm formation and others mechanisms as transporters could present an increase in their invasive capacity (development of bacteraemia) due to virulence factors (as the type VI secretion system) previously activated under pressure conditions.

In conclusion, this is the first study about the adaptive response to bile salts investigating the molecular and microbiological characteristics in response to bile salts of an isogenic model of *A. baumannii* ATCC 17978 (*A. baumannii ΔadeB* ATCC 17978) and clinical isolates of *A. baumannii* (clinical strains of ST79/PFGE-HUI-1) lacking the main RND efflux pump (AdeABC). The response to bile salts led to activation of the QS system and modulated virulence factors such as surface motility, biofilm and Type VI secretion system. Moreover, we observed a new clinical profile (increased invasiveness) of strains of *A. baumannii* with or without the AdeABC efflux pump (or in which the pump is inhibited by treatment) (Pannek et al., 2006; Blair and Piddock, 2009; López et al., 2014; Richmond et al., 2016) under others stress conditions (oxidative and osmotic) that may activate global mechanisms such as transporters that may activate global mechanisms such as the QS system, which modulates virulence factors.

**NUCLEOTIDE SEQUENCE ACCESSION NUMBER**

The genome sequence of Ab421 GEIH-2010 strain has been deposited at GenBank under accession number CP014266.1. This genome sequence was determined as part of a II Spanish multicenter study, GEIH-REIPI *A. baumannii* 2000–2010 project (PRJNA308422).

**AUTHOR CONTRIBUTIONS**

Funding acquisition: MT; Investigation: ML, LB, EG, LF, LM, FF, JR, AP (4th author), AP (9th author), GB, and MT; Methodology: ML, LB, EG, LF; Supervision: MT; Writing: MT.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00143/full#supplementary-material

Table S1 | Primers and probes used in this study (Supplementary material).
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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