Transcriptional analysis reveals new factors involved in the biofilm formation ability of *Acinetobacter baumannii*.

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CERTIFICAN:

Que Dña. Soraya Rumbo Feal, Licenciada en Biología por la Universidade da Coruña, ha realizado en el Servicio de Microbiología y en el Instituto de Investigación Biomédica (INIBIC) del Complejo Hospitalario Universitario A Coruña, bajo su dirección y tutela, el trabajo **"**Transcriptional analysis reveals new factors involved in the biofilm formation ability of *Acinetobacter baumannii***"**, el cual, reúne todas las condiciones para ser presentado como Tesis Doctoral con mención internacional.

Y para que así conste, y surta los efectos oportunos, firmamos el presente certificado en A Coruña, mayo del 2018.

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ABREVIATIONS

Ac-505: acinetin 505

ACB complex: *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex

ACP: acyl carrier protein

ADC: *Acinetobacter*-derived cephalosporinase

AHL: acyl-homoserine lactone

AMP: adenosine monophosphate

bp: base pair

Cas: CRISPR-associated

cDNA: complementary deoxyribonucleic acid

CFU: colony-forming unit

Cys: cysteine

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Da: dalton

DNA: deoxyribonucleic acid

G+D: guanine-cytosine

Gly: glycine

h: hour

IDSA: Infectious Diseases Society of America

kb: kilo base pairs

LB: Luria-Bertani

LQ/MS: liquid chromatography/mass spectrometry

MDR: multidrug resistant

MIC: minimal inhibitory concentration

mRNA: messenger ribonucleic acid

Abreviations

NGS: next-generation sequencing

NRPS: non-ribosomal peptide synthase

OD_{600}: optical density at 600 nanometers

OMPs: outer membrane proteins

OMVs: outer membrane vesicles

PBPs: penicillin-binding proteins

PCP: petidyl carrier protein

PDR: pandrug resistant

PLase: phospholipase

PNAG: poly-β-(1-6)-*N*-acetylglucosamine

RT-qPCR: reverse transcription quantitative polymerase chain reaction

RBS: ribosome binding site

RNA: ribonucleic acid

RNA-seq: RNA sequencing

RND efflux pump: resistance-nodulation-cell division type efflux pump

rRNA: ribosomal ribonucleic acid

RT-PCR: reverse transcription polymerase chain reaction

SEM: scanning electron microscopy

spp.: species

sRNA: small ribonucleic acid

TFP: type IV pili

TPS: two-partner secretion

µm: micrometer

RESUMO

Acinetobacter baumannii é un patóxeno nosocomial que posúe unha enorme capacidade de adaptarse a condicións desfavorables, o que o converte nun importante problema de saúde pública.

Os perfís de expresión xénica durante a formación de biopelículas en *A. baumannii* resultaron diferentes en comparación con células libres planctónicas en fase exponencial e estacionaria. Confirmouse que os xenes A1S_1507, A1S_3168, A1S_2042, A1S_0302 y A1S_0114 que codifican unha proteína de fimbria, una proteína dun pilus, un regulador transcripcional, unha proteína hipotética e un transportador de grupos acilo, respectivamente, están implicados na capacidade de formación de biopelículas en *A. baumannii*. Ademais, demostrouse que o xene A1S_0114 participa na adherencia a superficies bióticas e abióticas, en virulencia e na síntese dun metabolito denominado acinetin 505.

Así mesmo, atopáronse moléculas de sRNAs que se expresan diferencialmente nas biopelículas con respecto ás células planctónicas. Entre eles, destacou o sRNA 13573, altamente expresado en células sésiles, que resultou estar implicado na formación de biopelículas e na adherencia a células eucariotas.

Neste traballo, descríbense, dúas novas dianas terapéuticas que participan na patoxénese de *A. baumannii*: o xene A1S_0114 e o sRNA 13573.

RESUMEN

Acinetobacter baumannii es un patógeno nosocomial que posee una enorme capacidad de adaptarse a condiciones desfavorables, lo que lo convierte en un importante problema de salud pública.

Los perfiles de expresión génica durante la formación de biopelículas en *A. baumannii* resultaron diferentes en comparación con células libres planctónicas en fase exponencial y estacionaria. Se confirmó que los genes A1S_1507, A1S_3168, A1S_2042, A1S_0302 y A1S_0114 que codifican una proteína de fimbria, una proteína de un pilus, un regulador transcripcional, una proteína hipotética y un transportador de grupos acilo, respectivamente, están implicados en la capacidad de formación de biopelículas en *A. baumannii*. Además, se demostró que el gen A1S_0114 participa en la adherencia a superficies bióticas y abióticas, en virulencia y en la síntesis de un metabolito denominado acinetin 505.

Asimismo, se encontraron moléculas de sRNAs que se expresan diferencialmente en las biopelículas con respecto a las células planctónicas. Entre ellos, destacó el sRNA 13573, altamente expresado en células sésiles, que resultó estar implicado en formación de biopelículas y en la adherencia a células eucariotas.

En este trabajo, se describen, dos nuevas dianas terapéuticas que participan en la patogénesis de *A. baumannii*: el gen A1S_0114 y el sRNA 13573.

ABSTRACT

Acinetobacter baumannii is a nosocomial pathogen with a notable ability to adapt to stress conditions and develop resistance to multiple antimicrobial compounds, becoming a remarkable public health problem.

Gene expression during biofilm formation in *A. baumannii* showed different profiles compared to planktonic cells in exponential and stationary phase of growth. We could confirm that genes A1S_1507, A1S_3168, A1S_2042, A1S_0302 y A1S_0114 coding a fimbrial protein, a pilus assembly protein, a transcriptional regulator, a hypothetical protein and an acyl-carrier protein, respectively, are involved in biofilm formation ability of *A. baumannii*. Furthermore, the A1S_0114 gene showed to play a role in attachment to biotic and abiotic surfaces, in virulence, and in the biosynthesis of a metabolite named as acinetin 505.

Moreover, an important number of sRNAs differentially expressed in biofilm associated cells compared to planktonic cells were determined. Among them, the 13575 sRNA, highly expressed in biofilm, resulted to be involved in biofilm formation and in adherence to eukaryotic cells.

In the present work, two new therapeutic targets involved in the pathogenesis of *A. baumannii* are described: the A1S_0114 gen and the 13573 sRNA.

1. INTRODUCTION

According to World Health Organization, infectious diseases are responsible of almost 30% of the 56 million deaths estimated *per* year worldwide (1). By the end of World War II, it was thought that microbial infections were under control due to the discovery and mass-production of penicillin and the efficiency of vaccines available (2). Since then, the access to potent and safe antimicrobial agents reduced the morbidity and mortality associated with formerly fatal diseases (3).

However, nowadays some infectious diseases are considered as a remarkable public health problem, both in developing and developed countries. The misuse of antibiotics has been associated with the adaptation of bacteria to these drugs and the subsequent emergence of multidrug resistant (MDR) strains. This fact is especially dangerous in hospital environments, wherein patients with compromised immune system are particularly vulnerable to infectious diseases. About a million people acquire some type of nosocomial infection in the United States every year and 75,000 die as a result (4). The growing number of antimicrobial-resistant pathogens, which are associated with nosocomial infection, suppose a significant burden on healthcare systems and have important economic costs (5).

The pathogens that currently cause the majority of hospital infections and effectively avoid the action of antibacterial drugs are known as "ESKAPE" pathogens. This group is formed by *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species (3). Increasing rates of infection due to methicillin-resistant *S. aureus*, vancomycin-resistant *E. faecium* and pandrug resistant (PDR) Gram-negative pathogens such as *Acinetobacter* species or *P. aeruginosa*, leaves clinicians without therapeutic options being forced to use previously discarded drugs, such as colistin. Although this antibiotic causes nephrotoxicity, it is considered a last-line therapeutic option for infections caused by MDR *A. baumannii* (6, 7). The absence of new compounds effective against these pathogens would have a terrible effect on public health. Thus, it is necessary to find new targets that would allow us to design new therapeutic tools for treating infections caused by MDR bacteria.

1.1. Genus *Acinetobacter*

Acinetobacter is a genus of Gram-negative bacteria belonging to the family *Moraxellaceae* within the order of *Gammaproteobacteria*. It comprises strictly aerobic, non-fermenting, non-motile, catalase-positive, oxidase-negative organisms with a DNA G+C content of 39% to 47%. The cells are 1.5 µm in length, with a shape varying from coccoid to coccobacillary (8). Members of this genus are considered ubiquitous organisms and can be recovered from different kind of samples of soil and water. Most *Acinetobacter* strains are metabolically versatile and able to grow in a simple mineral medium. Many species of this genus have also been recovered from human clinical specimens and have some significance as human pathogens considering that they cause a number of nosocomial infections (9, 10).

The first organism of this genus was described in 1911 by Beijerinck, who named it as *Micrococcus calco-aceticus*, and it was isolated from soil by enrichment in a calcium-acetate-containing minimal medium (11). Since then, members of the genus have been classified under a variety of different names till 1954, when Brisou and Prévot proposed the current designation (12). However, when Baumann *et al*. published their comprehensive study based on nutritional and biochemical properties, this classification became more widely accepted (13). One of the biggest improvements on the history of this genus was in 1986, when Bouver and Grimont distinguished 12 genospecies based on DNA-DNA hybridization studies (14). Currently, the genus comprises 56 species with valid names (http://www.bacterio.net/acinetobacter.html, last accessed May 2018).

Six of these species, including *A. baumannii*, *A. calcoaceticus*, *A. nosocomialis*, *A. pittii*, *A. seifertii* and *A. dijkshoorniae*, are very closely related and difficult to distinguish by phenotypic properties so it has been proposed to refer to these species as the *A. calcoaceticus-A. baumannii* complex (ACB). Indeed, taxonomic studies based on DNA-DNA hybridization, as well as comparison of housekeeping gene sequences or genome-wide analysis, demonstrated that the ACB complex represents a phylogenetically well-defined subgroup within the genus (15-18). This group of organisms comprises the most clinically relevant species that have been implicated in the majority of both community acquired and nosocomial infections caused by these pathogens (9).

Acinetobacter spp. are among the most frequent causes of hospital-acquired bacterial infections, being considered responsible for 2-10% of all Gram-negative hospital infections (19). Furthermore, the incidence of infection due to MDR strains of this genus continues to increase globally (6). They have been involved in a wide range of infections, particularly in critically-ill patients with impaired host defences, including pneumonia, skin and soft-tissue infections, wound infections, urinary tract infections, meningitis, and bloodstream infections (20).

The most important human pathogen of this group is *A. baumannii*, which is often encountered in intensive care units and in long-term care facilities (21). As mentioned above, it has been classified by the IDSA (Infectious Diseases Society of America) as one of the six most important multidrug resistant microorganisms in hospitals worldwide (6).

1.2. *Acinetobacter baumannii*

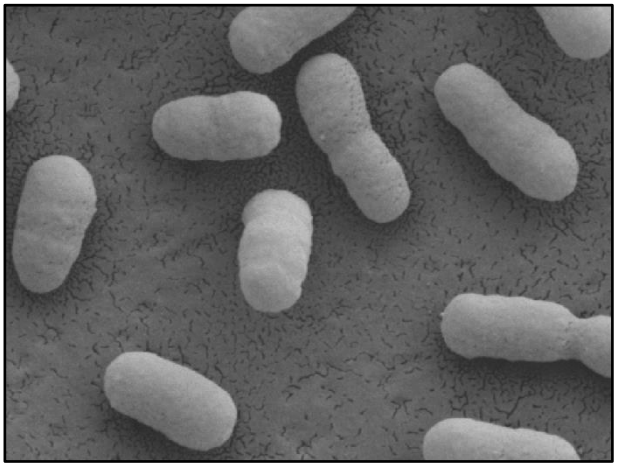

Figure 1. SEM image of *A. baumannii* ATCC 17978. 50000x

A. baumannii is a non-fermentative, oxidase-negative, non-flagellated Gram-negative coccobacillus. It is commonly isolated from medical environments and equipment, medical personnel, and hospitalized patients. This microorganism is considered as highly prevalent in nature and as well as a common component of human flora. However, *A. baumannii* is not a ubiquitous organism and strains from this species are mainly isolated from medical environments and hospitalized patients (22, 23). The ability of *A. baumannii* to resist desiccation and persist on hospital environments together with the facility to develop resistance to multiple antimicrobial agents were the main causes of the emergence of this bacterium as a relevant human pathogen (24).

Multiple studies indicate that *A. baumannii* intrinsically has a higher virulence potential than other *Acinetobacter* spp. Peleg *et al*. showed that *A. baumannii* strains were more letal to the wax moth larva of *Galleria mellonella* than strains of *A. baylyi* and *A. lwoffii* (25). In a different study, all the strains of *A. baumannii* tested were lethal in neutropenic mice, unlike *A. junii*, which was nonlethal (26). Comparison of clinical outcomes in patients infected with *A. nosocomialis*, *A. pittii* and *A.baumannii* resulted in a nearly 9-fold reduction in mortality in non-*baumannii* species compared to *A. baumannii*. Similar results were found using a *G. mellonella* model (27).

1.2.1. Clinical relevance

A. baumannii causes a wide variety of infections. Most of the cases involve the respiratory tract, although bacteraemia, urinary tract infections, endocarditis, meningitis, and wound infection may also occur (28). A study performed in Spain in 2010 showed that the predominant type of infection caused by this pathogen was pneumonia (38.4%), followed by skin infection (20.5%), tracheobronchitis (9.9%), urinary tract infection (9.9%), and intraabdominal infection (9.9%) (24). Infections are more common in patients suffering from an underlying disease or who have undergone major surgical problems. *A. baumannii* can easily enter the body through open wounds, intravascular catheters and mechanical ventilators, which facilitate the establishment of an infection (19).

Hospital-acquired pneumonia occurs most typically in patients receiving mechanical ventilation in the intensive care setting. The crude mortality of ventilator-associated pneumonia caused by *A. baumannii* has been reported to be between 40% and 70% (29-31), although it is controversial whether this mortality is directly attributable to this pathogen. However, several studies have concluded that nosocomial infection caused by *A. baumannii* is associated with increased attributable mortality (32, 33).

Mortality associated to severe *A. baumannii* infection is significantly high, especially when the isolate is resistant to carbapenems. Crude mortality for carbapenem-resistant *A. baumannii* infections ranges from 16 to 76% (34). In addition to clinical complications, the emergence of drug resistance has also resulted in an additional economic burden on

health systems (23). Risk factors contributing to mortality in patients with carbapenem-resistant *A. baumannii* bloodstream infections include the severity of illness, underlying malignancy, presence of a catheter, intubation, history of transplant, higher age, septic shock, concurrent pneumonia, inappropriate antimicrobial therapy, prolonged ICU stay, and renal failure (35-37).

A. baumannii is responsible for occasional sudden outbreaks that are difficult to control (38, 39). The local circumstances of clinical units and their environment determine the type of infection and the consequent risk of dissemination, which could cause an outbreak (40). Such outbreaks may be a reflection of the extreme pressure conditions that hospital emergency units have, leading to breakdowns in infection control procedures and to the epidemic spread of *A. baumannii* (19).

Less commonly, *A. baumannii* may cause community-acquired infections, including pneumonia and bacteraemia. These type of infections are usually associated with alcohol abuse, diabetes, cancer or bronchopulmonary disease and mortality rates can be as high as 60% (19, 20). *A. baumannii* has been also associated with infections subsequent to injuries in conflict areas, as Iraq or Afghanistan, or following natural disasters (40).

1.2.2. Antibiotic resistance

Multidrug resistant *A. baumannii* is recognized to be one of the most difficult antimicrobial resistant Gram-negative bacilli to control and treat. Aside from a big resistance island in its genome, *A. baumannii* has the capacity to rapidly acquire additional resistance genes from other bacterial species (41). This genetic plasticity allows this microorganism to adapt to stressful conditions and to develop antibiotic resistance, which may evolve into a multirresistant pattern following the acquisition of different resistance mechanisms (20, 42). The rapid global emergence of *A. baumannii* strains resistant to all β-lactams, including carbapenems, illustrates the potential of this organism to respond quickly to changes under selective environmental pressure (9). Reports describing infections caused by PDR strains that are resistant to all clinically used antibiotics are specially alarming and represent a challenge for clinicians treating infections caused by these strains (43-45).

Thanks to advances in sequencing and computational techniques, genomic comparative analyses of different strains have revealed the ability of *A. baumannii* to acquire genetic material that contributes to the pathobiology of the bacteria (23). The major resistance mechanisms that have been identified in *A. baumannii* are summarized in Table 1.

Table 1. Major mechanisms of antimicrobial resistance in *Acinetobacter baumannii*.

Antimicrobial agent	Resistance mechanism	Protein	References
β-Lactams	Inactivating enzymes	AmpC, CTX-M, OXA-23, -40, -51, NDM, VIM, IMP	(46-52)
	Decreased outer membrane protein expression	CarO, Omp33-36	(53-55)
	Efflux pumps	AdeABC	(56)
	Altered penicillin-binding protein	PBP2	(57)
Fluoroquinolones	Target modification	GyrA, ParC	(58)
	Efflux pumps	AdeABC, AdeFGH	(58, 59)
Aminoglycosides	Aminoglycoside modifying enzymes	AAC, ANT, APH	(60-62)
	Ribosomal methylation	ArmA	(63, 64)
	Efflux pumps	AdeABC	(65)
Tetracyclines	Ribosomal protection	TetM	(66)
	Efflux pumps	AdeABC, TetA, TetB	(67)
Rifampicin	Target modification	RpoB	(68)
Colistin	Target modification	PmrCAB	(69)

The most prevalent mechanism of β-lactam resistance is the enzymatic degradation by β-lactamases. As penicillins, cephalosporins, and carbapenemes are included in the preferential treatments for many infectious diseases, the presence of these enzymes play a critical role in the selection of appropriate therapy (70). Most of *A. baumannii* clinical isolates are now resistant to cephalosporins, including those of third- and fourth-

generation. The mechanism most frequently associated with resistance to cephalosporins is the over-expression of the chromosomal cephalosporinase AmpC (or ADC, *Acinetobacter*-derived cephalosporinase), related to the presence of the insertion sequence IS*Aba1* upstream of the gene (46, 71).

In the recent past, carbapenems used to be the most adequate antibacterial agent used to treat *A. baumannii* infections. However, the resistance to these compounds has been recently reported with increasing frequency. The most significant mechanism of carbapenem resistance is the production of carbapenemases. These enzymes are β-lactamases that can be sorted into class A, B, C, or D, following Ambler classification (72), being the class D carbapenemases the most frequently found (73). Several phylogenetic subgroups of class B β-lactamases have been identified, including the chromosomally encoded and naturally produced OXA-51/69 and five clusters of acquired carbapenemases (OXA-23, -40, -58, -143, and -235) (74). Non-OXA carbapenemases have also been acquired by *A. baumannii*, such as NDM-1, that has been detected worldwide since 2011 (75). Furthermore, the production of TEM-1, a class A β-lactamase unable to hydrolyse carbapenems, has been associated with resistance to sulbactam, a β-lactamase inhibitor used to mitigate the hydrolysis of ampicillin or cefoperazone by class A β-lactamases (76).

β-Lactam resistance has also been attributed to nonenzymatic mechanisms (9). Thereby, changes in outer membrane proteins (OMPs) reduce the transport into the periplasmic space, limiting the access to penicillin-binding proteins (PBPs) and leading to resistance to these compounds (77). For example, the decreased expression of the outer membrane porin Omp33-36 and the loss of the porin CarO have been associated with carbapenem resistance (53, 54).

The natural role of efflux pumps is to remove chemicals that could damage the membrane, but they have also the ability to expel a wide variety of antibiotics (77). The genome of a multidrug-resistant *A. baumannii* encodes also a wide variety of multidrug efflux systems (61), being the AdeABC a major mechanism of resistance with a broad substrate affinity that includes β-lactams, aminoglycosides, erythromycin, chloramphenicol, tetracyclines, fluoroquinolones, trimethoprim, or ethidium bromide (9, 78).

The presence of genes coding for aminoglycoside-modifying enzymes is highly-prevalent in multidrug resistant *A. baumannii* strains where different classes of enzymes have been described, such as acetyltranferases, nucleotidyltransferases, and phosphotransferases (60). Another aminoglycoside resistance mechanism is the production of 16S ribosomal RNA methyltransferases that protects the 16S rRNA through the methylation of a guanine residue in the aminoglycoside-binding site (79).

Also, single amino acid changes in proteins that are targets of different compounds may be responsible of a higher resistance profile. Thus, modifications in RpoB, GyrA and PmrCAB proteins cause resistance to rifampicin, fluoroquinolones or colistin, respectively, in *A. baumannii* (68, 69, 80).

1.2.3. Pathogenicity and virulence factors

A. baumannii has a unique ability in surviving in the hospital environment and in developing resistance to antibiotics, leading to troublesome hospital outbreaks and therapeutic challenges (81). New advances in genetics and molecular biology, such as genomic analyses, elaboration of gene knockout mutant strains, or transposon mutant libraries, have facilitated our understanding of its physiology and allow us to identify different virulence factors (82).

The success of *A. baumannii* can be attributed to several factors such as its ability to form biofilm and its resistance to desiccation on abiotic surfaces. Biofilms are sessile communities formed by cells embedded in an extracellular matrix that confers resistance to numerous antimicrobial agents and products of the immune system (83). Thus, organisms that are able to grow in this sessile lifestyle are extremely difficult to eradicate. Furthermore, under desiccation conditions, *A. baumannii* undergoes morphological changes, such as thicker cell walls, that contribute to its persistence on solid surfaces (84). Ethanol also enhances the growth of the bacteria under high concentrations of salts, which increases the virulence of *A. baumannii* (85, 86). The ability of *A. baumannii* to adhere to host cells is also an important virulence factor since it is considered the initial step of the colonization and subsequent infection processes (87). Despite the etymology of the term

Acinetobacter (*a-kineto*, nonmotile), motility has been reported several years ago in this genus and it has been related to virulence although its role remains unclear (88, 89).

1.2.3.1. Attachment and biofilm formation

Bacteria have the ability to adhere to solid surfaces leading to a slippery coat. This mode of existence constitute a major component of the bacterial biomass in many different conditions (90). This biofilm is a structured consortium of bacteria embedded in a self-produced polymer matrix consisting of polysaccharides, proteins and extracellular DNA (91). Biofilms are complex structures and constitute a protected mode of growth for bacteria that allows them to survive in a hostile environment. These sessile communities can promote the multiplication and dispersion of non-sessile individuals (83).

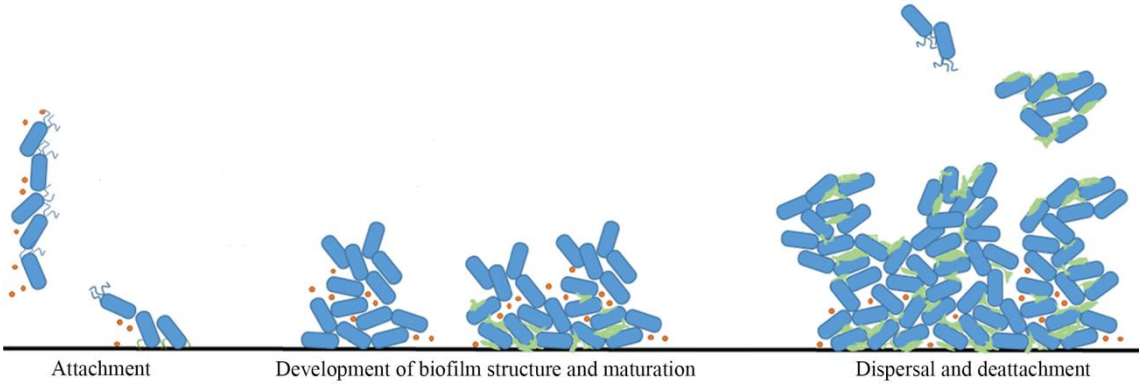

Figure 2. Biofilm formation steps. Modified from Coughlan *et al*. 2016 (92).

The process of biofilm formation is dynamic and complex and involves three stages (Figure 2). The first stage initiates with reversible attachment of bacteria to a favourable surface. Attachment occurs seconds after the bacterial cells detect the required environmental signals (93). During the maturation stage (second stage) the cell aggregates begin to grow in layers in a three-dimensional manner (94). This stage is mostly characterized by cell-to-cell interactions and the formation of important surface components that contribute to the structure of the biofilm (93). The third stage consists of detachment and dispersion. At this final step, sessile communities can give rise to planktonic bacteria that are able to rapidly multiply and disperse to colonize new surfaces.

The nutritional status of the environment usually dictates bacterial behaviour, including the biofilm dispersal response (95, 96).

Bacteria embedded in biofilms exhibit a set of properties that differ substantially from free-living bacteria, mainly due to the structural and functional characteristics of the matrix that protects them (97). Bacterial biofilms are resistant to antibiotics, to disinfectant chemicals, to phagocytosis and to other components of the innate and adaptive inflammatory defense system of the host (91). Thus, a high number of chronic bacterial infections involve bacterial biofilms, which are not easily eradicated by conventional antibiotic therapy. Biofilm infections share clinical characteristics: biofilms grow slowly and symptoms associated with the infection show up late, sessile bacterial cells release antigens and stimulate the production of antibodies, but even in healthy individuals biofilm infections are rarely resolved by the host defense mechanisms (83).

A. baumannii has a remarkable capacity to attach and adhere to medical devices, such as catheters, respiratory equipment, environmental surfaces, and has a notable ability to colonize both biotic and abiotic surfaces forming biofilms (98). Adherence to host cells represents the initial step of colonization or infection. Such capacity is in accordance with the reported ability of some strains to survive for a long time on abiotic surfaces under desiccation conditions (99).

The adherence of *A. baumannii* to biotic and abiotic surfaces usually results in biofilm formation which, in turn, is related with persistence in medical environments and antimicrobial resistance, causing disease (23). Adherence and biofilm formation to both abiotic and biotic surfaces have showed a high variability among different strains (100, 101). Furthermore, there are significant variations in the cell density and biofilm structures formed on these surfaces depending on the strains (101). These variations may be influenced by common factors such as nutrient availability, bacterial surface components, quorum sensing abilities, and regulatory networks (102). Growth temperature and concentration of extracellular free iron also affect the amount of biofilm formed on abiotic surfaces, as well as the presence of blue light and ethanol (86, 87, 103, 104).

Nowadays, a number of genes have been reported to play a role in adherence and biofilm formation on abiotic and biotic surfaces. However, there seems to be no direct correlation

between adhesion on host cells and biofilm formation on abiotic surfaces (98). A study comparing the ability to adhere to eukaryotic cells and to form biofilms of different strains reported that there is not a clear relationship between these two abilities (100).

- **Type I pili**

Type I pili are one of the most common protein structures present on the pathogen surfaces, playing a major role in adherence and/or biofilm formation of Gram-negative bacteria (105). One example is the *csuE* gene, which truncation causes a decrease in biofilm formation but does not affect the ability of the mutant strain to adhere to bronchial epithelial cells (106). This gene is part of the operon *csuA/BABCDE*, that encodes an usher-chaperone assembly system and mediates pilus production required for the initial steps of bacterial attachment on abiotic surfaces in *A. baumannii* ATCC 19606^{T} (107). This operon is controlled by a two-component regulatory system that includes a sensor kinase encoded by the *bfmS* gene and a response regulator encoded by the *bfmR* gene. Inactivation of *bfmR* resulted in abolition of the whole operon expression, no pili production and loss of biofilm formation on plastic (108). Accordingly, experiments performed with a *bfmS* knockout strain showed that this sensor kinase mediates not only biofilm formation, but also adherence to biotic surfaces, serum resistance, and antibiotic susceptibility (109).

Another type I pilus was found in the *A. baumannii* MAR002 clinical strain. This pilus showed to by encoded by the operon LH92_11070-11085, playing a role in biofilm formation and attachment to eukaryotic cells. The inactivation of the major subunit of this system, encoded by the LH92_11085 gene, significantly reduced bacterial attachment to human alveolar cells and biofilm formation on plastic surfaces (110). This gen, homologue to A1S_2091 from *A. baumannii* ATCC 17978, was described as one of the most abundant proteins present in the pellicle matrix of different clinical strains, together with proteins CsuA/B (111). The subunit encoded by the A1S_1510 gene, which is part of the fimbrial operon A1S_1510-1507, was also abundant in the pellicle samples (111). Taken together these data suggest that the above mentioned pili systems are essential for biofilm formation.

- **Type V secretion systems**

The type V secretion systems encompasses the autotransporter proteins, the two-partner secretion system (TPS) and the type Vc family proteins. It has been reported that the TPS pathway mediates the translocation across the outer membrane of large effector proteins involved in interactions between the pathogens and their hosts (112). However, most of the autotransporter proteins remain to be characterized (113).

The autotransporter Ata, a surface protein in the adhesion ability of *A. baumannii*, is critical for biofilm formation since its deletion resulted in a lower biofilm production (114). This protein also mediates the adhesion of bacterial cells to collagen type IV of eukaryotic cells, being demonstrated its role in virulence since mice infected with *A. baumannii* ATCC 17978 wild type strain resulted in higher levels of mortality than those mice infected with the *ata*-negative strains (114).

The TPS AbFhaB/FhaC of *A. baumannii* AbH12O-A2 clinical strain was reported to be involved in adhesion since the inactivation of this system significantly decreased bacterial attachment to human alveolar cells. Moreover, the TPS AbFhaB/FhaC is implicated in fibronectin-mediated adherence and plays a role in *A. baumannii* virulence (115).

- **Membrane-associated proteins**

The bacterial adhesin Bap (biofilm-associated protein) was identified by Loehfelm *et al*. as an important factor for the development of mature biofilm structures (116). Transposon-mediated inactivation of *bap* gene leaded to a remarkable decrease in the biofilm thickness produced by the clinical strain *A. baumannii* 307-0294 (116). A different study proved that biofilm formation on abiotic surfaces by *A. baumannii* strains is inhibited using a Bap-specific antibody, confirming that this protein is expressed at the cell surface and that it is associated with biofilm formation (117). Bap has also been described as a surface structure involved in adherence to human bronchial epithelial cells and neonatal keratinocytes (118).

One of the most abundant porins in the outer membrane, OmpA, has been described as implicated in attachment to plastic and in the interaction with human epithelial cells and *Candida albicans* filaments (119-121). In *A. baumannii*, OmpA is a well-characterized virulence factor as it has been shown to induce apoptosis in human epithelial cells, to

promote invasion of epithelial cells by interacting with fibronectin and to be necessary for persistence of the bacteria in the mouse lung (122-124). Furthermore, OmpA is involved in antimicrobial resistance and its disruption decreases the minimal inhibitory concentrations (MICs) of several antibiotics (125).

- **Surface polysaccharides**

Exopolysaccharides, apart from surface proteins, form a key component of *A. baumannii* biofilms. Poly-β-(1-6)-*N*-acetylglucosamine (PNAG) is the major polysaccharide polymer and it is synthesized by proteins encoded by the *pgaABCD* locus (126). Although the deletion of this locus has no effect on biofilm formation under static conditions, very striking differences were observed when cultures were grown with vigorous shaking, suggesting that PNAG may be essential for maintaining the integrity of the biofilm (126). PNAG has also been described as a virulence factor that protects bacteria against innate host defenses (102, 126).

- **Quorum sensing**

Cell population density is a mechanism used for bacteria to control adherence and biofilm formation (23). Quorum sensing is an important global regulatory system in bacteria that provides mechanism to coordinate the behaviour of individual bacteria in a population. In Gram-negative species, acyl-homoserine lactones (AHLs) are mainly employed as signalling molecules able to control biofilm formation (127). Luo *et al*. (128) showed that *A. baumannii* ATCC 19606^{T} cells cultured in the presence of *N*-Hexanoyl-L-homoserine lactone over-expressed the *csuA/BABCDE* genes and had higher biofilm densities than the control strain. Accordingly, the addition of a quorum quenching lactonase, an enzyme that degrades AHL, caused the disruption of biofilm formation in *A. baumannii* S1 strain (129).

An autoinducer synthase coded by *abaI* gene was proved to be responsible for the production of AHL in *A. nosocomialis* strain M2 (130). The deletion of the above mentioned gene caused a decrease in the ability to form biofilm in the mutant strain compared to the wild type strain. Furthermore, the addition of ethyl acetate extract from wild type cells restored the capacity of the mutant strain to form normal biofilms, suggesting that this biofilm defect was due to a loss of AHL signal (130).

The production of AbaI-dependent AHL activated the transcription of A1S_0112-A1S_0118 genes in *A. nosocomialis* strain M2 (131). This set of genes, along with A1S_0119, was predicted to be involved in the biosynthesis of a secondary metabolite (105, 131). Insertions in genes A1S_0112 and A1S_0115 in *A. baumannii* 17978hm made this strain unable to form pellicle, a specialised type of biofilm localised at the interface between air and liquid (132).

- **Environmental factors**

Different cues as temperature, light or concentration of extracellular free iron are relevant for the interaction of *A. baumannii* with the host and also affect the amount of biofilm formed (23). Blue light modulates biofilm and pellicle formation through *blsA* gene, which codes for a protein that carries a blue-light-sensing-using flavin domain and is an active photoreceptor protein (104). While the incubation of *A. baumannii* ATCC 17978 for 4 days in LB at 24°C under blue light produced no biofilm on glass tube, this strain formed biofilm on the tube walls as well as pellicles when incubated under the same conditions in darkness (104).

Other culture conditions as temperature, shaking or the surface material may vary the amount of biofilm formed by a strain, as Tomaras *et al*. reported (107). Thus, *A. baumannii* ATCC 19606^{T} formed more biofilm at 30°C than at 37°C, under stagnant conditions and in polystyrene or polypropylene tubes than in borosilicate ones (107). A recent study indicated that even though *A. baumannii* has the ability to form biofilm on different materials such as glass, porcelain, stainless steel, rubber, polycarbonate or polypropylene, the amount of biofilm formed varied significantly being polycarbonate the surface wherein more biofilm was developed (133).

1.2.3.2. Motility

The lack of flagellar genes in *A. baumannii* resulted in the assumption that this microorganism is unable to move or at least to show swarming motility, which is mediated by flagella (102). However, *A. baumannii* spreads rapidly over surfaces, probably due to twitching, and displays differential motility in response to different

factors (23). Movement on the surface of semi-solid media diminishes as the concentration of agar or agarose increases as well as different types of agar affect the motility response (101, 131, 134). Furthermore, different strains display different motility patterns and not all strains move on semi-solid surfaces (101, 131). Although the type of motility displayed by *A. baumannii* has not been fully elucidated, previous studies suggest that it may vary in response to illumination, quorum sensing or other factors (23, 104, 131).

- **Type IV pili**

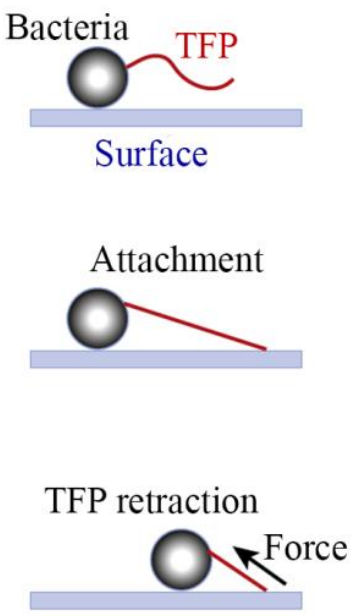

Figure 3. The type IV pili pulls the cell body along surfaces by cycles of polymerization, surface adhesion, and retraction. Modified from Maier and Wong. 2015 (135).

The type IV pili (TFP) are organelles with polymeric organization that extend several micrometres from the cell body (135). Due to their ability to assemble and disassemble rapidly, TFP participate in processes such as natural transformation, twitching motility, and adherence to abiotic and biotic surfaces (136). By cycles of polymerization, surfaces adhesion, and retraction, TFP pull the cell body along surfaces mediating twitching motility (Figure 3) (135). Thus, an insertion in *pilT*, coding for an ATPase required for the retraction of the TFP, impaired surface motility of *A. nosocomialis* strain M2 (131). Furthermore, *pilA*, *pilD*, and *pilT* mutants of this strain lost their ability to be naturally transformable and did not exhibit twitching motility (136). The major fimbrial subunit, PilA, is highly variable among *A. baumannii* strains and these differences are correlated with their motility abilities (100).

- **Lipopolysaccharide and exopolysaccharide**

Production of lipopolysaccharide and exopolysaccharide is required for motility in many Gram-negative bacteria. Mutations in genes involved in their synthesis may cause a decrease in motility (137-139). In *A. baumannii* 307-0294 strain the motility phenotype is affected by the activity of the glycosyltransferase LpsB (140), as well as by the protein tyrosine kinase PTK and the polysaccharide export outer membrane protein EpsA,

required for capsule polymerization and assembly (141). Mutant strains harbouring transposon insertions in *lpsB*, *epsA*, and *ptk* resulted in a reduction in motility. These strains were capable of some motility but their movement was significantly diminished and the pattern varied from the wild type strain (142). Furthermore, the presence of Congo red in the medium, a dye that inhibits the production of exopolysaccharide, produced a wide variety of changes in motility of several *A. baumannii* strains (142).

- **Quorum sensing**

Several bacterial pathogens utilize quorum sensing cell communication to coordinate multiple virulence factors, such as motility, once a population size threshold is reached (143). Accordingly, the addition of a non-native AHL enhances twitching motility in *A. baumannii* ATCC 19606^T strain (128). Furthermore, the loss of the autoinducer synthase AbaI, present in different strains of *A. baumannii* and *A. nosocomialis* strain M2, cause decrease in motility and the phenotype is restored when exogenous AHL is added (131). The altered expression of genes regulated by the AbaI quorum-sensing pathway results also in deficiencies in the motility pattern. Insertions in genes A1S_0112, A1S_0113 and A1S_0115, which are up-regulated by quorum sensing, cause non-motile phenotypes (131, 132).

The intercellular communication mediated by AHL requires protein receptors. This role seems to be played by AbaR in *A. baumannii*, a LuxR-type receptor that activates the transcription of QS target genes (144). Some non-native AHLs act as AbaR antagonists inhibiting *A. baumannii* motility (145). Furthermore, the presence of AidA, a hydrolase enzyme with quorum quenching activity, was recently related with a non-motile phenotype in several *A. baumannii* strains (146).

- **Environmental factors**

Motility is dependent upon different environmental signals that may vary its pattern (142). Light is a ubiquitous cue that may cause physiological changes in many organisms. Due to the production of light-sensing photoreceptors, different bacteria are able to detect and respond to light (104). Thereby, while *A. baumannii* ATCC 17978 cells grew only around the inoculation point in the presence of blue light, bacteria moved away from the

inoculation site covering almost half of the surface when motility plates were incubated in darkness (104).

Motility phenotype is dependent upon the medium richness, and extracellular stressful conditions applied to the bacteria can alter them (142). Accordingly, the addition of a sub-inhibitory concentration of antibiotics such as ampicillin and kanamycin or the disinfecting agent triclosan reduced the motility of different isolates of *A. baumannii* (142). Other stressful conditions, as an increased osmolarity or the addition of 1% ethanol reduced motility of all strains (142).

1.2.3.3. Iron acquisition

Competition for iron by potential pathogens is essential to establish an infection. Although iron is one of the most abundant elements in environmental and biological systems, ferric iron is not readily available for bacteria either in the environment or in the human host due to its low solubility under aerobic conditions and its chelation by compounds as heme, lactoferrin and transferrin (147). This micronutrient plays an essential role in diverse cellular processes such as electron transport, nucleic acid biosynthesis, and protection from free radicals (148).

Bacteria can survive and divide under iron-limiting conditions by exploiting different strategies for high-affinity iron acquisition, including production of siderophores, ferric iron chelators that are released outside cells, uptake of exogenous chelators, as heme and heterologous siderophores, and acquisition of ferrous iron (102). Low free-iron concentration in host organisms is used by pathogens as a stimulus to express not only iron-acquisition systems, but genes coding for virulence factors, such as hemolysins, toxins and proteases (149).

- **Siderophores**

Siderophores are low molecular weight compounds with high affinity for iron. Once they are secreted from the cell and sequester ferric iron from the extracellular environment, siderophores are transported back into the cell through specific outer membrane receptors (150). The best-characterized siderophore in *A. baumannii* is acinetobactin, which was

initially described in the ATCC 19606^T strain (151, 152). Impairment of acinetobactin biosynthesis and transport in *A. baumannii* ATCC 19606^T affects the virulence of the strain, significantly reducing its ability to persist and kill the host in a *G. mellonella* larvae infection model (153). The expression of *entA* gene, which is essential for the biosynthesis of the acinetobactin precursor 2,3-dihydroxybenzoic acid, is also needed for the full virulence of the ATCC 19606^T strain (154). Furthermore, a study showed that acinetobactin production occurred significantly more frequently in clinical isolates compared with avirulent isolates of *A. baumannii* (155).

Apart from acinetobactin, different gene clusters encoding siderophores, including cluster A1S_1647-1657, cluster A1S_2562-2581, cluster orn73-entD, and cluster ABAYE1888-1889, have been described in several *A. baumannii* strains (156). Cluster A1S_1647-1657 is as extended as acinetobactin among *A. baumannii* strains, being present in all genomes analysed except SDF. Cluster A1S_2562-2581 is found in *A. baumannii* ATCC 17978 and *A. baylyi* ADP1 strains. Cluster orn73-entD seems to be present only in strain 8399, while cluster ABAYE1888-1889 was studied in all strains except in ATCC 17978 and ADP1 (156-158).

- **Other iron-uptake systems**

The production and secretion of siderophores is an energy intensive mechanism of iron uptake, but bacteria can adapt to iron limited environment through the expression of a range of iron acquisition mechanisms (156). A major route for bacterial iron assimilation is the direct uptake of ferrous iron by using the Feo system, which consists of the cytosolic FeoA protein, the inner membrane permease FeoB, and the putative transcriptional repressor FeoC (159). Sequenced genomes of *A. baumannii* showed the presence of genes encoding for a Feo transport system in these specie (160, 161).

A. baumannii also uses heme as an iron source since two clusters encoding functions related to heme uptake have been identified (160). The chromosomal cluster annotated as A1S_1608-1614 in ATCC 17978 could be involved in the transport of heme from the periplasm into the cytoplasm (23). Genes of this putative operon encode two heme-binding protein A precursors and ABC-type transporter proteins that could be implicated in iron acquisition from heme (162). The second cluster, present in ACICU, AB0057 and SDF strains, includes an outer membrane receptor gene, the *hemO* gene involved in

oxidative cleavage of heme to release iron, as well as a *tonB* gene, which is part of a protein complex that mediates energy transduction from the inner to the outer membrane (160).

1.2.3.4. Other virulence factors

Multiple virulence factors that promote bacterial colonization and infection of the host are required for the pathogenesis of the bacteria (102). Despite extensive research into the virulence potential of *A. baumannii*, little is still known about its pathogenic repertoire. Although an increasing number of genomes have been sequenced and phenotypic analysis have allowed us to identify of several factors responsible of its pathogenicity, relatively few virulence factors have been described to date (147).

Probably the best-characterized virulence factor of *A. baumannii* is OmpA (23). OmpA is a porin, an outer membrane protein responsible of modulating cellular permeability. As previously mentioned, this protein is not only involved in attachment to biotic and abiotic surfaces, but also in pathogenesis of this microorganism. Purified OmpA binds to host epithelial cells, targets mitochondria, and induces apoptosis (119, 122). The *ompA* gene was shown to be involved in persistence of *A. baumannii* in the lung, assessed using a murine pneumonia model (124), as well as in biofilm formation and surface motility, which highlights its role on the pathogenesis of *A. baumannii* (121, 131).

The Omp33-36, which acts as a water passage channel, is also associated with *A. baumannii* cytotoxicity. Its deletion reduces adherence and invasion of human lung epithelial cells (163). Purified Omp33-36 induces apoptosis and modulates autophagy in human cells (164). Decreased expression of genes encoding the porins CarO and OprD-like are also associated with attenuated virulence in a mouse model (165).

The cellular envelope is associated with several factors that may contribute to pathogenicity in mammalian infection disease models (166). The production of capsular polysaccharides occurs in many isolates from patients with *A. baumannii* infections (167). Experiments with capsule-defective mutants have demonstrated the role of capsular exopolysaccharide of *A. baumannii* in virulence in a mouse septicaemia model as well as

in a rat soft tissue infection model (141, 168). Capsular polysaccharides are also involved in antimicrobial resistance as mutants have lower intrinsic resistance to peptide antibiotics (166). In addition, the presence of antibiotics induces hyperproduction of capsular polysaccharides which increases virulence in a mouse model of systemic infection (166).

Lipopolysaccharide is the major component of the outer leaflet of the outer membrane and it is an immunostimulatory molecule that plays an important role in bacterial resistance to external stressful conditions (169). In *A. baumannii*, lipopolysaccharide plays a major role in virulence and survival of the bacteria as assessed by animal model experiments (147). Mutant cells with truncated lipopolysaccharide showed a decreased resistance to human serum and a reduced survival rate in a rat model of soft tissue infection (140). Furthermore, modification of lipopolysaccharide contributes to resistance to many clinical antibiotics, such as colistin (69).

Some Gram-negative bacterial species secrete outer membrane vesicles (OMVs) during bacterial growth. OMVs are 20-200 nm diameter spherical vesicles that have been shown to participate in quorum sensing, biofilm formation, gene transfer, and transport of virulence factors (170, 171). Pathogen-host interactions may be mediated by OMVs through the delivery of diverse virulence factors to the interior of host cells, allowing the pathogens to interact with the host without a close contact (172). A study showed that an *A. baumannii* strain that produced abundant OMVs containing virulence factors was able to induce more cytotoxicity and stronger innate immune response in host cells (173). Due to the importance of OMVs in virulence, these structures have been used as candidates for acellular vaccines (171).

Phospholipases (PLases) are lipolytic enzymes that catalyse the cleavage of phospholipids in bacterial membranes and are considered virulence factors in many bacteria. Degradation of phospholipids affects the stability of host cell membranes, and may interfere in the host immune response (174). According to the cleavage site on their substrate, PLases are classified into different groups: carboxyl ester acyl hydrolases, PLases C, and PLases D. Carboxyl ester acyl hydrolases include PLase A, PLaseB and lysophospholipase A (174). PLases C and D have been described in several pathogenic bacteria, playing a role in virulence (175, 176). Disruption of *A. baumannii* PLase D causes a reduction in its ability to thrive in serum, a deficiency in epithelial cell invasion,

and diminished pathogenesis in a murine model of pneumonia (177). Accordingly, the inactivation of a PLase C gene causes a modest decrease in the cytotoxic effect caused by *A. baumannii* on epithelial cells (178).

PBPs are commonly involved in resistance to β-lactam antibiotics. However, PBPs contribute to bacterial cell stability by participating in the biosynthesis of the peptidoglycan layer (23). PBPs are usually classified into high-molecular-mass and low-molecular mass. High-molecular-mass PBPs enable peptidoglycan polymerization and insertion into the cell wall (179). Low-molecular-mass PBPs, such as PBPs 7 and 8, contribute to cell separation and peptidoglycan remodelling (180). The inactivation of PBPs 7 and 8 caused a significant decrease in the survival of the *A. baumannii* mutant strain in rats' soft tissue and pneumonia infection models (181). β-Lactamase PER-1 has also been suggested to be a virulence factor as several PER-1-producing strains adhered to eukaryotic cells while all PER-1-negative strains showed a lack in cell adhesion (182).

1.2.4. Animal models

Animal models are a necessary tool in order to evaluate novel treatments, characterize the host immune response, and identify bacterial virulence factors. The first report describing an animal model for *A. baumannii* infection was published in 1997 (183). Since then, the development of new animal models allowed an increase of information available about *A. baumannii* pathogenesis (23). The use of animal models allows researchers to investigate human diseases resembling the progression of the infection as occurs in human patients. However, it is utopic to hope that laboratory animals can truly reproduce human diseases. Performing animal models requires complex facilities to maintain and handle large number of animals, thus non-mammalian models have emerged recently as useful tools to study microbial pathogenesis (81). Many research teams all over the globe have been implementing a simple model system for the host-pathogen interaction assays. Accordingly, invertebrate models have become popular as they are less expensive, less ethically challenging, and more efficient, as well as they allow the assessment of a larger number of bacterial mutant strains than the common murine models in the same experiment (184). The most commonly used invertebrate models include the

Caenorhabditis elegans worm model, the *Drosophila melanogaster* fly model, the *G. mellonella* caterpillar model, and the *Dictyostelium discoideum* amoebic model (81). The identification of virulence determinants in a wide range of human pathogenic bacteria have been described through the use of these models worldwide (185).

Non-mammalian models have been used to study *A. baumannii* pathogenesis since 2004, when a *C. elegans* killing assay was implemented to measure survival of worms after exposure to this pathogen with and without ethanol (85). Following this work, *C. elegans* and *D. discoideum* were used to screen an *A. baumannii* transposon-generated library for isolating mutants with attenuated virulence in presence of ethanol (162). Instead of using a killing assay, mutants of this study were tested for the reduction of brood size in *C. elegans* and the inhibition of *D. discoideum*. A recent study from Vallejo *et al*. (186) showed that the *C. elegans* fertility model was able to detect virulence differences between several *A. baumannii* strains and the results obtained were validated in a murine sepsis model. Furthermore, *C. elegans* models have been performed not only to examine the virulence features of different *A. baumannii* strains (187-189), but also to study the interaction between this pathogen and *C. albicans*. Therefore, this model can be used to investigate the complex dynamics of a polymicrobial infection (190).

G. mellonella, the larval stage of the wax moth, has also been used as model for infection caused by *A. baumannii* (25). In this model, caterpillars are infected by injecting the pathogen into the hemocel of the animal. Peleg *et al*. showed that the rate of *G. mellonella* killing was dependent on the infection inoculum and on the virulence of the strains injected. Furthermore, treatment of lethal *A. baumannii* infection with proper antibiotics prolonged the survival of *G. mellonella* caterpillars (25). This model has been used to evaluate the *in vivo* efficacy of different treatments against multidrug-resistant *A. baumannii* strains (191, 192). Different proteins that play a role in pathogenesis have also been identified using *G. mellonella* models, such as the acinetobactin-mediated iron acquisition system or the proteins involved in the synthesis of 1,3-diaminopropane or phospholipase D (153, 193, 194).

Despite the advantages of non-mammalian models, mammalian infection model systems remain the gold standard for pathogenesis-based research. Mammalian models of *A. baumannii* infection allow the characterization of a host response similar to that found in

human infections and permit the study of antibiotic regimens for the treatment of infections (23).

Due to the low virulence of most *A. baumannii* strains in mice, high infection doses are required to establish infection. Alternatively, several studies have employed immunocompromised mice to facilitate infection or porcine mucin mixed in the inoculum, as it protects bacteria from rapid innate immune clearance (23). Although, these models may mask host-microbe interactions that are important in determining host outcome, they likely expose virulence functions that become relevant in the context of an infection (195).

Lung infection models in small rodents, produced by either intratracheal or intranasal inoculation, have been thoroughly reported in *A. baumannii* since this pathogen mainly causes pneumonia (81). Earlier mouse models of pneumonia were performed to test the pharmacodynamics properties of various antibiotics where animals were infected through intratracheal instillation (183, 196). One of the first studies using intranasal inoculation in a murine pneumonia model was carried out to identify the importance of the lipopolysaccharide-induced inflammatory response to *A. baumannii* infection (197).

The pneumonia model has also been employed for characterizing virulence factors involved in respiratory infections. Thus, the role of OmpA on *A. baumannii* pathogenesis was determined infecting C57BL/6 mice intratracheally in order to perform an experimental pneumonia model. The bacterial burden in mice infected with an OmpA defective strain was significantly lower than in those infected with the wild type strain (120). Even though the most of the animal models have employed mice, models using other species have also been implemented. Hraiech *et al*. compared the fitness and lung pathogenicity of two isogenic *A. baumannii* strains in a rat model of pneumonia, concluding that mutations in the *pmrA* gene, related to colistin resistance, lead to a reduced virulence (198).

Animal models for studying soft tissue infection by *A. baumannii* have also been performed. Rat soft tissue infection has proved to be an efficient model to discriminate among the differences in virulence abilities of various *A. baumannii* isolates (199). The utilization of mutant strains for challenge in this type of infection model allowed the identification of genes essential for growth *in vivo* (199). Above mentioned studies

performing rat soft tissue infections demonstrated that a capsule-positive phenotype and an integral lipopolysaccharide are important for *in vivo* survival of *A. baumannii* (140, 141).

Models of *A. baumannii* causing sepsis have also been reported recently. Although the source of infection, usually intraperitoneal instillation, is an important difference between this model and the human disease process, this model has been extensively performed due to its easy inoculation, high mortality, and the possibility of measuring bacterial loads in multiple organs (23). Several *A. baumannii* virulence factors, such as the acinetobactin, the Omp33-36 or the autotransporter Ata, have been identified through intraperitoneal mouse sepsis models (114, 153, 163). Mouse septicemia models have also been employed to characterize the efficacy of vaccines and to evaluate the effect of antibiotic resistance on virulence and fitness (200, 201).

1.3. Small RNAs in bacteria

Bacteria have specific regulatory molecules for controlling gene expression. RNAs transcripts are well-known regulators and their roles cannot be ignored in any organism. In bacteria, an important number of these RNA regulators exist as short transcripts (50-300 nucleotides) that act on independently expressed targets and that are commonly referred to as small RNAs (sRNAs) (202). Regulatory RNAs can modulate transcription, translation, mRNA stability, and DNA maintenance or silencing through a variety of mechanisms, such as changes in RNA conformation, protein binding, base pairing with other RNAs, or interactions with DNA (203).

First sRNA was discovered in 1981. It was 108 nucleotide long, named as RNA I, that blocked ColE1 plasmid replication by base pairing with the RNA that is cleaved to produce the replication primer (204). This sRNA and some others were identified by gel analysis due to their abundance or simply by chance (203). Since 2001, new sRNAs have been discovered by systematic computational searches or by direct detection using cloning-based techniques or microarrays using probes in intergenic regions (205, 206). The availability of bacterial genome sequences have led to the identification of an

increasing number of regulatory RNAs. Recent technical advances, including multilayered computational searches and deep sequencing, have allowed the prediction of hundreds of candidate regulatory RNAs (207, 208).

Riboswitches, the simplest bacterial RNA regulatory elements, are defined as sequences found at the 5' end region of mRNA, or less frequently at the 3' end region, that adopt different conformations in response to environmental signals that act as ligands (209). They usually consists of two parts: the aptamer region, which binds the ligand, and the expression platform, which regulates gene expression through alternative RNA structures that affect transcription or translation (210). Upon binding of the ligand, the riboswitch changes conformation, creating alternative hairpin structures which form or disrupt transcriptional terminators or antiterminators, or which occlude or expose ribosome binding sites (203). Generally, the riboswitches in Gram-positive bacteria affect transcriptional attenuation, while more frequently inhibit translation in Gram-negative (211).

Some sRNAs interact with proteins regulating their activity by sequestering them from normal targets, particularly RNA binding proteins, or producing more complex outcomes, as the modification of an enzymatic activity (202). Small RNAs that regulate RNA binding proteins typically work by mimicry, since those sRNAs contain the protein recognition sequence. Noted examples are CsrB and CsrC sRNAs of *Escherichia coli*, which modulate the activity of CsrA, an RNA-binding protein that regulates mRNA translation and stability (212). Other sRNAs bind proteins with enzymatic activity, inhibiting, activating or modifying their function, such as the *E. coli* 6S RNA, which binds to the housekeeping form of RNA polymerase regulating transcription (213).

Most characterized sRNAs regulate gene expression by base pairing with mRNAs. Some of them, known as *cis*-encoded sRNAs, have extensive potential for base pairing with their target mRNA while *trans*-encoded sRNAs have more limited complementarity with their targets (203). The first group is encoded in *cis* on the DNA strand opposite the target RNA and share extended regions of complete complementarity with their target. Regulatory mechanisms employed by these sRNAs encompass transcriptional attenuation, inhibition of translation or promotion of RNA degradation or cleavage (Figure 4) (214).

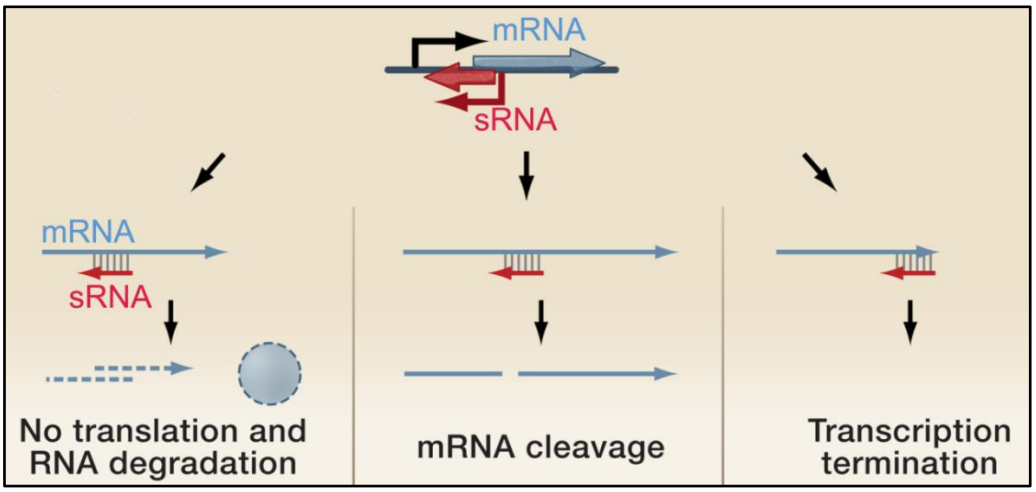

Figure 4. *Cis*-encoded sRNAs. Modified form Waters and Storz. 2009 (203).

Early identified *cis*-encoded antisense RNAs were found opposite to replication genes of plasmids or to transposase genes, controlling maintenance and stability of mobile genetic elements (215). Many of these sRNAs are components of type I toxin-antitoxin systems. These two-gene elements consist of a stable toxin and an unstable *cis*-encoded RNA antitoxin that base pairs with the toxin mRNA, inhibiting its translation and leading to degradation (216).

Trans-encoded sRNAs consist on a diverse family of short, usually untranslated, transcripts that share only limited complementarity with their target mRNAs regulating their translation and/or stability (203). Most of them are global regulators of gene expression and are often induced in response to stress or inside of host cells (217). In contrast to *cis*-encoded sRNAs, each *trans*-encoded sRNA typically base pairs with multiple mRNAs. Thus, a single sRNA may globally modulate a particular physiological response, in the same manner as a transcription factor does, but at a post-transcriptional level (203).

The majority of the regulation mediated by *trans*-encoded sRNAs blocks gene expression. Base pairing between the sRNA and its target mRNA usually leads to repression of protein levels through translational inhibition, mRNA degradation, or both (Figure 5) (218). However, sRNAs can also activate expression of their target through an anti-antisense mechanism whereby base pairing of the sRNA disrupts an inhibitory secondary structure which sequesters the ribosome binding site (RBS) (219). In many cases, the RNA chaperone Hfq is required for *trans*-encoded sRNA-mediated regulation,

presumably to facilitate RNA-RNA interactions due to the limited complementarity between the sRNA and the mRNA target (203).

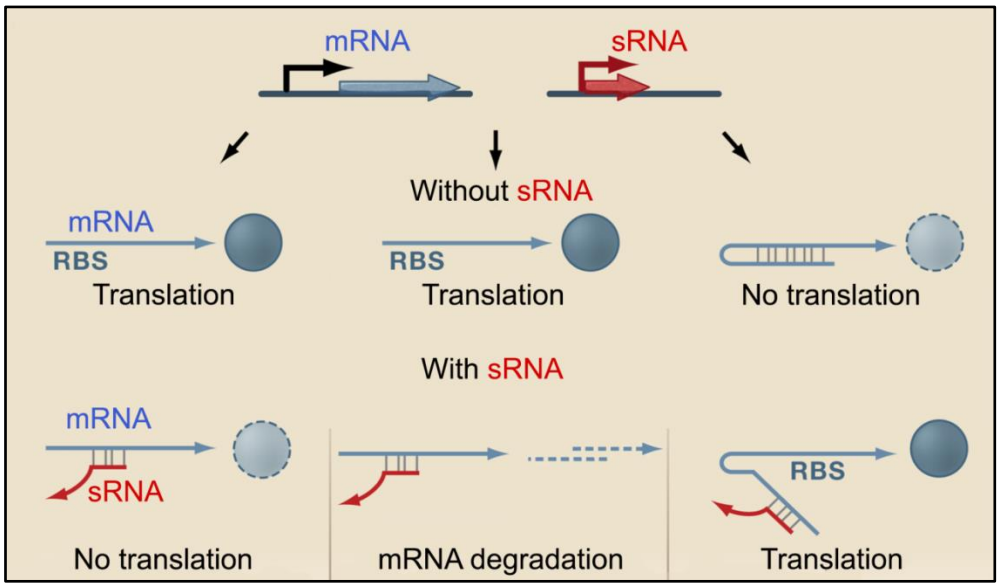

Figure 5. *Trans*-encoded sRNAs. Modified from Waters and Storz. 2009 (203).

Recently, an adaptive microbial immune system named Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), has been identified and provides resistance to viruses as well as prevent plasmid conjugation in bacteria and Archaea (220). Immunity is acquired by integrating short fragments of foreign DNA into CRISPR loci. Following transcription and processing of these loci, the CRISPR RNAs guide the CRISPR-associated (Cas) proteins to invading complementary nucleic acid, which results in target interference (221).

CRISPR-Cas systems are highly diverse, which is probably a result of the dynamic selective pressure that is imposed by invading mobile genetic elements (221). CRISPR sequences are variable DNA regions which consist of a ~550 bp leader sequence followed by series of repeat-spacer units (222). Spacer regions contain sequence homologous not only to foreign DNA but also to endogenous chromosomal regions of the bacteria. This self-targeting was proposed to lead to a form of autoimmunity, suggesting a role in regulation of endogenous gene expression (223). Adjacent to the CRISPR DNA array are several Cas genes. Two to six core Cas genes seem to be associated with most CRISPR systems (222).

The functionality and the role of CRISPR-Cas system are not fully established. It may promote bacterial fitness through protection against bacteriophages increase indirectly the survival rate and the ability to infect host cells. Alternatively, CRISPR-Cas system might have a direct influence by regulating endogenous virulence factors (224).

1.3.1. Regulatory roles of small RNAs

Bacteria live in between markedly different environments, which require detection, integration, and response to different conditions and subsequent realignment of the bacterial physiology and metabolism. Signals detected include changes in temperature, nutrient availability, or cues from the host and neighbouring bacteria (215). Gene expression is regulated at many different levels, beyond transcriptional control at the DNA level. Regulatory RNAs are recognized as important factors in many physiological and adaptive responses in bacteria (225). Some of them have been identified as previous missing links in the regulatory pathways that allow bacteria to sense population density, to modulate and to modify cell-surface properties, to fine-tune their metabolism during cell growth, and to regulate virulence gene expression (226).

1.3.1.1. Role of small RNAs in biofilm formation

Biofilm structure protects bacteria from a damaging environment. Biofilm formation is one of the most complex physiological processes in bacteria. Thus, the decision-making process of bacteria to form biofilm or not should be precisely and dynamically regulated in response to environmental changes (227). Although transcriptional regulation is the most well studied form of controlling biofilm production, post-transcriptional regulation allows bacteria to rapidly adjust to the changing environment. Post-transcriptional regulatory mechanisms monitoring biofilm formation include RNA-binding proteins and *cis*- and *trans*-acting small RNAs (93).

The c-di-GMP RNA is implicated in controlling several cellular functions including virulence, motility, and adhesion, but its principal role consists on modulating the switch

that controls the transformation from motile planktonic lifestyle to the sessile biofilm state (228). High c-di-GMP levels promote the synthesis of exopolysaccharides, as well as the auto-aggregation and the surface adhesion, which locks the cells in a sessile state. In contrast, low c-di-GMP concentrations inhibit biofilm formation and the production of adhesive surface organelles, stimulating swimming and swarming motility (229).

One of the best characterized regulatory elements is CsgD, the global transcription factor that integrates signals to control biofilm formation in *E. coli* and *Salmonella* (93). CsgD induces the expression of the *csgBAC* operon, required for the production of curli fimbriae and cellulose, two factors needed for biofilm formation, as well as the production of c-di-GMP or the repression of flagellar operons favouring the biofilm-formation phenotype (230). CsgD can be repressed by several sRNAs, such as OmrA, OmrB, GcvB, McaS, and RprA, that respond to various stress conditions (231). The non-coding RNA McaS disfavours biofilm formation by binding and exerting inverse control on *csgD* and the *flhCD* operon, the master regulator of flagella expression in *E. coli* and related bacteria (232). In the absence of McaS, *csgD* upregulation occurs and an increased McaS expression activates the synthesis of *flhDC* (232, 233). However, the McaS-dependent regulation of biofilm seems to be complex, and also activates PNGA leading to a CsgD independent pathway of biofilm formation (233).

RpoS, the general stress response sigma factor, is activated by the sRNA RprA, which targets several branches of the CsgD network in response to environmental cues (234). Cell transition into stationary phase activates RprA and subsequently *csgD* expression and curli biosynthesis (234). The non-coding RNAs OmrA and OmrB are induced during high osmolarity conditions and their over-expression inhibits curli and cellulose production (235).

Although most regulatory RNAs directly interact with mRNA targets, this is not the only mode of action of sRNAs. The above mentioned small RNAs CsrB and CsrC interact with the protein CsrA and prevent the latter from binding to several biofilm-relevant mRNAs (236). Cells entering into stationary phase induce CsrB and CsrC, which sequester and inactivate CsrA. This fact switches from the production of flagella to the synthesis of the biofilm matrix component PNAG (237).

As described above, biofilm formation and development is an intricate process involving finely altered gene expression, requiring complex and well-coordinated regulation. Small RNAs can contribute to post-transcriptional regulation and make to rapidly adjust and fine-tune gene expression required for the cell during biofilm formation (93).

1.3.1.2. Role of small RNAs in metabolism

Changes in nutrient abundance trigger major switches in gene expression and require coordination of global regulatory networks. These calculations that depend on the kind and amount of nutrients usually implicate transcriptional regulators and RNA regulators (238). One of them is the previously mentioned Csr (carbon storage regulatory) system, which ensures connections between carbon metabolism and other traits in many bacteria. In *E. coli*, CsrA is the repressor that regulates carbon starvation and glycogen biosynthesis, biofilm formation, motility, virulence, quorum sensing, and stress response system by binding to conserved sequences in its target mRNA that alter their translation and/or turnover (237). The sRNAs CsrB and CsrC, antagonize its activity during the exponential growth phase and repress metabolic pathways related to the stationary phase (212).

Amino acids may also be used as energy sources by bacteria and the regulation of their catabolism may involve sRNA. In *Bacillus subtilis*, the sRNA Sr1 controls AhrC, a transcriptional activator of *rocABC* and *rocDEF* (239). These operons encode catabolic and transport enzymes required for the use of arginine. Sr1 transcription is stimulated by the presence of L-arginine or stationary-phase entrance and it is repressed when sugars are used as the energy source (240). The sRNA GcvB is also involved in the regulation of amino acid catabolism. It is induced by high concentrations of glycine and regulates *oppA* and *dppA* mRNAs, which encode oligopeptide and dipeptide periplasmic binding proteins, respectively (241).

As a nutrient, iron is required for the operation of many important enzymes in central metabolism, as well as synthesis processes. The sRNA RyhB of *E. coli* is a key regulator of iron homeostasis that modulates cellular physiology under iron-starvation conditions by regulating a large set of genes that primarily encode iron-containing enzymes (242).

sRNAs with similar functions have been identified in different Gram-negative species, such as PrrF1 and PrrF2 of *P. aeruginosa* (238). *In E. coli* and *P. aeruginosa*, the production of sRNA associated with iron assimilation is regulated by the Fur repressor (243).

1.3.1.3. Role of small RNAs in virulence

Pathogenic bacteria possess intricate regulatory networks that temporally control the production of virulence factors, and enable bacteria to survive and proliferate after host infection. Regulatory RNAs are now recognized as important components of these networks (226). Small RNAs have an impact on bacteria pathogenicity as they participate in regulation of biofilm formation, motility, and cellular metabolism. Moreover, several sRNAs have been identified as regulators of different virulence factors (238).

Vibrio cholerae pathogenicity as well as its life cycle are considered well studied models for the understanding of molecular processes involving virulence gene expression. Two sRNAs regulators that affect pathogenicity of *V. cholerae* are TarA and TarB, both of which are controlled by the master virulence regulator ToxT (226). TarA negatively regulates the major glucose transporter PtsG (244), whereas TarB down-regulates the secreted colonization factor TcpF and the transcription regulator VspR (245, 246). VspR modulates the expression of several genes, including *dncV*, which encodes a di-nucleotide cyclase and is required for efficient intestinal colonization (246). A mutant strain lacking TarA is compromise for mouse colonization in competition with wild type, suggesting its role in the *in vivo* fitness showed by *V. cholerae* (244). However, the *tarB* knockout strain outcompetes the parental strain, indicating that TarB is a negative regulator of virulence of *V. cholerae* (245).

The expression of a variety of genes that play an important role in the virulence and survival of *P. aeruginosa* is affected by the sRNAs RsmY and RsmZ, analogous to *E. coli* CsrB and CsrC, which act sequestering the transcriptional regulator RsmA (247). RsmA controls the switch between acute and chronic infection through the regulation of motility and biofilm (248).

These and many other examples indicate that sRNAs are required for a coordinated response and play a crucial role in virulence. Despite the increasing number of identified sRNAs, only a few of them have been functionally characterized (238). Thus, further studies are required in order to fully decipher the role that regulatory RNAs play in pathogenicity.

1.3.1.4. Role of small RNAs in antibiotic resistance

Traditionally, efforts to find novel therapeutic treatment options have been focussed on bacterial proteins as drug targets. However, regulatory RNAs have proven to be important factors for the bacterial response and resistance to antibiotics (249). Mechanisms controlling antibiotic resistance genes at the post-transcriptional level generates an immediate response, which is beneficial when antibiotic concentrations increase rapidly (249). sRNAs play a key role in regulatory circuits controlling antibiotic resistance through various processes, including antibiotic uptake, modifications of the cell envelope, drug efflux pumps, and DNA mutagenesis mechanisms that facilitate the emergence of novel mechanisms of resistance (250-253).

Antimicrobial resistance in *E. coli* is modulated by the sRNAs MicF, GcvB, and RyhB, which regulate genes required for antibiotic uptake. MicF represses translation of the porin OmpF, a major antibiotics uptake pathway. Deletion of *micF* gene increases the susceptibility to cephalosporins and norfloxacin whereas its over-expression increases the resistance of *E. coli* against these antibiotics (250). GcvB represses the mRNA of the serine transporter CycA and the iron-responsive sRNA RyhB sensitizes *E. coli* to colicin Ia (254).

Several sRNAs regulate genes coding for drug efflux pumps, active transporters that pump toxic substances, including antibiotics, out of the cell. The AcrAB-TolC multidrug efflux pump is negatively regulated by the sRNA SdsR. This sRNA binds and represses the *tolC* mRNA, which encodes the porin of AcrAB system, preventing the efflux of lipophilic antibiotics (252). Thus, over-expression of SdsR reduces resistance to novobiocin and several quinolone antibiotics (250, 252).

Recently, a library of *E. coli* strains overproducing and lacking sRNAs was screened for altered susceptibility to various antibiotics. Over-expression of 17 out of 26 sRNAs affected resistance or susceptibility to antibiotics suggesting that sRNAs are important elements in controlling antibiotic resistance genes (250).

1.3.2. Small RNAs in *A. baumannii*

Small RNAs have proved to be a class of gene regulators of several cellular processes in bacteria. Although expression patterns and regulatory mechanisms employed by sRNAs are well understood in *Enterobacteriaceae* such as *E. coli* or *Salmonella* (255, 256), researchers are still not able to confirm whether sRNA-mediated regulation in *A. baumannii* is analogous to these well-studied systems (257).

The first study reporting sRNAs in *A. baumannii* was performed by Sharma *et al*. (258), wherein a total of 31 putative sRNA were predicted by a bioinformatic approach. Three of them, named AbsR11, AbsR25 and AbsR28, were detected by Northern blot and did not show similarity with previously described sRNAs. Differential expression profile of AbsR25 in presence of varying amounts of ethidium bromide suggested an expression influenced by environmental or internal signals.

Recently, Weiss *et al*. have identified 78 sRNAs in the *A. baumannii* AB5075 strain based on RNA sequencing (RNA-seq) results (259). Using conservation analysis six types of similar sRNAs were identified, with one found to be particularly abundant and homologous to regulatory C4 antisense RNAs found in bacteriophages. Additionally, two sRNAs were found to be antisense to phage-derived transcripts, giving them the potential to exert broad regulatory influence.

Only the sRNA Aar has been studied as a regulatory RNA in *A. baylyi*. Over-expression of Aar in *trans* did not affect bacterial growth but seven mRNA targets were upregulated in stationary growth phase. All those seven mRNAs were shown to be involved in *A. baylyi* amino acid metabolism (260). This sRNA is conserved in *A. baumannii*, as Aar homologs were found in *A. baumannii* strains SDF, AB307-00294, AB5075, ACICU, AYE, and ATCC 17978 (259, 260).

The role of the RNA chaperone Hfq, involved in the regulation of diverse genes *via* sRNAs, has been studied in *A. baumannii* ATCC 17978 (261). In the absence of the *hfq* gene, the mutant strain exhibited retarded cell growth and reduced biofilm formation, airway epithelial cell adhesion and invasion, and survival in macrophage. The loss of *hfq* gene also caused a decrease in outer membrane vesicles secretion and fimbriae production and affected transcription of genes involved in stress response. Taken together, these data indicate that Hfq plays a critical role in environmental adaptation and virulence in *A. baumannii* by modulating stress response and virulence factors (261).

So far, very few regulatory RNAs have been defined in *A. baumannii* and the mechanisms of RNA-mediated gene regulation remain unknown. Thus, multiple efforts are required to understand how genes are regulated by sRNAs in this pathogen.

2. OBJECTIVES

A. baumannii is one of the most important pathogens that cause hospital infections and constitutes a remarkable public health problem due to its ability to develop antibiotic resistance and to adapt to stressful conditions. The capacity of *A. baumannii* to form biofilm allows its resistance to desiccation, to antimicrobial agents, and to survive to the host immune system. The understanding of the different steps that regulate *A. baumannii* biofilm formation may offer the key to explain its virulence mechanisms.

Thus, the objectives of the present study were the following:

1. The study of the gene expression patterns of *A. baumannii* planktonic and sessile cells.
2. The identification of genes involved in biofilm formation in *A. baumannii*.
3. The characterization of the A1S_0114 gene and the study of its role in different biological processes including attachment to eukaryotic cells, virulence, and biosynthesis of metabolites.
4. The description of sRNAs in *A. baumannii* and the comparison of their expression in different lifestyles, including biofilm.
5. The description of sRNAs implicated in biofilm regulation.
6. The study of the role of the 13573 sRNA in biofilm formation and attachment.

3. CHAPTERS

3.1. CHAPTER I: Whole transcriptome analysis of *Acinetobacter baumannii* assessed by RNA-sequencing reveals different mRNA expression profiles in biofilm compared to planktonic cells

A. baumannii has become an important opportunistic pathogen because of its genetic plasticity, which enables its adaptation to stressful conditions and to develop antibiotic resistance. Furthermore, the *A. baumannii* ability to colonize both biotic and abiotic surfaces and to grow in a sessile lifestyle plays an important role in the capacity of the microorganism to persist and spread in the hospital environment. A deep understanding of the mechanisms that promote biofilm formation is essential to identify new therapeutic targets. Thus, transcriptome sequencing is a goal tool to gain insight into the differences in genes expressed under diverse conditions.

This chapter is focused on a transcriptome analysis performed over different *A. baumannii* ATCC 17978 lifestyles. Transcripts from sessile biofilm cells, as well as planktonic cells from exponential and stationary phase, were analysed by Illumina RNA-seq in order to recognise distinct expression patterns and to detect genes that were differentially expressed.

Although many genes were constitutively expressed, results showed clearly particular patterns in biofilm and in planktonic cells. A total of 1621 genes were over-expressed in biofilm compared to planktonic cells and 55 genes were exclusively expressed in sessile cells. Some of these genes were involved in transcriptional regulation, amino acid metabolism and transport, and iron acquisition. Genes encoding efflux pumps, a fimbrial protein, Csu proteins or an acyl carrier protein were also detected.

Five genes encoding an acyl carrier protein (ACP) (A1S_0114), a hypothetical protein (A1S_0302), a fimbrial protein (A1S_1507), a pilus assembly protein (A1S_3168), and a transcriptional regulator (A1S_2042) have been demonstrated to play a role in biofilm formation as their disruption caused a decrease in this capacity of the mutant strains compared to the wild type ATCC 17978 strain.

The gene A1S_0114 was deleted from the genome to obtain a stable mutant (Δ0114). This knockout strain produced significantly less biofilm than the wild type strain. The

role of the A1S_0114 gene in the ability of *A. baumannii* to form biofilm was confirmed and the mutant strain was complemented with a plasmid harbouring A1S_0114 gene, restoring the wild type phenotype. The loss of the A1S_0114 gene caused a reduction of the expression level of the surrounding genes A1S_0109, A1S_0112, and A1S_0113 during the stationary phase of growth. Since A1S_0109 encodes the only homoserine lactone synthase described to date in *A. baumannii*, these results also confirmed that quorum sensing plays a role in biofilm formation. Moreover, the A1S_0112-0118 cluster of genes could constitute an operon involved in biofilm formation and quorum sensing as all these genes are over-expressed in biofilm-associated cells compared to planktonic cells.

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Whole Transcriptome Analysis of *Acinetobacter baumannii* Assessed by RNA-Sequencing Reveals Different mRNA Expression Profiles in Biofilm Compared to Planktonic Cells

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Abstract

Acinetobacter baumannii has emerged as a dangerous opportunistic pathogen, with many strains able to form biofilms and thus cause persistent infections. The aim of the present study was to use high-throughput sequencing techniques to establish complete transcriptome profiles of planktonic (free-living) and sessile (biofilm) forms of *A. baumannii* ATCC 17978 and thereby identify differences in their gene expression patterns. Collections of mRNA from planktonic (both exponential and stationary phase cultures) and sessile (biofilm) cells were sequenced. Six mRNA libraries were prepared following the mRNA-Seq protocols from Illumina. Reads were obtained in a HiScanSQ platform and mapped against the complete genome to describe the complete mRNA transcriptomes of planktonic and sessile cells. The results showed that the gene expression pattern of *A. baumannii* biofilm cells was distinct from that of planktonic cells, including 1621 genes over-expressed in biofilms relative to stationary phase cells and 55 genes expressed only in biofilms. These differences suggested important changes in amino acid and fatty acid metabolism, motility, active transport, DNA-methylation, iron acquisition, transcriptional regulation, and quorum sensing, among other processes. Disruption or deletion of five of these genes caused a significant decrease in biofilm formation ability in the corresponding mutant strains. Among the genes over-expressed in biofilm cells were those in an operon involved in quorum sensing. One of them, encoding an acyl carrier protein, was shown to be involved in biofilm formation as demonstrated by the significant decrease in biofilm formation by the corresponding knockout strain. The present work serves as a basis for future studies examining the complex network systems that regulate bacterial biofilm formation and maintenance.

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Introduction

Acinetobacter baumannii are non-fermentative, oxidase negative, non-flagellated gram-negative bacilli. Although this species is a normal inhabitant of the human skin flora, intestinal tract, and respiratory system, it has been shown to cause severe disease, including bacteremia and pneumonia, especially in patients hospitalized in intensive care units and reanimation wards [1–5]. Consequently, *A. baumannii* was recently listed as one of the six most dangerous opportunistic

pathogens [6,7]. Its high genetic plasticity allows it to rapidly adapt to stressful or otherwise unfavorable conditions by acquiring mutations, plasmids, or transposable elements. Moreover, *A. baumannii* species exhibit a remarkable ability to develop antibiotic resistance, which may quickly evolve into a multiresistant pattern following the acquisition of different resistance mechanisms, including β-lactamases, efflux pumps, porins, penicillin-binding proteins (PBPs), and methylase enzymes [1,2,8–17].

Bacteria often adopt sessile lifestyles in the form of matrix-enclosed habitats referred to as biofilms [18]. These are dynamic structures in which transitions between planktonic and sessile modes of growth occur in response to different environmental signals. The bacterial species inhabiting biofilms differ physiologically and behaviorally from their free-living counterparts [19]. Importantly, the structural characteristics of biofilms make them resistant to most antibiotics and host defenses [19–24]. This persistence provides a source of recurrent infections. In the case of *A. baumannii*, the infection of mucous surfaces and bacterial contamination of medical devices, such as intravascular catheters or endotracheal intubation, may result in biofilm formation, increasing the risk of bloodstream and respiratory infections [9].

An understanding of the ability of *A. baumannii* to form biofilms that adhere to and persist on a broad range of surfaces may offer the key to revealing its pathogenic mechanisms. Biofilm formation in *A. baumannii* has been shown to involve several regulatory processes, including those based on the sensing of bacterial cell density, the presence of different nutrients, and the concentration of free cations available to bacterial cells. Some of these extracellular signals may be sensed by two-component regulatory systems such as BfmRS. This transcriptional regulatory system activates expression of the usher-chaperone assembly apparatus responsible for the production of pili, which are needed for cell attachment and subsequent biofilm formation on polystyrene surfaces [25–27]. Recently, three new two-component sensor/regulator systems involved in biofilm formation in *P. aeruginosa* were identified [28]. In *A. baumannii*, a homolog of the biofilm-associated protein (Bap) of *Staphylococcus aureus* has been described [29] and the involvement of the membrane protein OmpA in the development of solid biofilms on abiotic surfaces and in virulence was demonstrated [30,31].

Several studies have reported changes in amino acid metabolism during biofilm development [32–35]. In their analyses of planktonic and sessile cells from biofilms of *A. baumannii* ATCC 17978, Cabral et al. [34] found differences in their proteomic profiles. In general, processes involved in bacterial adhesion and the formation and development of biofilms engage complex regulatory networks that coordinate the temporal expression of genes related to adhesion, motility, and the synthesis of matrix components. Although the ability of *A. baumannii* to form biofilms on abiotic surfaces contributes to the unique survival pattern of this pathogen in hospital settings, little is known about the mechanisms that promote and support biofilm formation. However, this knowledge is essential to the identification of new therapeutic targets and thus to the design of drugs effective against persistent diseases caused by multi-resistant biofilm-forming clones of *A. baumannii*.

Microarray technology has been used to obtain the complete transcriptional profiles of different microorganisms and offers an approach to studying biofilm formation [36,37]. For example, Whiteley et al. [38] found significant differences in gene expression between sessile and planktonic cells in *Pseudomonas aeruginosa*. Moreno-Paz et al. [19] demonstrated different profiles in cells of the iron-oxidizing bacteria *Leptospirillium* grown in biofilm vs. planktonic modes. In *A. baumannii*, Hood et al. described the distinct transcriptional profile of the bacterium in response to NaCl [39] while Eijkelkamp et al. [40] were able to analyze its transcriptome in cultures grown under iron-limiting conditions (which prevents biofilm formation), reporting major transcriptional changes mostly related to iron acquisition but also to motility processes.

Among the more recent techniques used to analyze the genome-wide RNA profiles of a number of organisms is deep sequencing, using the platforms 454 GS_FLX (Roche), Genome Analyzer or HiSeq (Illumina Inc.), and ABI SOLID (Life Technologies). These are open platforms not limited to the study of previously known genes, and they are sensitive as well as fast [41–48]. RNA sequencing using the Illumina system has developed as an extremely informative technique for the study of transcriptional profiles of microbes [45,49,50].

The aim of the present study was to use bacterial mRNA and the Illumina RNA-sequencing technologies to gain insight into the mechanisms behind the remarkable ability of *A. baumannii* to form biofilms. We therefore obtained whole transcriptomes from planktonic and biofilm cells of *A. baumannii* strain ATCC 17978 and then compared them for differences in their gene expression profiles.

Materials and Methods

Strains and culture conditions

A. baumannii ATCC 17978 was routinely grown in Mueller-Hinton (MH) broth. *E. coli* TG1, used for cloning procedures, was grown in Luria-Bertani (LB) broth. Agar was added to a final concentration of 2% when necessary. All strains were grown at 37 °C with shaking (180 rpm), and stored at -80 °C in LB broth containing 10% glycerol. Kanamycin (50 μg/mL) and rifampicin (50 μg/mL) were from Sigma-Aldrich (St. Louis, MO) and were added to select transformant strains. Cultures of planktonic cells originated from a single colony of *A. baumannii* strain ATCC 17978 isolated in MH agar and then grown in 5 mL of MH broth overnight as described above. The resulting culture was diluted 100-fold in 500 mL of MH broth in 1-L flasks and again grown as described above, measuring the optical density at 600 nm (OD600nm) every 30 min. Cells were harvested during the exponential (OD600nm = 0.4) and late stationary phases (OD600nm = 2.0) of growth, 48 h after inoculation. Planktonic and sessile cells (obtained as described below) were resuspended in RNA Later reagent (Sigma-Aldrich), frozen using liquid nitrogen, and stored at -80 °C.

Biofilm generation in Pyrex plates

A. baumannii ATCC 17978 biofilms were obtained in the Fermentation Laboratory of the Agrobiotechnology Institute (Navarra, Spain). A sample from an overnight culture of *A. baumannii* grown in MH broth was used to inoculate 60-mL microfermentors (Institute Pasteur, Paris, France), which were then maintained at 37 °C for 24 h. The bacterium was grown in MH broth medium under a continuous-flow culture system and continuous aeration consisting of 40 mL of compressed, sterile air/h. Submerged Pyrex slides served as the growth substratum. Biofilms that formed on the Pyrex slides were removed with a cell scraper and frozen in liquid nitrogen at -80 ˚C.

Isolation of mRNA

Three samples, corresponding to exponential and stationary phase cells and sessile cells from biofilms, were reduced to powder under liquid nitrogen while grinding using a mortar and pestle. Total RNA was then isolated using the mirVana miRNA isolation kit (Ambion) following the manufacturer's protocols. Ten µg of each total RNA was further processed by removing 23S and 16S rRNAs using the MICROBExpress bacterial mRNA enrichment kit (Ambion). The rRNA-depleted samples (free of 16S and 23S rRNA) of exponentially growing, stationary phase, and biofilm cells were treated with DNAse I (Invitrogen), purified using phenol-chloroform, and concentrated by ethanol precipitation. Final concentrations and purity grades of the samples were determined using a NanoDrop ND-1000 (Thermo Scientific) and a BIOANALYZER 2100 (Agilent Technologies Inc., Germany).

Transcription assays

A cDNA synthesis kit (Roche) was used to obtain double-stranded cDNA (ds-cDNA), following the manufacturer's instructions. The rRNA-depleted samples together with 5'-phosphorylated degenerated hexamers and the AMV reverse transcriptase (both from Roche) were used to obtain the first cDNA strand. The second cDNA strand was then generated and treated with RNase. The ds-cDNA products were purified using the High Pure PCR purification kit (Roche). The samples were further quantified using a Nanodrop ND-1000 (Thermo Scientific) and a BIOANALYZER 2100 (Agilent Technologies Inc., Germany). The ds-cDNAs were then used in subsequent steps of the study.

Deep-sequencing procedures

To characterize the complete transcriptomes of the studied samples, mRNA libraries from three cellular conditions (exponential and stationary phase planktonic cells and sessile cells from biofilms) were prepared following the Truseq RNA sample preparation protocols from Illumina Inc. at CIC bioGUNE's genome analysis platform (Derio, Spain). Two biological replicates were studied for each sample.

Read processing and comparisons of gene expression profiles

Fifty nucleotide reads from each mRNA library were obtained using HiScanSQ (Illumina Inc., CIC bioGUNE, Bilbao, Spain). Short reads were aligned against the complete genome of *A. baumannii* ATCC 17978 and plasmids pAB1 and pAB2 (GenBank accession codes: NC_009085.1, NC_009083.1 and NC_009084.1, respectively) using Bow tie [51], allowing a maximum of three mismatches within the first 50 bases. Reads were annotated with the R Bioconductor Genominator package and differences in expression levels estimated with the R DESeq package [52]. DESeq performs a count normalization to control the variation in the number of reads sequenced across samples. After normalization, fold changes and their significance (*p* values), indicating differential expression, were determined after a negative binomial distribution. Those mRNAs with *p* values (adjusted for a false discovery rate of 0.1%) < 0.001 were considered to be differentially expressed. Raw sequences were deposited at the NCBI Sequence Read Archive, under Bioproject accession number PRJNA191863 (experiment accessions codes SRX263965, SRX263966, SRX263968 and SRX263969 to SRX263977). Blast2GO [53] was used for the functional re-annotation of genes, the mapping of gene ontology terms, and the description of biological processes, molecular functions, cellular components, and metabolic pathways associated with the biofilm expression profiles.

Quantitative biofilm assay

Biofilm formation was quantified using the procedure described by Tendolkar et al. [54], with slight modifications. A colony of *A. baumannii* was grown on MH agar medium for 18 h at 37 ˚C and used to inoculate 25 mL of liquid MH medium, supplemented with 50 µg kanamycin /mL when necessary. The culture was maintained overnight at 37 ˚C and 180 rpm. Cells were harvested by centrifugation (3500 *g*, 10 min), washed three times with 0.9% NaCl, and resuspended in fresh liquid medium without antibiotic. From this suspension, 100 µL (containing 10^8 CFU) were dispensed into each well of a 96-well flat-bottom polypropylene microtiter plate containing MH medium and then incubated at 37 ˚C for 24–48 h. Next, the cells were stained with 25 µL of a 1% w/v crystal violet solution for 15 min at room temperature, washed twice with sterile 0.9% w/v NaCl, solubilized with 200 µL of a 4: 1 v/v mixture of ethanol and acetone, and finally quantified at 570 nm. All biofilm assays were performed with at least six replicates for each strain. ANOVA tests were used to evaluate the statistical significance of the measured differences.

Gene disruption

Plasmids were inserted into the target genes as previously described [55], with slight modifications. Briefly, kanamycin- and zeocin-resistant plasmid pCR-BluntII-TOPO (Invitrogen), unable to replicate in *A. baumannii*, was used as a suicide vector. An internal fragment (~ 500 bp) of the target gene was PCR-amplified using the primers listed in Table 1 and genomic DNA from *A. baumannii* ATCC 17978 as template. The PCR products were cloned into the pCR-BluntII-TOPO vector and

the recombinant plasmids (0.1 μg) were introduced into kanamycin- and zeocin-susceptible *A. baumannii* ATCC 17978 by electroporation. Mutants were selected on kanamycin-containing plates. Inactivation of the target gene by insertion of the plasmid *via* single-crossover recombination was confirmed by sequencing the PCR-amplified products using the primers listed in Table 1.

Construction of knockout strains

Knockout strains were constructed using the plasmid pMo130 (Genbank accession code EU862243), containing the genes *xylE*, *sacB* and a kanamycin resistance marker, as a suicide vector [56]. Briefly, 851–932 bp upstream and downstream of the *A. baumannii* ATCC 17978 (Genbank accession code NC_009085.1) gene of interest were cloned into the pMo130 vector using the primers listed in Table 1. The resulting plasmid was used to transform *A. baumannii* by electroporation. Recombinant colonies representing the first crossover event were obtained using a combination of kanamycin selection and visual detection of XylE activity following the cathecol-based method described by Hamad et al. [56]. Bright yellow and kanamycin-resistant colonies were grown overnight in LB supplemented with 15% sucrose and then plated on the same agar medium. The second crossover event was confirmed by PCR using the primers listed in Table 1. Quantitative biofilm assays were used to determine the phenotype of the mutants.

Complementation of stable knockout mutant

To complement the stable knockout mutant, the target gene was amplified from *A. baumannii* ATCC 17978 genomic DNA using the primers listed in Table 1 and then cloned into the *Xba*I restriction site of the pET-RA plasmid under the control of the β-lactamase CXT-M-14 gene promoter, as described by Aranda et al. [57]. The new construction was used to transform the mutant strain. Transformants were selected on rifampicin- and kanamycin-containing plates and confirmed by PCR using the primers listed in Table 1. The mutant strain containing the pET-RA plasmid was used as the control.

Real-time RT-PCR

Real-time reverse transcription-PCR (RT-PCR) was carried out to determine the expression levels of a collection of genes using Taqman probes (TIB Mol Biol) listed in Table 1. In all cases, the expression levels were standardized relative to the transcription levels of the housekeeping gene *gyrB*. The primers used were those listed in Table 1. Total RNA was isolated from exponentially growing and stationary phase cultures and from the biofilms using the High Pure RNA isolation kit (Roche, Germany) and then treated with RNase-free DNase I (Invitrogen Corporation, CA). The samples were further purified using the RNeasy MinElute Cleanup kit (Qiagen, Germany). For qRT-PCR, the LightCycler 480 RNA Master hydrolysis probes kit and a LightCycler 480 RNA instrument (both from Roche, Germany) were used together with the following protocol: initial incubation of 65 °C, 3 min, followed by a denaturation step at 95 °C for 30 s, 45 cycles at 95 °C, 15 s and 60 °C, 45 s, and a final elongation step at 40 °C,

Table 1. Oligonucleotides and probes used in the present work.

Primer/Probe name	Sequence	Use in the present study
0114intF	actggagcgcaatcattcgt	Disruption of gene A1S_0114
0114intR	atgaagcaactccctgctgc	Disruption of gene A1S_0114
0114extF	caaggagtttgaaacgat	Confirm disruption of gene A1S_0114
0114extR	ctcgcagcaatagaccaa	Confirm disruption of gene A1S_0114
0302intF	cggaagcagtggtaaacttgc	Disruption of gene A1S_0302
0302intR	tggtgaaaacacgcgagagc	Disruption of gene A1S_0302
0302extF	acaccaactatttccgtg	Confirm disruption of gene A1S_0302
0302extR	cccaaaatcagtcaccct	Confirm disruption of gene A1S_0302
1507intF	ccacaccaactccgtttgct	Disruption of gene A1S_1507
1507intR	acttgcaaccgtgccaatga	Disruption of gene A1S_1507
1507extF	tgtgtgtgatcatttgac	Confirm disruption of gene A1S_1507
1507extR	aagagcggtttactcatc	Confirm disruption of gene A1S_1507
3168intF	atctcgagcagcttgtgcag	Disruption of gene A1S_3168
3168intR	attaagccgtggtgcaggtg	Disruption of gene A1S_3168
3168extF	actcttattgccaaaacc	Confirm disruption of gene A1S_3168
3168extR	cttgcttaatgatggagg	Confirm disruption of gene A1S_3168
2042intF	tgactggatttacacagaaga	Disruption of gene A1S_2042
2042intR	tgttccatcattaataactcc	Disruption of gene A1S_2042
2042extF	ccagagcactagccttaa	Confirm disruption of gene A1S_2042
2042extR	ttgagtgagtgcagctaa	Confirm disruption of gene A1S_2042
0114UpFNotI	cccgcggccgcgggttggtacgtgagcaactc	Construction of stable knockout strain ΔA1S_0114
0114UpRBamHI	gggggatcccccggggtaatctccttttaacc	Construction of stable knockout strain ΔA1S_0114
0114DownFBamHI	cccggatccgggacaaccttgcacgactagaa	Construction of stable knockout strain ΔA1S_0114
0114DownRXbaI	gggtctagaccttcaagtcgacctgctacg	Construction of stable knockout strain ΔA1S_0114

Table 1 (continued).

Primer/Probe name	Sequence	Use in the present study
pMo130 site2 F	attcatgaccgtgctgac	Confirm construction of stable knockout strain ΔA1S_0114
pMo130 site2 R	cttgtctgtaagcggatg	Confirm construction of stable knockout strain ΔA1S_0114
0114XbaIF	ccctctagaggggttattcgctcgtattgctg	Cloning of the gene A1S_0114 into the pET-RA plasmid for complementation of the stable knockout strain ΔA1S_0114
0114XbaIR	ccctctagaggggactggttgaccttcacatc	Cloning of the gene A1S_0114 into the pET-RA plasmid for complementation of the stable knockout strain ΔA1S_0114
pETRAF	ttcttcgtgaaatagtgattttt	Confirm complementation of stable knockout strain ΔA1S_0114
pETRAR	ctgtttcatatgatctgggtatc	Confirm complementation of stable knockout strain ΔA1S_0114
A1S_0109F	caaacatcgaatatccatcaatcgtc	qRT-PCR
A1S_0109R	cagccgtagatttttcaaatccg	qRT-PCR
A1S_0109 Taqman probe	cctctagcagtcaggctgtgtcatcacc	qRT-PCR
A1S_0112F	accagaagatgttggcctga	qRT-PCR
A1S_0112R	gagccgatcaaccccata	qRT-PCR
A1S_0112 Taqman Probe	gctgcctg	qRT-PCR
A1S_0113F	tggctttaacaacgctgaaa	qRT-PCR
A1S_0113R	aaccctgaccttcttcacc	qRT-PCR
A1S_0113 Taqman Probe	tgccctga	qRT-PCR
A1S_0114F	gtagagcctgagacgattgatcca	qRT-PCR
A1S_0114R	gttggctcaagttctaatttcgtca	qRT-PCR
A1S_0114 Taqman Probe	ttctaaatccccagacacagacaaagcaa	qRT-PCR
A1S_0302F	gcaggtaaagcaataatatcgaaag	qRT-PCR
A1S_0302R	ttatcaactaaggagaagctagcaagt	qRT-PCR
A1S_0302 Taqman Probe	ggaagcag	qRT-PCR
A1S_1507F	acaccaactccgtttgcttt	qRT-PCR
A1S_1507R	ctgacacttcaaatagccaggtt	qRT-PCR
A1S_1507 Taqman Probe	tcagcagc	qRT-PCR
A1S_3168F	tcgcatctcgagcagctt	qRT-PCR
A1S_3168R	cgcagctggtaattttgctt	qRT-PCR
A1S_3168 Taqman Probe	cagccacc	qRT-PCR

Table 1 (continued).

Primer/Probe name	Sequence	Use in the present study
A1S_2042F	tggtatattgactggatttacacaga	qRT-PCR
A1S_2042R	catcattaataactccatcgagg	qRT-PCR
A1S_2042 Taqman Probe	tggctctatgagcttgtttttctatttt	qRT-PCR
gyrBF	tctctagtcaggaagtgggtacatt	qRT-PCR
gyrBR	ggttatattcttcacggccaat	qRT-PCR
gyrB Taqman Probe	tggctgtg	qRT-PCR

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30 s. All assays were performed in triplicate. The statistical significance of the determined differences was confirmed by ANOVA tests.

Results

Determination of the complete transcriptomes of planktonic and biofilm cells

The mRNA fractions purified from exponentially growing (Exp) and stationary-phase (Sta) cultures and from biofilms (Bio) of *A. baumannii* ATCC 17978 were analyzed to determine the respective gene expression level profiles and to identify differentially expressed genes. Six libraries, including two biological replicates *per* sample, were constructed (Exp 1, Exp 2, Sta 1, Sta 2, Bio 1, Bio 2) and paired-end sequenced using Illumina technology (50 bpx 2). Insert average sizes in the above mentioned libraries were 208, 240, 230, 239, 209 and 253 bp, respectively. Reads were aligned against the chromosome and plasmids of *A. baumannii* ATCC 17978. The number of reads that mapped against the genome is detailed in Table 2. Gene level read counts are shown in Figure S1, and MD plots and correlation between samples in Figure S2. The complete mRNA transcriptomic profiles of exponentially growing and stationary-phases cultures and from biofilm cells were obtained by Illumina procedures. Gene expression values are provided in the Supporting Information (Tables S1, S2, and S3). Table S1 shows the gene expression profile of cells obtained in exponential growth phase vs. stationary phase cultures. Table S2 and Table S3 show the gene expression profile of biofilm cells vs. exponentially growing and stationary phase cultures, respectively. Overall, the data confirmed the complete description of the whole transcriptome of each stage of growth. Approximately 97% of the genes described in the *A. baumannii* ATCC 17978 genome database (NC_009085.1) were transcribed using the method described herein.

Different mRNA expression patterns of cells grown in exponential phase, stationary phase and in biofilms

The expression patterns of exponentially growing vs. stationary phase cells, stationary phase cells vs. biofilm cells, and exponentially growing cells vs. biofilm cells were compared to identify differentially expressed transcripts. Up-regulated and down-regulated genes were determined based on differences

Table 2. Total number of reads aligning with the regions of interest (coverage) of the six libraries constructed from the mRNA samples.

Exp 1	Exp 2	Sta 1	Sta 1	Bio 1	Bio 2
8189422	9497363	7707791	6317554	9084229	3111192

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for which the *p* values were below 0.001. The results are shown in Supporting Information (Tables S4, S5, S6, S7, S8, S9). Although, as expected, many genes were constitutively expressed, the comparisons indicated the association of each cellular condition with a specific expression profile, with significant differences in the expression level of a large number of genes. Thus, in biofilm vs. stationary-phase cells, 31 genes were down-regulated and 35 up-regulated; in biofilm vs. exponentially growing cells 15 genes were down-regulated and 116 up-regulated, and in stationary-phase vs. exponentially growing cells 130 genes were up-regulated and 33 down-regulated in ($p < 0.001$ in all cases).

The gene expression profile of biofilm cells

A comparison of gene expression levels in biofilms vs. stationary phase cells without applying any *p* value filter indicated that among the 1621 genes over-expressed in biofilms there were 408 genes whose expression was at least four-fold higher in sessile cells or completely inhibited in planktonic cells but with an expression level value of at least 2 in biofilm. With the aim of describing gene expression profile differences in terms of gene ontology, the complete proteome of *A. baumannii* strain ATCC1 7978 was re-annotated using Blast2GO. Biological processes, molecular functions, and cellular components associated with the set of 1621 up-regulated genes in biofilms, as determined using Blast2GO, are shown in Figure 1. The results showed that the largest group, made up of 129 genes, was involved in transcriptional regulation. Many genes were those involved in acyl carrier protein biosynthetic processes, amino acid metabolism, fatty acid metabolism, ion transport, carbohydrate biosynthesis, translation, transmembrane transport, and the stress response, among other biological processes. The cellular location of the majority of the proteins encoded by these 1621 genes was in most cases consistent with the proteins being integral to the inner membrane or members of a transcription factor complex. Fewer proteins were located in the outer membrane periplasmic space, in the cell outer membrane, or in a transcriptional repressor complex. Moreover, there were small groups of genes that encoded proteins associated with the peptidoglycan-based cell wall, the type II protein secretion system complex, the fatty acid synthase complex, or cell projection, among other cellular components. According to the molecular function ontology, most of the genes over-expressed in biofilms were related to transferase, hydrolase, and oxidoreductase activities and, to a lesser extent, to metal ion, ATP, coenzyme, or DNA binding activities, among other molecular functions.

When the comparison of gene expression levels in biofilms *vs.* exponentially growing and stationary phase planktonic cells was filtered by *p* value (< 0.001), similar results were obtained (Figure S3). A list of genes differentially expressed ($p < 0.001$) in the biofilm vs. exponential and stationary cultures is presented in Table 3. Among them, genes coding an acyl carrier protein, an allophanate hydrolase and the RND efflux pump AdeT (A1S_0114, A1S_1278 and A1S_1755, respectively) were only expressed in biofilms and were totally inhibited in planktonic cells. Genes corresponding to hypothetical proteins (A1S_0302, A1S_0644, A1S_1293, and A1S_2893), a transmembrane arsenate pump protein (A1S_1454), the CsuD, CsuC, and CsuA/B proteins (A1S_2214, A1S_2215, and A1S_2218), the BasD protein (A1S_2382), a ferric acinetobactin binding protein (A1S_2386), a sulfate transport protein (A1S_2534), and maleylacetoacetate isomerase (A1S_3415) were also highly expressed in biofilms but totally inhibited in stationary cells. Many genes involved in amino acid metabolism and transport (such as A1S_0115, A1S_0429, A1S_1357, A1S_3134, A1S_3185, A1S_3402, A1S_3404, A1S_3405, A1S_3406, A1S_3407, or A1S_3413), or related to iron acquisition and transport (A1S_0653, A1S_0742, A1S_0980, A1S_1631, A1S_1657, A1S_2385, or A1S_2390, encoding a ferrous iron transport protein, an iron-regulated protein, a ferric enterobactin receptor, an iron-binding protein, a siderophore biosynthesis protein, a ferric acinetobactin receptor, and an acinetobactin biosynthesis protein, respectively), transcriptional regulators (A1S_1377 and A1S_1687), or encoding efflux pumps (A1S_0009 and A1S_0538) were also up-regulated in biofilm vs. planktonic cells. A gene coding for a fimbrial protein (A1S_1507) was highly up-regulated and the outer membrane protein A (A1S_2840) was down-regulated in biofilm cells vs. either exponential or stationary phase planktonic cells. An operon containing a group of genes related to phenylacetate metabolism (with identifiers A1S_1335 to A1S_1340) was up-regulated in biofilm cells vs. exponential cells but down-regulated vs. stationary cells. A homoserine lactone synthase (A1S_0109) was over-expressed in biofilms vs. either growth form of planktonic cells, as was a group of seven genes (from A1S_0112 to A1S_0118). Among the latter, A1S_0114 was an extreme case because of its very high level of expression in the biofilm (*ca.* 127) and lack of detectable expression in planktonic cells.

Genes only expressed in biofilm associated cells

Fifty-five genes were exclusively expressed in sessile cells (Table 4), including 12 genes assigned to uncharacterized proteins and nine encoding transcriptional regulators (A1S_0547, A1S_1256, A1S_1430, A1S_1763, A1S_1958, A1S_2042, A1S_2151, A1S_2208 and A1S_3255). Other genes in this group belonged to the Csu operon (A1S_2216 and A1S_2217), encoded a membrane protein (A1S_0595), or were related to iron acquisition systems (A1S_0945, A1S_1719, A1S_2380, and A1S_2388). Genes coding for a DNA polymerase (A1S_2015), a DNA helicase (A1S_1585), an extracellular nuclease (A1S_1198), and an endonuclease (A1S_2408) as well as two genes involved in DNA methylation

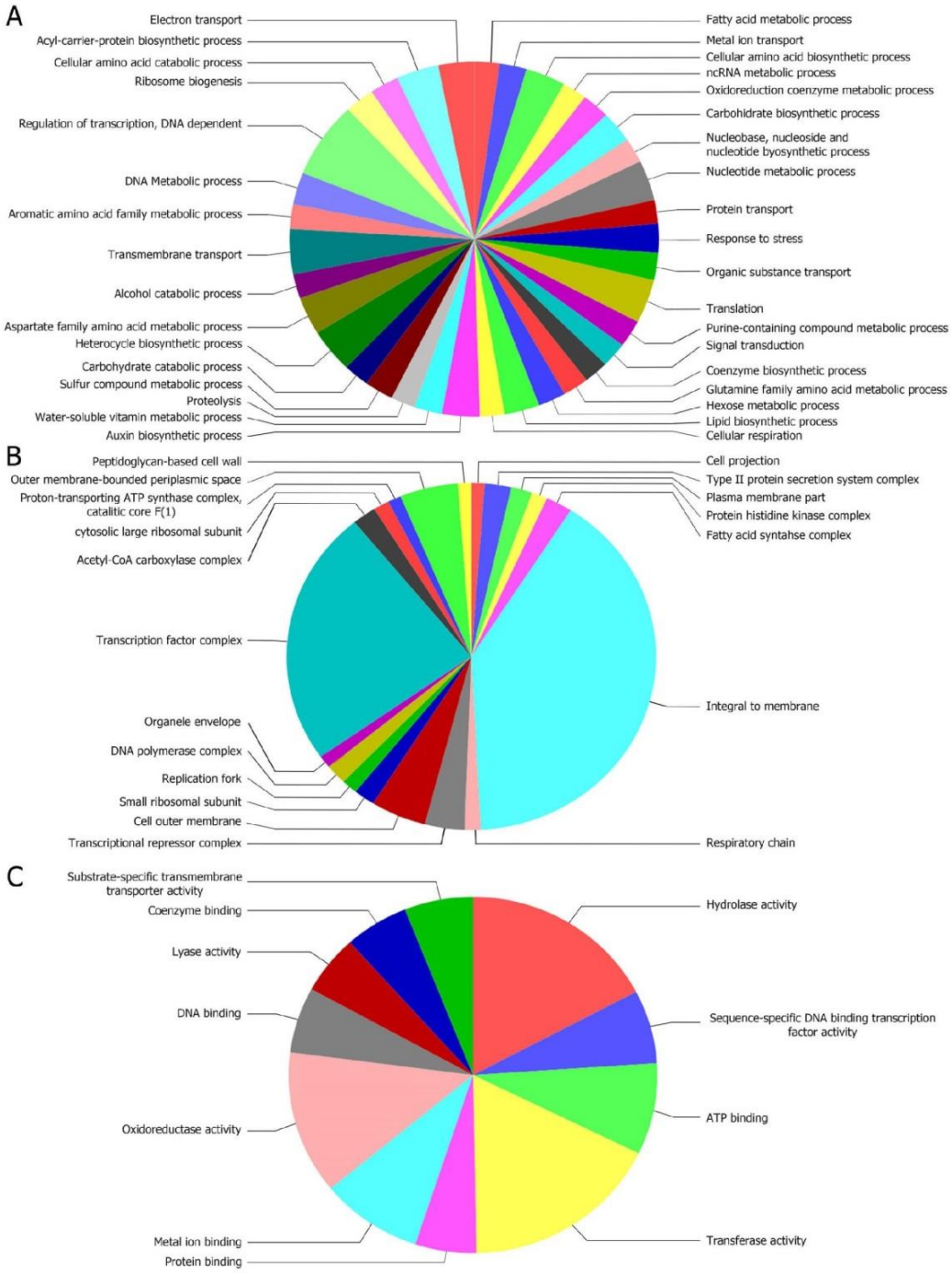

Figure 1. Sequence distribution of the 1621 genes identified in the present work as up-regulated in biofilm vs. stationary phase cells. Genes involved in: A) biological processes, B) cellular components, and C) molecular functions. The results were filtered by the number of sequences (cutoff = 40, 5, and 80, respectively).

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Table 3. Differentially expressed genes in biofilm-associated cells vs. both exponentially growing and stationary-phase cells.

Gene Id*	Gene description	Fold change Biofilm vs. exponential phase cells	Fold change Biofilm vs. stationary phase cells
A1S_0004	DNA gyrase	0.44	0.19
A1S_0009	RND type efflux pump	2.57	4.84
A1S_0032	signal peptide	32.18	4.82
A1S_0073	2-methylisocitrate lyase	6.60	1.61
A1S_0087	short-chain dehydrogenase	2.57	6.37
A1S_0103	3-hydroxyisobutyrate dehydrogenase	61.82	3.81
A1S_0107	enoyl-CoA hydratase	5.51	5.97
A1S_0109	homoserine lactone synthase	60.22	16.74
A1S_0112	acyl-CoA synthetase/AMP-acid ligases II	75.17	53.14
A1S_0113	acyl-CoA dehydrogenase	135.15	42.91
A1S_0114	acyl carrier protein	from zero to 127.96**	from zero to 127.96**
A1S_0115	amino acid adenylation	151.37	32.08
A1S_0116	RND superfamily exporter	56.18	79.67
A1S_0117	hypothetical protein	23.97	8.73
A1S_0118	hypothetical protein	9.31	5.26
A1S_0151	F0F1 ATP synthase subunit B	1.90	4.77
A1S_0153	F0F1 ATP synthase subunit alpha	1.13	3.75
A1S_0154	F0F1 ATP synthase subunit gamma	1.65	4.77
A1S_0155	F0F1 ATP synthase subunit beta	1.06	4.55
A1S_0156	F0F1 ATP synthase subunit epsilon	1.00	4.54
A1S_0179	NADPH-dependent FMN reductase	0.01	0.71
A1S_0279	elongation factor Tu	1.18	3.08
A1S_0283	50S ribosomal protein L11	1.99	1.94
A1S_0285	50S ribosomal protein	2.31	5.91
A1S_0292	outer membrane protein W	0.53	0.08
A1S_0302	hypothetical protein	23.30	from zero to 27.09**
A1S_0360	30S ribosomal protein S15	0.74	0.14
A1S_0429	DAACS family glutamate:aspartate symporter	3.04	1.84
A1S_0445	hypothetical protein	0.53	0.14
A1S_0449	coniferyl aldehyde dehydrogenase	0.16	0.12
A1S_0481	phosphate acetyltransferase	3.92	2.49
A1S_0482	acetate kinase	3.31	0.75
A1S_0496	phosphatidylglycerophosphatase B	10.13	1.05
A1S_0528	preprotein translocase subunit SecB	0.67	0.14
A1S_0538	RND efflux transporter	6.59	11.03
A1S_0570	hypothetical protein	0.70	0.12
A1S_0591	acyl-CoA synthetase	6.17	0.74
A1S_0628	transposase	4.32	2.80
1S_0644	hypothetical protein	18.27	from zero to 33.44**
A1S_0653	ferrous iron transport protein B	4.46	1.58
A1S_0661	phage integrase family protein	0.59	0.07
A1S_0670	protein tyrosine phosphatase	0.65	0.06
A1S_0671	protein tyrosine phosphatase	0.53	0.04

Table 3 (continued).

Gene Id*	Gene description	Fold change Biofilm vs. exponential phase cells	Fold change Biofilm vs. stationary phase cells
A1S_0675	dihydropteroate synthase	0.88	0.09
A1S_0736	hypothetical protein	79.49	2.76
A1S_0737	methyltetrahydropteroyltriglutamate/ homocysteine S-methyltransferase	25.32	2.26
A1S_0742	iron-regulated protein	2.50	1.74
A1S_0745	hypothetical protein	30.81	4.28
A1S_0869	elongation factor Tu	1.22	2.52
A1S_0884	outer membrane protein	0.50	4.35
A1S_0971	B12-dependent methionine synthase	0.05	0.07
A1S_0980	ferric enterobactin receptor precursor	4.38	4.01
A1S_1032	hypothetical protein	4.41	2.63
A1S_1077	hypothetical protein	9.41	2.43
A1S_1104	chlorogenate esterase	0.35	0.01
A1S_1266	hypothetical protein	1.09	11.88
A1S_1278	allophanate hydrolase subunit 2	from zero to 20.35**	from zero to 20.35**
A1S_1293	hypothetical protein	15.25	from zero to 30.54**
A1S_1316	major facilitator superfamily transporter cyanate permease	14.40	4.83
A1S_1319	hypothetical protein	22.56	50.37
A1S_1335	bifunctional aldehyde dehydrogenase/enoyl-CoA hydratase	21.33	0.22
A1S_1336	phenylacetate-CoA oxygenase subunit PaaA	93.43	0.28
A1S_1337	phenylacetate-CoA oxygenase subunit PaaB	22.63	0.45
A1S_1338	hypothetical protein	34.73	0.41
A1S_1339	phenylacetate-CoA oxygenase PaaJ subunit	196.37	0.78
A1S_1340	phenylacetate-CoA oxygenase/reductase PaaK subunit	161.34	0.77
A1S_1341	enoyl-CoA hydratase/carnithine racemase	28.43	0.56
A1S_1344	thiolase	14.31	0.40
A1S_1357	alanine racemase	4.59	1.23
A1S_1370	oxidoreductase	2.67	0.80
A1S_1376	acyl-CoA dehydrogenase	11.34	3.14
A1S_1377	acrR family transcriptional regulator	4.28	0.56
A1S_1385	hypothetical protein	9.16	8.68
A1S_1454	transmembrane arsenate pump protein	54.33	from zero to 27.07**
A1S_1507	fimbrial protein	17.73	18.49
A1S_1530	SSS family major sodium/proline symporter	0.29	1.03
A1S_1541	hypothetical protein	8.27	9.19
A1S_1572	30S ribosomal protein S1	1.74	0.88
A1S_1617	30S ribosomal protein S20	4.08	1.15
A1S_1631	iron-binding protein	0.71	0.13
A1S_1637	DNA-binding protein HU-beta	1.13	5.12
A1S_1657	siderophore biosynthesis protein	13.61	2.89
A1S_1687	transcriptional regulator	1.84	0.00
A1S_1726	aspartate ammonia-lyase	0.33	0.22

Table 3 (continued).

Gene Id*	Gene description	Fold change Biofilm vs. exponential phase cells	Fold change Biofilm vs. stationary phase cells
A1S_1731	acetoacetyl-CoA transferase subunit beta	29.63	3.12
A1S_1732	acetoacetyl-CoA transferase subunit alpha	78.74	5.03
A1S_1736	hypothetical protein	6.21	1.47
A1S_1755	RND efflux pump AdeT	from zero to 17.27**	from zero to 17.27**
A1S_1924	cytochrome d terminal oxidase polypeptide subunit I	0.22	0.21
A1S_1925	cytochrome d terminal oxidase polypeptide subunit II	0.27	0.22
A1S_1926	hypothetical protein	0.17	0.03
A1S_1932	hypothetical protein	1.88	0.11
A1S_1965	UDP-N-acetylglucosamine acyltransferase	3.57	1.48
A1S_2072	universal stress family protein	0.52	0.21
A1S_2091	hypothetical protein	24.78	3.28
A1S_2093	hypothetical protein	1.13	0.01
A1S_2098	alcohol dehydrogenase	13.14	130.77
A1S_2102	aldehyde dehydrogenase 1	2.59	8.55
A1S_2148	acetyl-CoA synthetase/AMP-(fatty) acid ligase	12.90	0.51
A1S_2149	acyl CoA dehydrogenase oxidoreductase protein	8.68	3.11
A1S_2150	oxidoreductase	5.52	1.13
A1S_2164	phosphoenolpyruvate synthase	1.04	0.28
A1S_2183	signal peptide	0.59	0.03
A1S_2214	protein CsuD	180.04	from zero to 89.72**
A1S_2215	protein CsuC	201.23	from zero to 33.43**
A1S_2218	protein CsuA/B	164.40	from zero to 1122.03**
A1S_2261	cold shock protein	5.09	1.12
A1S_2289	signal peptide	20.61	2.63
A1S_2322	elongation factor Ts	1.50	4.89
A1S_2382	BasD (iron acquisition systems)	72.89	from zero to 24.22**
A1S_2385	ferric acinetobactin receptor	6.48	6.48
A1S_2386	ferric acinetobactin binding protein	9.10	from zero to 48.57**
A1S_2390	acinetobactin biosynthesis protein	34.11	18.97
A1S_2447	EsvD ABC transporter	7.56	14.72
A1S_2449	aromatic amino acid APC transporter	16.58	1.15
A1S_2450	pyruvate decarboxylase	8.17	0.22
A1S_2452	NAD-dependent aldehyde dehydrogenases	1.71	0.15
A1S_2458	fatty acid desaturase	0.24	0.15
A1S_2496	phosphoserine phosphatase	0.3	0.01
A1S_2534	sulfate transport protein	21.12	from zero to 24.66**
A1S_2696	hypothetical protein	1.35	0.20
A1S_2705	hypothetical protein	0.21	0.09
A1S_2718	succinyl-CoA synthetase subunit beta	1.14	7.65
A1S_2719	succinyl-CoA synthetase subunit alpha	1.07	5.76

Table 3 (continued).

Gene Id*	Gene description	Fold change Biofilm vs. exponential phase cells	Fold change Biofilm vs. stationary phase cells
A1S_2753	hypothetical protein	1.66	3.36
A1S_2840	outer membrane protein A	0.60	0.74
A1S_2889	signal peptide	46.50	25.85
A1S_2893	hypothetical protein	64.83	from zero to 32.31**
A1S_3043	hypothetical protein	3.91	1.5
A1S_3055	50S ribosomal protein L17	2.54	3.88
A1S_3056	DNA-directed RNA polymerase subunit alpha	1.89	3.04
A1S_3057	30S ribosomal protein S4	1.89	2.99
A1S_3058	30S ribosomal protein S11	2.00	2.58
A1S_3061	preprotein translocase subunit SecY	2.64	1.71
A1S_3062	50S ribosomal protein L15	2.79	2.07
A1S_3063	50S ribosomal protein L30	2.54	3.31
A1S_3064	30S ribosomal protein S5	2.97	3.54
A1S_3065	50S ribosomal protein L18	3.37	3.34
A1S_3066	50S ribosomal protein L6	2.55	2.29
A1S_3068	30S ribosomal protein S14	2.90	2.27
A1S_3069	50S ribosomal protein L5	2.13	1.66
A1S_3070	50S ribosomal protein L24	2.26	2.05
A1S_3073	50S ribosomal protein L29	2.01	3.00
A1S_3074	50S ribosomal protein L16	2.05	3.56
A1S_3075	30S ribosomal protein S3	1.73	3.06
A1S_3077	50S ribosomal protein L2	1.75	1.77
A1S_3079	50S ribosomal protein L4	1.87	1.59
A1S_3080	50S ribosomal protein L3	2.13	1.29
A1S_3104	ATP-dependent RNA helicase	1.64	0.23
A1S_3108	coproporphyrinogen III oxidase	0.28	0.33
A1S_3113	hypothetical protein	0.90	0.04
A1S_3134	glutamate dehydrogenase	1.26	3.21
A1S_3161	50S ribosomal protein L19	2.57	2.14
A1S_3185	glutamate synthase subunit alpha	0.40	0.48
A1S_3231	acetyl-CoA hydrolase/transferase	3.42	0.96
A1S_3297	outer membrane protein	1.17	3.80
A1S_3300	acetate permease	17.44	1.23
A1S_3301	hypothetical protein	5.77	0.52
A1S_3303	hypothetical protein	5.78	0.47
A1S_3309	acetyl-CoA synthetase	4.17	1.96
A1S_3328	pyruvate dehydrogenase subunit E1	0.50	0.92
A1S_3350	hypothetical protein	0.38	0.89
A1S_3402	arginase/agmatinase/ formimionoglutamate hydrolase	3.72	7.33
A1S_3404	amino acid APC transporter	3.88	4.18
A1S_3405	histidine ammonia-lyase	3.13	3.94
A1S_3406	urocanate hydratase	3.92	0.85
A1S_3407	urocanase	4.52	2.63
A1S_3413	APC family aromatic amino acid transporter	66.30	18.38
A1S_3414	fumarylacetoacetase	60.10	20.87
A1S_3415	maleylacetoacetate isomerase	24.49	from zero to 77.30**

Table 3 (continued).

Gene Id*	Gene description	Fold change Biofilm vs. exponential phase cells	Fold change Biofilm vs. stationary phase cells
A1S_3416	glyoxalase/bleomycin resistance protein/dioxygenas	24.26	1.02
A1S_3418	4-hydroxyphenylpyruvate dioxygenase	78.62	12.69
A1S_3463	diaminopimelate decarboxylase	0.41	0.08
A1S_3473	hypothetical protein	0.67	0.20
A1S_3475	hypothetical protein	1.15	0.19

The data were filtered based on a p value < 0.001.

*. Genes that significantly differed in their expression values with a p value below 0.001 in at least one of the two profile comparisons are listed in this table.

**. In these cases the expression values are absolute and no expression was detected under planktonic conditions.

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(A1S_1146 and A1S_1147) were likewise only expressed in biofilms. Other groups of genes comprised those involved in efflux systems (A1S_1117, A1S_1751, and A1S_1755), or amino acid metabolism and transport (A1S_0956 and A1S_2302), or encoded an acyl carrier protein (A1S_0114). The complete list is shown in Table 4.

Further analysis of these 55 genes using Blast2GO revealed that most were involved in regulation of transcription and fewer to processes such as electron transport, acyl carrier biosynthesis, transmembrane transport, DNA replication, and siderophore biosynthesis (Figure 2a). The main molecular functions ascribed to the 55 genes are shown in Figure 2b, with oxidoreductase, transporters, DNA binding, and transcription factors activities predominating. The cellular location of the proteins encoded by the 55 genes is illustrated in Figure 2c, which shows that most of the proteins were located in a transcription factor complex or in the cell membrane.

Decrease in biofilm formation ability by gene disruption and knockout mutants

Five genes over-expressed in the biofilm vs. planktonic cells, as previously confirmed by qRT-PCR (Table 5), were selected for gene disruption by insertion of the plasmid pCR-Blunt-II-TOPO *via* single crossover recombination, as described in Materials and methods. These genes were A1S_0114 (encoding an acyl carrier protein expressed only in biofilms and inhibited in planktonic cells), A1S_0302 (encoding a hypothetical protein whose expression was *ca*. 27-fold higher in biofilms than in stationary-phase cells), A1S_1507 (encoding a fimbrial protein with *ca*. 18-fold higher expression in biofilms than in planktonic cells), A1S_3168 (encoding a pilus assembly protein PilW expressed in biofilms and repressed in stationary-phase cells, see Table S3), and A1S_2042 (a transcriptional regulator of the TetR family expressed in biofilms but inhibited in planktonic cells). The resulting mutant strains were used to evaluate their ability to form biofilms compared to the wild-type strain. As shown in Figure 3, biofilm formation ability was

Table 4. List of genes expressed only in biofilm cells and inhibited in planktonic cells.

Gene Id	Expression value in biofilm cells	Gene description
A1S_0079	0.47	N-acetyltransferase GNAT family (98% Ab SDF)*
A1S_0114	127.96	acyl carrier protein
A1S_0547	1.22	transcriptional regulator
A1S_0595	0.56	membrane protein (100% Ab MDR-TJ)*
A1S_0648	1.41	hypothetical protein
A1S_0741	0.19	hypothetical protein
A1S_0945	0.75	ferredoxin
A1S_0946	1.13	hypothetical protein
A1S_0956	1.13	L-aspartate dehydrogenase
A1S_0969	0.19	transketolase
A1S_1116	1.03	vanillate O-demethylase oxygenase subunit
A1S_1117	2.16	MFS superfamily vanillate transporter
A1S_1121	0.19	lipase/esterase
A1S_1125	0.38	transferase
A1S_1133	0.75	flavin-binding monooxygenase
A1S_1146	1.41	site-specific DNA-methyltransferase
A1S_1147	1.88	DNA methylase-like protein
A1S_1198	0.19	extracellular nuclease
A1S_1256	0.38	transcriptional regulator
A1S_1276	0.28	hypothetical protein
A1S_1278	20.35	allophanate hydrolase subunit 2
A1S_1349	0.47	thioesterase
A1S_1366	1.03	transporter LysE family
A1S_1430	0.28	LysR family malonate utilization transcriptional regulator
A1S_1452	0.94	arsenate reductase
A1S_1583	0.84	hypothetical protein
A1S_1585	0.56	replicative DNA helicase
A1S_1590	0.94	peptidase U35 phage prohead HK97
A1S_1622	1.13	hypothetical protein
A1S_1699	3.28	pyruvate/2-oxoglutarate dehydrogenase complex
A1S_1719	0.38	4Fe-4S ferredoxin iron-sulfur binding
A1S_1751	2.34	AdeA membrane fusion protein
A1S_1755	17.27	AdeT
A1S_1763	1.50	transcriptional regulator
A1S_1853	0.38	hypothetical protein
A1S_1887	0.28	major facilitator superfamily permease
A1S_1958	0.38	transcriptional regulator
A1S_2015	0.38	DNA-directed DNA polymerase
A1S_2028	0.19	phage putative head morphogenesis protein
A1S_2029	11.30	hypothetical protein
A1S_2033	0.47	hypothetical protein
A1S_2035	0.28	hypothetical protein
A1S_2042	2.72	transcriptional regulator (TetR family)
A1S_2151	0.75	transcriptional regulator (AraC family)
A1S_2208	0.38	transcriptional regulator
A1S_2216	11.96	CsuB
A1S_2217	3.84	CsuA
A1S_2302	0.75	ABC lysine-arginine-ornithine transporter
A1S_2380	8.81	acinetobactin biosynthesis protein

Table 4 (continued).

Gene Id	Expression value in biofilm cells	Gene description
A1S_2388	1.69	putative ferric acinetobactin transport system
A1S_2408	0.09	HNH endonuclease (93% Ab MDR-TJ)*
A1S_2580	1.40	23-dihydro-2,3-dihydroxybenzoate synthetase, isochorismatase
A1S_3120	0.09	hypothetical protein
A1S_3255	0.09	transcriptional regulator AraC/XylS family protein
A1S_3260	1.59	hypothetical protein

*. These genes are annotated in strain ATCC 17978. Similarities to sequences in the databases are indicated.

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severely hindered (~8-fold reduction) in all of the mutant strains.

To obtain a stable mutant free of antibiotic resistance markers or potential polar effects, the A1S_0114 gene was deleted from the genome of *A. baumannii* ATCC 17978 using the pMo130 vector, as described in Material and methods. As shown in Figure 4, the biofilm formation ability of the stable A1S_0114 knock-out (KO) mutant was significantly reduced (< 3-fold) compared to the wild-type strain.

The relationship of the gene A1S_0114 to genes related to homoserine lactone synthesis (A1S_0109, A1S_0112 and A1S_0113) was examined in qRT-PCR assays. As shown in Figure 5, genes A1S_0109, A1S_0112, A1S_0113 and A1S_0114 were over-expressed in the late stationary phase of growth compared to the exponential phase in the wild-type strain. When gene A1S_0114 was deleted from the chromosome (yielding the stable A1S_0114 KO mutant strain), the expression levels of genes related to homoserine lactone synthesis (A1S_0109, A1S_0112 and A1S_0113) were considerably reduced in the late stationary phase of growth (83, 68 and 73%, respectively) of the resulting mutant compared with the wild-type strain.

Discussion

In the present work, we successfully used Illumina RNA-sequencing to establish the complete transcriptional profile of *A. baumannii* strain ATCC 17978 grown in planktonic and sessile (biofilm) modes. To obtain an overview of the temporal regulation of gene expression, planktonic cells were harvested during the exponential and late stationary phases of growth. A similar strategy was previously used in a proteomic study demonstrating the growth-dependent regulation of many proteins [58]. In another proteomic study of *A. baumannii* ATCC 17978 [34], planktonic and sessile cells were shown to exhibit distinct proteomic profiles, indicating that biofilms are not simply surface-attached stationary-phase cells.

Our data revealed that although many genes were constitutively expressed in both biofilm and planktonic cells, others differed in their growth-dependent expression, with clearly distinct and specific expression patterns between sessile biofilm cells and cells in either phase of planktonic growth. Among the 1621genes over-expressed in biofilms, 55 genes were only expressed in sessile cells and were totally inhibited in planktonic cells. The majority of the 55 genes encoded proteins involved in functions and mechanisms already known to be related with biofilm formation and maintenance whereas others were detected in this study for the first time. The presence of 12 genes encoding uncharacterized proteins further highlights the deficits in our knowledge of the specific genes associated with biofilm in *A. baumannii*. One of these genes (A1S_0302) was selected for gene disruption procedures because of its high level of expression in biofilm cells; indeed, the corresponding mutant strain was significantly deficient in biofilm formation. In addition, nine transcriptional regulators were found to be expressed only by biofilm cells, suggesting that biofilm formation and maintenance is controlled by specific molecules that are either not expressed, silenced, or not operative in planktonic cells. Gaddy and Actis [26] suggested that the regulatory process associated with biofilm formation includes the sensing of bacterial density, the presence of nutrients, and the concentration of free cations. Some of these extracellular signals are controlled by two-component regulatory systems such as BfmR/S. The *bfmS* gene encodes a sensor kinase that receives extracellular signals and phosphorylates the product of the *bfmR* gene, a response regulator. Tomaras et al. [27] studied the BfmR/S system in *A. baumannii* strain 19606, where this two-component regulator is required for the activation of the usher-chaperone assembly system involved in pili formation, a feature of biofilms. Based on their study of *P. aeruginosa*, Petrova et al. [59] proposed a role for BfmR in biofilm development, by limiting bacteriophage-mediated lysis and subsequent DNA release. According to our data, expression of the *bfmR* gene, identified as A1S_0748 by Liou et al. [60], was *ca*. five-fold higher in biofilm cells than in stationary cells. However, BfmR cannot be claimed as a biofilm-specific molecule of the strain 17978 since it was also expressed in the planktonic cells. No significant similarities were found in the databases for the nine transcriptional regulators described herein as biofilm specific. The mutant strain generated by the disruption of one of these genes (A1S_2042) showed an important decrease in biofilm formation ability relative to the wild-type strain. A1S_2042 appears to be a transcriptional regulator of the TetR family and could play an important role in biofilm regulation. Together with the other uncharacterized biofilm-specific transcriptional regulators, all of which were expressed at low but significant levels in biofilms, A1S_2042 merits further study to gain insight into the complex regulatory networks involved in biofilm formation and maintenance.

The CsuA/BABCDE chaperone-usher pili assembly system is involved in the adherence of *A. baumannii* strain 19606 biofilm to abiotic surfaces [26,27]. In the present work, Csu A/B, C, D and E were highly over-expressed in biofilms vs. planktonic cells. Of particular interest is CsuA/B, which is predicted to form part of the type I pili rod [40]. While the gene encoding CsuA/B was not expressed in stationary cells, its expression was greatly enhanced (value of 1122) in biofilms, although it was also expressed in exponentially growing cells

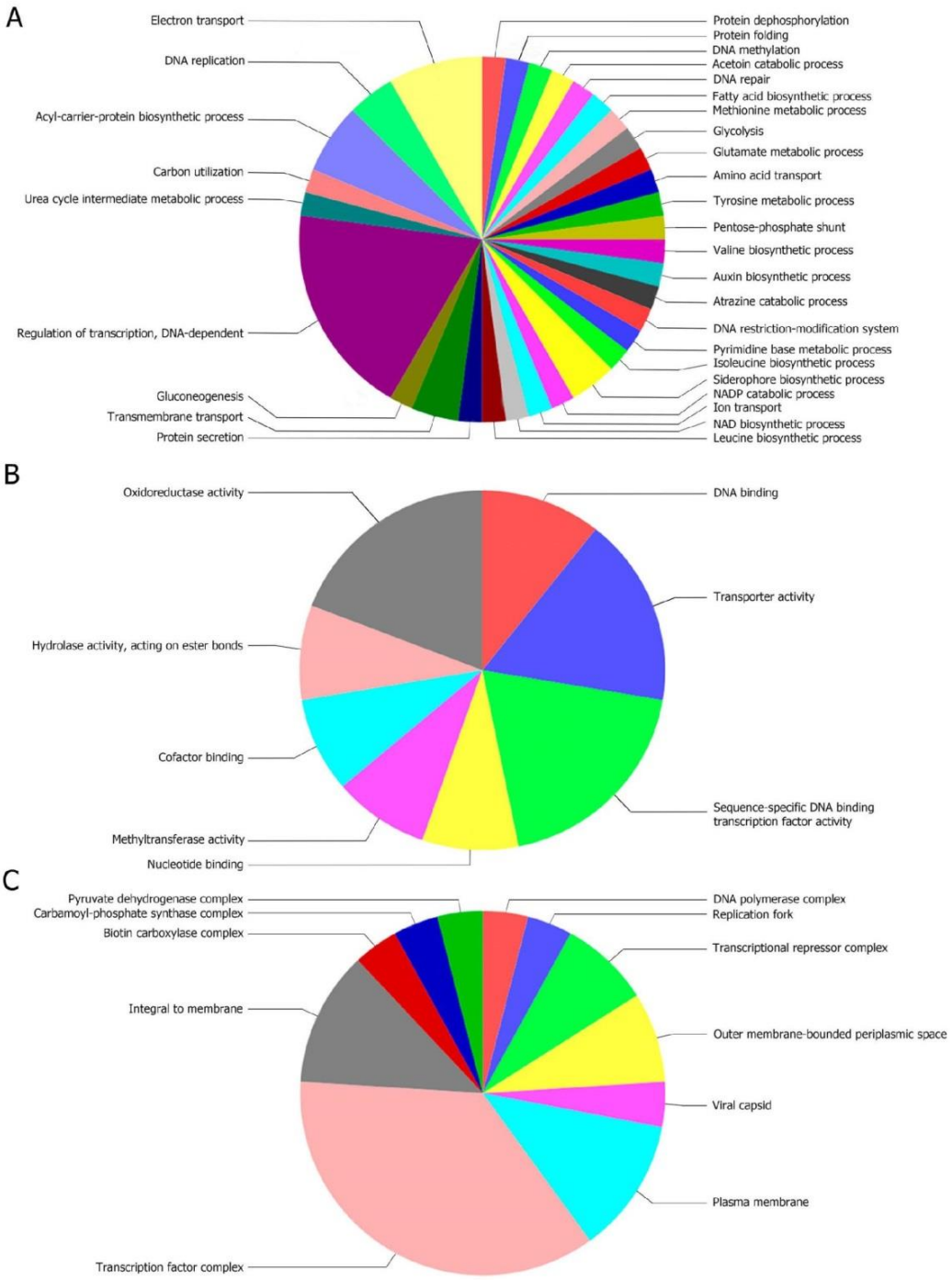

Figure 2. Sequence distribution of genes expressed only in biofilm-associated cells and inhibited in planktonic cells. Genes involved in A) biological processes, B) molecular functions, and C) cellular components. The results were filtered by the number of sequences (cutoff = 1, 4, and 1, respectively).

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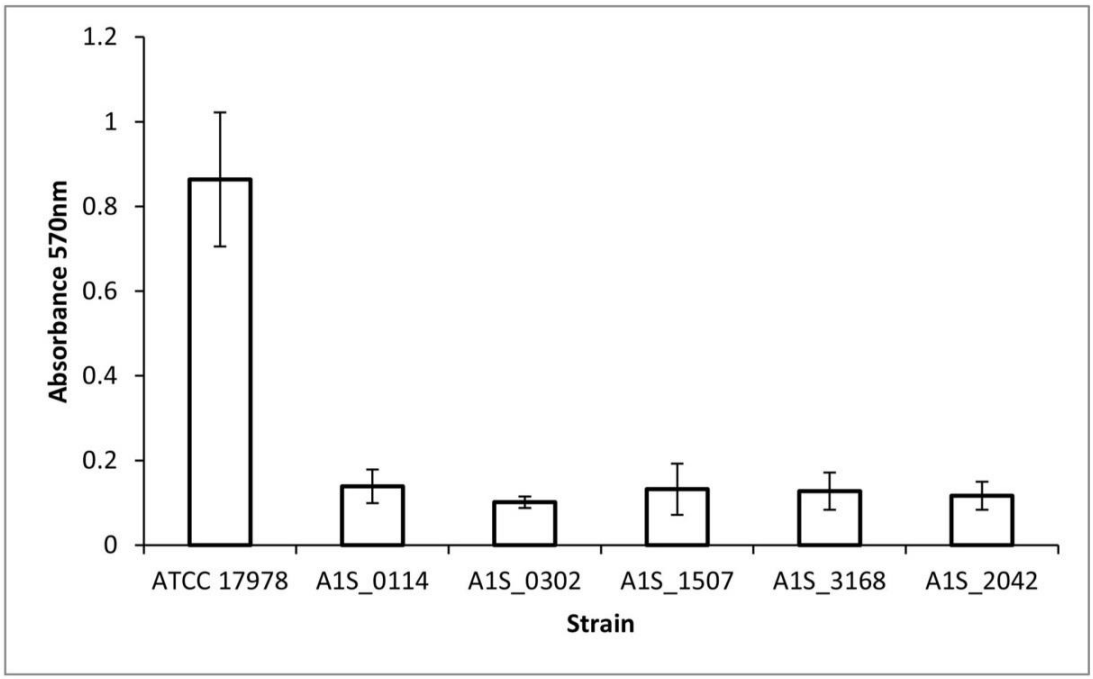

Figure 3. Quantification of biofilm formation by the wild-type strain (ATCC 17978) and strains with chromosomal disruptions in the genes A1S_0114, A1S_0302, A1S_1507, A1S_3168 and A1S_2042.
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Table 5. Expression levels of genes A1S_0114, A1S_0302, A1S_1507, A1S_2042, and A1S_3168 in biofilm and planktonic cells as measured by qRT-PCR.

Gene Id	Expression level in exponential phase cells*	Expression level at the stationary phase cells	Expression level in biofilm cells
A1S_0114	1 ± 0.319	0.219 ± 0.234	6.023 ± 1.493
A1S_0302	1 ± 0.339	3.099 ± 0.847	4.069 ± 0.599
A1S_1507	1 ± 0.073	1.535 ± 0.215	7.761 ± 0.719
A1S_2042	1 ± 0.488	4.589 ± 2.152	39.35 ± 12.670
A1S_3168	1 ± 0.158	1.237 ± 0.076	2.087 ± 0.522

*. The expression levels of each of the five genes were determined with respect to the exponential growth phase value, defined as 1.
doi: 10.1371/journal.pone.0072968.t005

(value of *ca* 164). Moreover, we detected CsuA (A1S_2216) and CsuB (A1S_2217) transcripts only in biofilms and not in planktonic cells. These results indicate that the complete Csu operon is highly active in the biofilms analyzed in this study. Nevertheless, it should be noted that MacQueary and Actis [61], found strong variations in the CsuA/BABCDE chaperone-usher pili assembly system and other motility factors among different strains of *A. baumannii* attached to abiotic surfaces. This finding may pose a challenge in the treatment of the infections caused by this bacterium, if biofilm formation on abiotic surfaces is chosen as a target for the development of new antimicrobial agents.

Two genes coding for a fimbrial protein (A1S_1507) and a pilus assembly protein PilW (A1S_3168), different from the CsuA/BABCDE chaperone-usher pili assembly system, were over-expressed in biofilm vs. planktonic cells. The disruption of these two genes in the genome of *A. baumannii* revealed their involvement in biofilm formation and suggested that the biofilm analyzed here could require multiple pili systems to maintain its cohesive structure. Pilus and fimbriae are important for the initial step of bacterial adhesion, which is followed by the production of exopolysaccharides, an important constituent of mature biofilms that suppresses neutrophil activity and contributes to resistance. Variation in the expression of factors involved in these pathways may account for the different capacity of bacterial strains to form biofilms and therefore to colonize or infect the host environment [62].

A. baumannii secretes a variety of molecules involved in iron acquisition including siderophores such as acinetobactin. The iron concentration in the medium acts as an important environmental signal that induces the expression of adhesion factors, thus playing a critical role in biofilm formation [63]. However, there is wide variability in the expression of iron uptake molecules, even between strains isolated during the

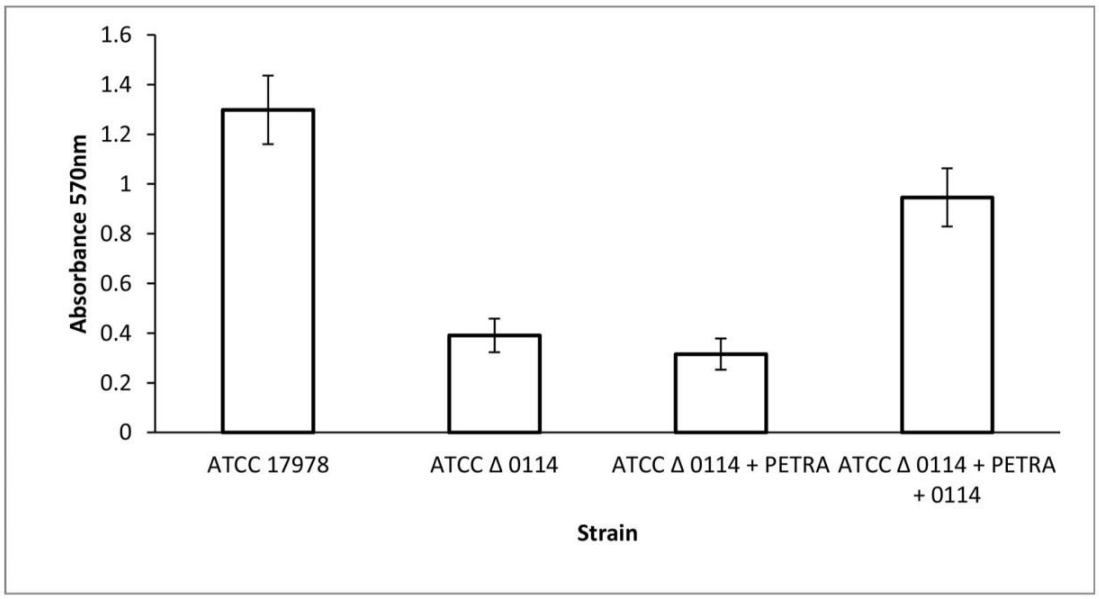

Figure 4. Quantification of biofilm formation by the wild-type strain (ATCC 17978), a stable knockout mutant strain lacking the gene A1S_0114 (ATCC Δ0114), the same mutant strain containing the pET-RA plasmid (ATCC Δ0114 + PETRA), and a mutant strain containing the pET-RA plasmid harboring the A1S_0114 gene (ATCC Δ0114 + PETRA + 0114).

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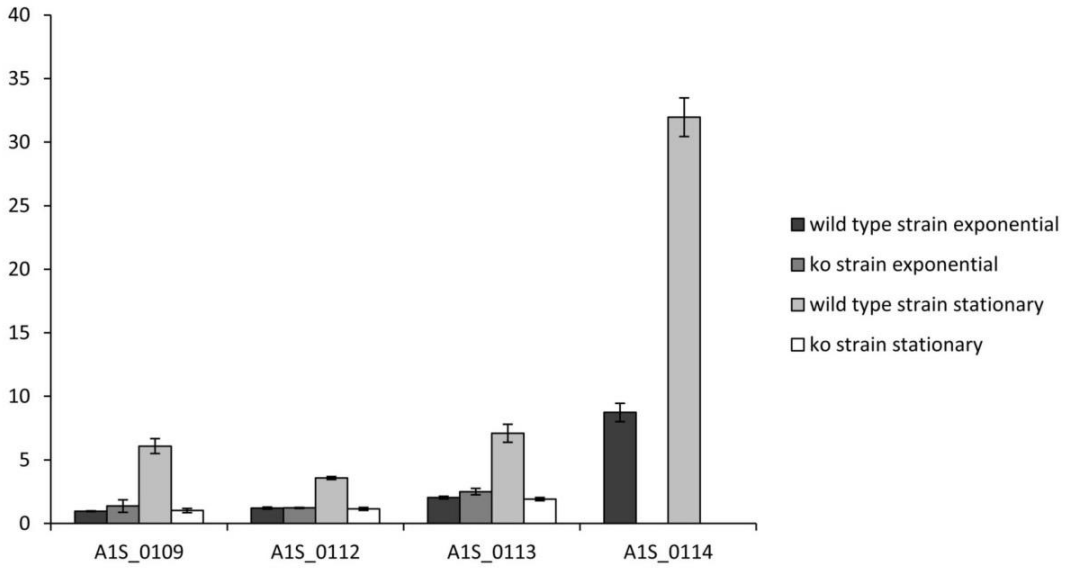

Figure 5. Comparison of the expression levels of genes related to homoserine lactone synthesis (A1S_0109, A1S_0112 and A1S_0113) in the wild-type strain *A. baumannii* 17978 and in the A1S_0114 knock-out (KO) strain as determined by real-time qRT-PCR assays.

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same outbreak [61]. In our experimental model, several genes involved in iron acquisition were over-expressed in biofilm vs. planktonic cells, while some genes related to acinetobactin (A1S_2380 and A1S_2388) and ferredoxin (A1S_0945 or A1S_1719) were expressed only in biofilms and totally inhibited in planktonic cells. The exclusively expression of acinetobactin genes in biofilm cells could be explained in terms of iron starved conditions in the sessile cells compared with an iron-rich medium used for growing planktonic cells. Eijkelkamp et al. [40] found transcriptional changes in genes involved in motility when *A. baumannii* was grown under iron-limiting conditions. As shown by our data, the biofilm is a resistance mode where cells clearly over-express many genes related to iron acquisition systems. It is known that the ability of *A. baumannii* to obtain and utilize resources such as iron is an important factor for bacterial survival but it seems to also be essential for biofilm formation and maintenance, given that bacteria able to form biofilms actively search for iron [63]. This scenario was reflected in our study by the over-expression of many genes involved in iron acquisition and transport.

The detection of two genes involved in DNA methylation and expressed exclusively in biofilms suggests a role for DNA methylation in the regulation of biofilm-associated processes. In addition, several genes encoding efflux system components were activated in the biofilm cells, including the gene encoding resistance-nodulation-cell division type efflux pump (RND pump), involved in bacterial resistance to a number of antibiotics. Our results indicate that the up-regulation of efflux pumps is a mechanism of antibiotic resistance that operates in the mature biofilm [34].

Another factor previously described as involved in biofilm formation is the homolog of the staphylococcal protein Bap, studied in *A. baumnannii* 307-0294. The protein is a surface adhesin that mediates primary attachment to both biotic and abiotic surfaces and is involved in intercellular adhesion within the mature biofilm [29]. The *A. baumannii* 17978 genome (NC_009085.1) contains two loci homologous to the 5' and 3' ends of the *bap* locus defined in *A. baumannii* strain 307-0294 [29]. These two regions correspond to genes A1S_2724 and A1S_2696 (annotated as a hemaglutinin/hemolysin like protein and a hypothetical protein, respectively) [46] that were over-expressed in biofilms vs. exponential cells, suggesting that *A. baumannii* Bap-related proteins in the strain 17978 could also enhance the cell to cell interactions that support biofilm maturation.

Amino acid metabolism also clearly differed in our biofilm experimental design with respect to planktonic cells, as several genes involved in the metabolism and transport of amino acids were differentially expressed. Our results not only corroborate the hypothesis formulated by Cabral et al. [34] regarding the importance of histidine metabolism in biofilm formation but also extend it, based on our detection of genes involved in amino acid metabolism that were differentially expressed in biofilm cells and were not previously detected by proteomic analysis.

Cell surface membrane proteins may be essential to biofilm formation. Some of these proteins were differentially expressed in our biofilm cells, such as CarO (A1S_2538) and OprD-like (A1S_0201), which were up-regulated, while OmpA (A1S_2840) was down-regulated. These results conflict somewhat with those of Cabral et al. [34], who found that OmpA was up-regulated in biofilm cells. Gaddy et al. [30] also described the importance of OmpA in biofilm formation in *A. baumannii* strain 19606. The discrepancy in the results can be explained by strain-dependent variations or different adhesion phenomena in response to diverse biotic or abiotic surface materials, as previously described [64]. However, Marti et al. [63], analyzed the proteome of *A. baumannii* strain 77 and found three mass isoforms identified as OmpA. In accordance with our results, OmpA was down-regulated in the biofilm, leading the authors to suggest that this porin participates in the initiation step of biofilm formation and that the subsequent iron starvation conditions encountered during biofilm maturation trigger a decrease in its expression. This may have been the case in our experimental model. The extracellular matrix that surrounds the biolfim protects the resident bacterial cells against a number of agents but it also limits bacterial access to fresh nutrients. Accordingly, an increase in the expression of transmembrane channels may be essential for the entrance of important nutrients. In the present work, the under-expression of OmpA was complemented by an over-expression of the porins OprD-like and CarO, which may have helped to maintain the permeability of the cells in the biofilm.

Although little is known about the factors involved in biofilm regulation, cell to cell signaling mediated by N-acyl-homoserine lactones has been implicated in gram-negative bacteria [65–67]. Indeed, we identified a group of genes (identifiers A1S_0112 to A1S_0118) over-expressed in biofilms vs. planktonic cells. This group of genes has been described as an operon related to quorum sensing and may be involved in the expression of the protein encoded by A1S_0109, the only homoserine lactone synthase described thus far in *A. baumannii* [46,68–70]. In our experimental model, this homoserine lactone synthase (A1S_0109) was over-expressed in biofilm vs. planktonic cells. Among the genes contained in the above-mentioned operon, A1S_0114 was exclusively expressed at high levels in biofilms but totally inhibited in planktonic cells. This gene encodes a small acidic acyl carrier protein (ACP) that is very abundant in bacteria, where it serves as an important acyl donor. ACP is first synthesized in its inactive form (apo-ACP) and then activated by an acyl carrier protein synthase [71]. In its activated form, ACP is essential for the synthesis of N-acyl-homoserine lactone, which is a substrate for the homoserine lactone synthase [72]. In this work, proteins encoded by the genes A1S_0112 and A1S_0113 were identified as an acyl-CoA synthetase and an acyl-CoA dehydrogenase, respectively, both of which are necessary for ACP activation. In the gene disruption and in the stable knock out A1S_0114 (ACP) mutants there was a notable decrease in biofilm formation ability compared to the wild-type strain, demonstrating the importance of this gene in biofilm formation. Our qRT-PCR results indicated reduced expression of the genes A1S_0112, A1S_0113 as well as the N-acyl-homoserine lactone synthase gene A1S_0109 in the stable A1S_0114 KO mutant, which presumably could affect quorum sensing and biofilm formation. Moreover, since our results were consistent with alterations in fatty acid metabolism in biofilms

vs. planktonic cells an alternative explanation for the decrease in biofilm formation ability of the A1S_0114 mutant is that the encoded ACP acts as an acyl donor associated with general fatty acid metabolism.

Concluding remarks

The main goal of this study was to provide insight into the molecular mechanisms underlying the ability of *A. baumannii* to form biofilms. The expression profiles described herein allow the definition of many genetic elements involved in the sessile lifestyle of *A. baumannii*, including 55 genes exclusively expressed in biofilm. Five genes were disrupted in the chromosome and the corresponding mutant strains were significantly hindered in their biofilm formation ability, demonstrating their involvement in biofilm development. An ACP-encoding gene that belongs to an operon involved in quorum sensing mediated by a homoserine lactone was highly over-expressed in our biofilm experimental model and its inactivation significantly limited biofilm formation by cells of the corresponding mutant strain.

The results described in this work constitute a basis for the identification of new therapeutic targets and the design of new drugs able to prevent infectious diseases related to biofilm production by *A. baumannii*. It also serves as a starting point for future studies of the complex network systems involved in biofilm formation and maintenance, as well as the regulation of these processes.

Supporting Information

Figure S1. Gene level counts. Left: boxplot (median, first and third quartiles and standard deviation) of the number of reads per gene. Right: density functions of the number of reads *per* gene.
(TIF)

Figure S2. MD plots and correlation between samples. Upper right: MD plots showing (countsA+countsB)/2 against (countsA-countsB), with A and B being the samples shown on the diagonal.
(TIF)

Figure S3. Sequence distribution of genes up-regulated in biofilm-associated cells. The data were filtered based on $p <$ 0.001 and with respect to biological processes. A) Exponentially growing cells, filtered by the number of sequences (cutoff 6). B) Stationary phase cells, filtered by the number of sequences (cutoff 1).
(TIF)

Table S1. Gene expression data from the complete transcriptome analysis of *Acinetobacter baumannii* ATCC 17978, showing gene expression levels in exponentially growing vs. stationary phase cells. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA, log2Fold-change: $\log_2$ baseMeanB/baseMeanA, pval: *p* value, padj: *p* value adjusted for multiple testing, resVarA: variance of A, resVarB: variance of B.A: stationary phase cells. B: exponential phase cells. NA, non-applicable because of zero expression.
(XLSX)

Table S2. Gene expression data from the complete transcriptome analysis of *Acinetobacter baumannii* ATCC 17978, showing gene expression levels in biofilm-associated cells vs. exponentially growing cells. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: exponential phase cells. B: biofilm-associated cells. NA, non-applicable because of zero expression.
(XLSX)

Table S3. Gene expression data from the complete transcriptome analysis of *Acinetobacter baumannii* ATCC 17978, showing gene expression levels of biofilm-associated vs. stationary phase cells. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: biofilm-associated cells. NA: non-applicable because of zero expression.
(XLSX)

Table S4. The expression levels of genes down-regulated in biofilm-associated vs. stationary phase cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: biofilm-associated cells.
(XLSX)

Table S5. The expression levels of genes up-regulated in biofilm-associated vs. stationary phase cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: biofilm-associated cells.

(XLSX)

Table S6. The expression levels of genes down-regulated in exponentially growing vs. stationary phase cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: exponential phase cells.
(XLSX)

Table S7. The expression levels of genes up-regulated in exponentially growing vs. stationary phase cells, filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: exponential phase cells.
(XLSX)

Table S8. The expression levels of genes down-regulated in biofilm-associated vs. exponentially growing cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: exponential phase cells. B: biofilm-associated cells.
(XLSX)

Table S9. The expression levels of genes up-regulated in biofilm-associated vs. exponentially growing cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value, padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: exponential phase cells. B: biofilm-associated cells.
(XLSX)

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Author Contributions

Conceived and designed the experiments: MP CG GB. Performed the experiments: SRF CG MP LAF MPC JV. Analyzed the data: AMA NRE AF MJG. Contributed reagents/materials/analysis tools: AMA NRE AF MJG MT. Wrote the manuscript: MP MJG.

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3.2. CHAPTER II: Contribution of the *A. baumannii* A1S_0114 gene to the interaction with eukaryotic cells and virulence

The previous transcriptomic analysis described in chapter I allowed us to identify and characterize the A1S_0114 gene of *A. baumannii* ATCC 17978 as a key factor involved in biofilm formation, due to its high expression level in biofilm-associated cells compared to planktonic cells. Furthermore, several genes from the A1S_0112-A1S_0119 cluster which expression is activated through a quorum sensing pathway, have been shown to be involved in biofilm biogenesis and surface motility. This gene cluster was predicted to act as a non-ribosomal peptide synthase (NRPS) and to participate in the biosynthesis of an uncharacterized secondary metabolite.

Thus, the aim of this chapter was to deepen the role of the A1S_0114 gene in different biological processes such as attachment to eukaryotic cells, virulence or synthesis of metabolites as well as decipher the relationship with its neighbouring genes. Firstly, *in silico* analysis of the region containing A1S_0112 to A1S_0119 genes indicated that they could form a polycistronic operon. RT-PCR assays confirmed these results and a review of their functions suggested that they could be involved in the production of secondary metabolites.

The implication of the A1S_0114 gene in biofilm formation was confirmed by scanning electron microscopy (SEM). SEM Images showed that the ATCC 17978 cells attached and formed multicellular structures on abiotic surfaces unlike the A1S_0114 defective strain, which was unable to form three-dimensional structures.

Adhesion experiments to A459 human epithelial alveolar cells were also performed. Adherence of the strain lacking the A1S_0114 gene was significantly lower than the wild type strain. Complementation of the knockout strain resulted in a restored attachment ability. SEM images supported these observations since while the ATCC 17978 strain could damage the surfactant layer and many bacteria were adhered to eukaryotic cells, only a few mutant cells were observed in the case of the defective strains lacking the A1S_0114 gene. These data suggest that the A1S_0114 gene plays an important role not

only in attachment to abiotic surfaces but also in adhesion to eukaryotic cells, which remarks its potential role in pathogenesis.

Thus, the role of the A1S_0114 gene in virulence was tested through three different animal models. A *C. elegans* fertility assay showed that the number of viable eggs was almost twice higher in worms infected with the knockout mutant strain compared to those infected with the wild type. Similar results were observed using a *G. mellonella* killing assay. A higher survival rate was obtained when larvae were infected with the mutant strain. The murine pneumonia model showed that lungs from mice infected with the mutant strain had a lower bacterial burden than those infected with the ATCC 17978 strain, which confirms and extends the previous results demonstrating the role of the A1S_0114 gene in virulence.

A liquid chromatography/mass spectrometry (LQ/MS) analysis of culture supernatants allowed us to detect the presence of a 505.28 Da molecule, named as acinetin 505 (Ac-505), that was present in the ATCC 17978 strain and absent in samples from the knockout strain. Ac-505 ($C_{23}H_{43}N_3O_7$) resembles a three-amino acid lipopeptide with non-standard linkages between amino acids as well as to the hydrocarbon moiety.

Altogether, data support the implication of the A1S_0114 gene of *A. baumannii* in virulence and suggest its involvement in the biosynthesis of the Ac-505 metabolite.

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Contribution of the *A. baumannii* A1S_0114 Gene to the Interaction with Eukaryotic Cells and Virulence

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Genetic and functional studies showed that some components of the *Acinetobacter baumannii* ATCC 17978 A1S_0112-A1S_0119 gene cluster are critical for biofilm biogenesis and surface motility. Recently, our group has shown that the A1S_0114 gene was involved in biofilm formation, a process related with pathogenesis. Confirming our previous results, microscopy images revealed that the ATCC 17978 Δ0114 derivative lacking this gene was unable to form a mature biofilm structure. Therefore, other bacterial phenotypes were analyzed to determine the role of this gene in the pathogenicity of *A. baumannii* ATCC 17978. The interaction of the ATCC 17978 parental strain and the Δ0114 mutant with A549 human alveolar epithelial cells was quantified revealing that the A1S_0114 gene was necessary for proper attachment to A549 cells. This dependency correlates with the negative effect of the A1S_0114 deletion on the expression of genes coding for surface proteins and pili-assembly systems, which are known to play a role in adhesion. Three different experimental animal models, including vertebrate and invertebrate hosts, confirmed the role of the A1S_0114 gene in virulence. All of the experimental infection assays indicated that the virulence of the ATCC 17978 was significantly reduced when this gene was inactivated. Finally, we discovered that the A1S_0114 gene was involved in the production of a small lipopeptide-like compound herein referred to as acinetin 505 (Ac-505). Ac-505 was isolated from ATCC 17978 spent media and its chemical structure was interpreted by mass spectrometry. Overall, our observations provide novel information on the role of the A1S_0114 gene in *A. baumannii*'s pathobiology and lay the foundation for future work to determine the mechanisms by which Ac-505, or possibly an Ac-505 precursor, could execute critical functions as a secondary metabolite.

Keywords: ***Acinetobacter baumannii*, biofilm, attachment, virulence, electron microscopy, secondary metabolite**

INTRODUCTION

Traditionally, *Acinetobacter baumannii* has been considered a low-virulence pathogen since its pathogenicity is influenced by the clinical condition of the patients it colonizes and infects. However, this epithet is often ignored by physicians due to its frequent and increasing occurrence as a multi-drug resistant (MDR) nosocomial pathogen around the world (Perez et al., 2008). In specific environments, such as intensive care and burn units where there is a remarkable selective antibiotic pressure, *A. baumannii* colonizes new niches because of its noteworthy ability to adapt to stressful conditions by modulating the expression of several virulence factors (Beceiro et al., 2013). Despite the importance of this microorganism, only a few virulence factors have been described to date (McConnell et al., 2013). This situation now can be addressed because of the development of inexpensive and convenient high-throughput sequencing methods, which have led to the description of numerous *A. baumannii* genomes and have facilitated the comparative analysis of entire genomes (Merino et al., 2014; Álvarez-Fraga et al., 2015; Ou et al., 2015). Furthermore, improved strategies for functional analysis of bacterial genes and the use of relevant animal models have provided novel insights into the virulence traits of this pathogen, which could lead to potential targets for the treatment of human infections (McConnell et al., 2013).

The inhibition of bacterial functions involved in quorum sensing, adhesion, colonization, iron acquisition and/or resistance to host defenses are possible strategies that could be used to fight bacterial infections, particularly those caused by MDR pathogens (Escaich, 2010). In the case of *A. baumannii*, one such strategy includes targeting capsular polysaccharides that have been identified as a virulence factors; capsule-deficient strains showed lower pathogenicity in a rat model (Russo et al., 2010). The acinetobactin-mediated iron acquisition system and related iron-mediated metabolic functions also play a role in *A. baumannii* ATCC 19606^T virulence as assessed using *ex vivo* and *in vivo* infection models (Gaddy et al., 2012; Zimbler et al., 2012, 2013). The ability of this pathogen to attach to different types of surfaces is also essential for its spread within the hospital environment and among patients as well as to colonize host tissues and medical devices. The outer membrane protein A (OmpA) and the biofilm-associated protein (Bap) are critical in host-pathogen interactions as well as in the interaction of bacteria with abiotic and biotic surfaces including human epithelial cells and neonatal keratinocytes (Choi et al., 2005, 2008; Iacono et al., 2008; Loehfelm et al., 2008; Kim et al., 2009). The *A. baumannii* ATCC 19606^T Type I pili assembled by the CsuA/BABCDE usher-chaperone assembly system were the first cellular appendages shown to be crucial for adherence and biofilm formation on abiotic surfaces under different experimental conditions (Tomaras et al., 2003). Our group previously identified the gene A1S_1507, which is part of a second Type I pili cluster, the disruption of which caused a significant decrease in biofilm formation by *A. baumannii* ATCC 17978 (Rumbo-Feal et al., 2013).

Comparative transcriptional studies of *A. baumannii* ATCC 17978 planktonic and sessile cells showed that expression of the A1S_0114 gene had the highest fold-change in biofilm-associated cells as compared to planktonic cells (Rumbo-Feal et al., 2013). Accordingly, deletion of this predicted gene led to a substantial decrease in biofilm formation (Rumbo-Feal et al., 2013). Random transposon mutagenesis of *A. nosocomialis* M2 resulted in the isolation of the M2-2 and M2-11 derivative mutants, which displayed a significant reduction in surface motility and harbored insertions in the *A. baumannii* ATCC 17978 A1S_0113 and A1S_0115 orthologs (Clemmer et al., 2011). Further RNA-Seq analysis and A1S_0112-*lacZ* fusion assays showed that the expression of the A1S_0112-A1S_0118 genes in *A. nosocomialis* M2 is transcriptionally activated by an AbaI-dependent quorum-sensing pathway (Clemmer et al., 2011). More recently, random insertion mutagenesis of the *A. baumannii* ATCC 17978hm, a hyper-motile derivative that harbors an IS insertion within the *hns*-like gene (Eijkelkamp et al., 2013; Giles et al., 2015), resulted in the isolation of the A1S_0112::Tn and A1S_0115::Tn mutants (Giles et al., 2015). Both mutants displayed no surface motility and a significant reduction in pellicle formation with an increased biofilm formation phenotype, observations suggesting a further link between motility and biofilm/pellicle formation (Giles et al., 2015). Based on all these observations, it was proposed that the A1S_0112-A1S_0118/0119 genes constitute a seven- or eight-gene operon, which is predicted to be involved in the biosynthesis of an uncharacterized secondary metabolite, such as a non-ribosomally synthesized lipopeptide (Clemmer et al., 2011; Eijkelkamp et al., 2013, 2014; Giles et al., 2015). Interestingly, this operon has been identified in many of the available genomic sequences of *A. baumannii*, including MDR strains (Adams et al., 2008; Iacono et al., 2008; Zhu et al., 2013), with the exception of the nonpathogenic *A. baumannii* SDF strain (Vallenet et al., 2008).

In this report we have established that the *A. baumannii* ATCC 17978 (referred to as 17978 in the rest of this work) A1S_0112-A1S_0119 gene cluster is indeed a polycistronic operon that includes eight genes predicted to code for proteins with functions involved in the production of bacterial secondary metabolites. Furthermore, a 17978 A1S_0114 isogenic deletion derivative (Δ0114) showed a significant reduction in cell adherence and virulence, as confirmed using three animal models. Mass spectrometry analysis of spent culture supernatants showed that the deletion of the A1S_0114 gene is associated with the absence of acinetin 505 (Ac-505), a 505-Da lipopeptide in which a hydroxylated-C_{15} acyl moiety is linked to both a Gly and a Cys-Gly containing moiety and has a non-standard peptide linkage. These observations indicate that the A1S_0114 gene could play a critical role in the pathobiology of *A. baumannii*, knowledge that could aid in the design of alternative therapeutic tools needed for the treatment of infections caused by emerging MDR isolates.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

A. baumannii ATCC 17978 and *Escherichia coli* strains listed in **Table 1** were routinely grown or maintained in Luria-Bertani (LB) broth with 2% agar added for plates. All strains were grown at 37°C with shaking (180 rpm) and stored at -80°C

TABLE 1 | Bacterial strains and plasmids used in this work.

Strain or plasmid	Relevant characteristic(s)	Sources or references
STRAINS		
A. baumannii		
ATCC 17978	Clinical isolate	ATCC
Δ0114	ATCC 17978 A1S_0114 deletion derivative	This study
Δ0114.C	17978 Δ0114 harboring pWH1266-Km-0114; Km^r	This study
Δ0114.E	17978 Δ0114 harboring pWH1266-Km; Km^r, Tc^r	This study
E. coli		
TG1	Used for DNA recombinant methods	Lucigen
OP50	Used for maintenance of *C. elegans*; Ura^-, Str^r	CGC
PLASMIDS		
pCR-Blunt II-TOPO	Cloning vector; Km^r, Zeo^r	Invitrogen
pWH1266	*A. baumannii* shuttle vector; Ap^r, Tc^r	Stiernagle, 2006
pWH1266-Km	*A. baumannii* shuttle vector; Km^r, Tc^r	This study
pWH1266-Km-0114	pWH1266-Km harboring A1S_0114; Km^r	This study
pMo130	Suicide vector for construction of *A. baumannii* isogenic derivative; Km^r, SacB, XylE	Hamad et al., 2009

Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tet^R, tetracycline resistance; Str^R, streptomycin resistance; Zeo^R, zeocin resistance.

in LB broth containing 10% glycerol. Swimming broth (SB) containing 10 g/L of tryptone and 5 g/L of NaCl was used for some phenotypic analyses and 0.3% of agarose were added for plates (Harding et al., 2013). When appropriate, cultures were supplemented with kanamycin (Km) at a final concentration of 50 μg/mL. Bacterial growth curves were determined in sextuplet using 96-well microtiter plates containing either LB or SB inoculated with 17978 or Δ0114 cells under the aforementioned culturing conditions over a 24-h time period (**Figure S2**). OD_{600} values of these cultures were recorded hourly.

Construction of Isogenic Deletion Derivatives

Plasmid pMo130 (**Table 1**), a suicide vector containing the genes *xylE*, *sacB,* and a Km resistance marker, was used as described by Hamad et al. (2009). Briefly, 900–1,000 bp upstream and downstream regions flanking the genes selected for deletion in 17978 were PCR-amplified and cloned into the pMo130 vector using primers listed in **Table S1**. The resulting plasmid (pMo130-0114, shown in **Table 1**) was transformed into 17978 cells by electroporation (Rumbo-Feal et al., 2013). Recombinant colonies representing the first crossover event were selected by resistance to Km and visual detection of XylE activity following the catechol-based method (Hamad et al., 2009). Bright yellow Km resistant colonies were then grown overnight in LB supplemented with 15% sucrose and then plated on LB agar without antibiotics. The second crossover event leading to gene deletion was then confirmed by PCR using primers listed in **Table S1**. The Δ0114 isogenic deletion derivative of 17978 was constructed by deleting a region encompassing the A1S_0114 gene (Hamad et al., 2009).

Complementation of the Δ0114 Deletion Derivative

A kanamycin resistance marker was PCR-amplified from the pCR-BluntII-TOPO plasmid (from Invitrogen) using the primers listed in **Table S1**. The resulting product was inserted in the *Pst*I site of the pWH1266 plasmid (Hunger et al., 1990), obtaining the pWH1266-Km plasmid. To complement the Δ0114 strain, the A1S_0114 wild type allele was PCR-amplified from 17978 genomic DNA using the primers listed in **Table S1**. The resulting product was cloned into the *Eco*RV and *Bam*HI restriction sites of the pWH1266-Km plasmid (**Table 1**). The parental A1S_0114 allele was cloned as a *Bam*HI-*Eco*RV amplicon into the pWH1266-Km under the control of the tetracycline resistance gene promoter using the primers listed in **Table S1**. The complementing plasmid pWH1266-Km-0114 (**Table 1**) was transformed into Δ0114 cells by electroporation (Rumbo-Feal et al., 2013). Transformants were selected on Km-containing plates and the presence of pWH1266-Km-0114 was confirmed by PCR using primers listed in **Table S1**. Δ0114 cells harboring empty pWH1266-Km were used as a negative control.

RNA Extraction and Transcript Quantification

Cultures of 17978 and its derivatives were grown for 48 h at 37°C in SB. RNA was extracted with the Maxwell 16 LEV simplyRNA Cells Kit (Promega). The total RNA samples were treated using the DNA-free DNA Removal Kit (Ambion). The integrity of the RNA samples was checked using an Agilent 2100 Bioanalyzer and qPCR. cDNA was obtained from RNA samples using the iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's recommendations. The level of expression of particular genes was tested by real time PCR (qRT-PCR) using cDNA as a template and the KAPA SYBR FAST qPCR kit (Kapa Biosystems) following the instructions of the manufacturer with the primers listed in **Table S1**. Three independent biological replicates were each tested in triplicate. The expression level was standardized relative to the transcription level of the housekeeping gene *recA* which was established as 1. PCR reactions lacking cDNA were used as negative controls. The statistical significance of the differences was determined using a Student's *t*-test.

To confirm the polycistronic nature of the A1S_0112-A1S_0119 gene cluster, cDNA, obtained from total RNA through reverse transcription, was used as a template in PCR reactions with Taq DNA Polymerase (New England Biolabs) using pairs of primers designed to anneal to the 3′-end of every gene and the 5′-end of the next one (**Figure 1** and **Table S1**). Genomic DNA and total RNA without reverse transcription were used as templates for positive and negative controls, respectively, and the amplicons

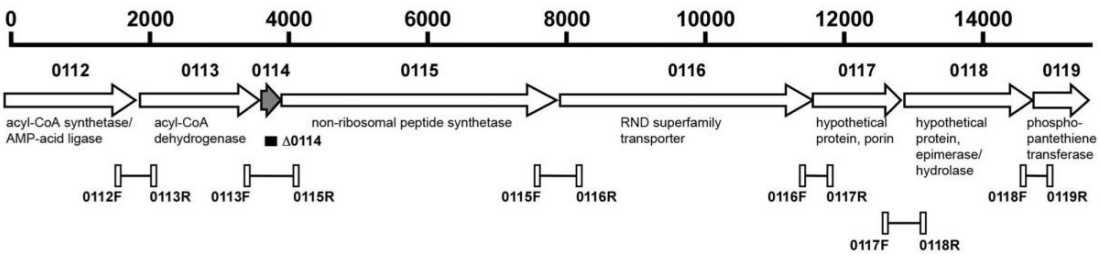

FIGURE 1 | Genetic organization of the A1S_0112-A1S_0119 operon of *A. baumannii* ATCC 17978. The horizontal arrows indicate the location and direction of transcription of the predicted genes. The numbers above the arrows represent the A1S_ genes located within this operon. The black rectangle indicates the region deleted within the A1S-0114 gene represented by the hatched arrow. The connected open rectangles and the number underneath of each of them identify the primers, listed in **Table S1**, and the regions amplified by PCR and RT-PCR assays using genomic DNA and DNA-free total RNA, respectively. Putative functions of the corresponding proteins are shown.

were detected by standard 1% agarose gel electrophoresis (Sambrook and Russell, 2001). These analyses were done in triplicate.

LC-MS Analysis of Bacterial Supernatants

The 17978 and Δ0114 strains were grown in static cultures and cell-free spent media was analyzed by liquid chromatography (LC)—mass spectrometry (MS) and LC-MS/MS with further internal source collision induced dissociation (ISCID) fragmentation. In brief, bacterial samples were cultured overnight in SB with shaking (200 rpm) at 37°C and used to inoculate SB at a 1:100 ratio. Then, 1-mL aliquots were dispensed into 12 × 75 mm polystyrene culture test tubes for incubation at 37°C for 48 h without shaking. Bacteria for isotopic labeling were cultured under the same conditions in unlabeled and U-^{15}N, 98% and U-^{15}N/^{13}C, 98% Bioexpress Cell Growth Media (Cambridge Isotope Laboratories, Inc.). Samples were vortexed, transferred to microcentrifuge tubes and centrifuged at 21,000 × g to remove cells. Aliquots were prepared for LC-MS by mixing 300 μL of spent media with 1:1 volume of 100% methanol (HPLC grade) containing 0.2% formic acid. LC-MS and LC-MS/MS (ISCID) data were collected on a micrOTOF Bruker Daltonics MS with Agilent Technologies 1200 Series LC and an analytical C_{18} column (100 × 2.1 mm, 2.6 mm particle size, Phenomenex Kinetex) operated at a column temperature of 35°C with a 0.2 mL/min flow rate. Solvent A was 100% water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. The elution method was a 30-min gradient from 100% solvent A to 90% solvent B in 25 min, followed by 5 min at 90% solvent B before returning to 100% solvent A for equilibration. Electrospray ionization (ESI)-MS was used in positive ion mode (*m/z* 50–2,000). Nitrogen was used as the drying gas with a temperature and nebulizing gas pressure of 190°C and 5.8 psi, respectively. The ESI capillary voltage of 4,500 V and Hexapole RF of 80 V_{pp} were used. ISCID energy of 80 eV (200 V) was applied for CID fragmentation. A sodium formate solution was employed as the external calibrant by direct infusion at the end of each run. LC-MS and LC-MS/MS (ISCID) data were analyzed using DataAnalysis 4.0 (Bruker Daltonics) software.

High-Resolution MS and MS^n Analysis of Ac-505

The 17978 strain was grown for 48 h at 37°C in SB in 2 L static cultures in flat-bottom flasks after a 1:100 dilution of innoculated shaken overnight culture. After centrifugation at 5,000 × g for 20 min to remove cells, 15 g of adsorptive XAD-7 resin was added to the culture supernatant and incubated at room temperature for 3 h. The resin was removed on glass filter paper in a Büchner funnel and then washed with 1 L deionized H_2O. The adsorbed material was then eluted with 300 ml of 100% methanol, dried on a rotary evaporator, and resuspended in 2 ml of methanol. Concentrated samples in methanol were kept at −80°C. One-ml injections were made on a BioCAD family vision Workstation equipped with a UV-detector, fraction collector, and a reversed-phase C_{18} preparative column (250 × 10.0 mm, 10 um particle size, Phenomenex Synergi Fusion-RP) operated at a 5 ml/min flow rate of the solvents A and B described above. The elution method was a gradient from 0 to 100% B in 10-column volumes, followed by an isocratic step with 100% solvent B for 5-column volumes. Eluted fractions of 1 ml were analyzed by analytical LC-MS on a Bruker micrOTOF (described above) and fractions containing purified Ac-505 were combined and dried using a stream of nitrogen.

High resolution LC-MS/MS in positive ion mode was performed at the Ohio State University (OSU) Mass Spectrometry and Proteomics Facility on a Bruker MaXis ESI Ionization Quadrupole Time-of-Flight Mass Spectrometer (ESI MaXis QTOF) with a Dionex U3000 RSLC system using a Waters Xbridge BEH C_{18} (3.5 μm, 1.0 × 100 mm) column and a gradient of H_2O and acetonitrile with 0.1% formic acid. Additionally, direct-infusion MS/MS in negative ion mode was performed on the ESI MaXis QTOF instrument. Ultra high resolution MS/MS was performed on the OSU ESI 15 tesla fourier transform ion cyclotron resonance (15T FT-ICR) instrument with electron-capture dissociation (ECD) of the

506.3 *m/z* peak in positive ion mode by direct infusion. MS^n fragmentation in positive ion mode was measured on the OSU Bruker amaZon, which has nominal mass resolution, using direct infusion of 5-µl samples of HPLC-purified Ac-505 in 50% methanol with 0.1% formic acid.

Bacterial Adhesion to A549 Human Alveolar Cells

A549 human alveolar epithelial cells were routinely maintained in 25-cm^2 tissue culture flasks in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated fetal bovine serum and 50 U/mL of penicillin and 50 µg/mL of streptomycin (DMEM) as described before (Gaddy et al., 2009). Confluent cultures were washed, trypsinized and transferred to 24-well plates to get a monolayer of 10^5 A549 cells *per* well. After 24 h of incubation under the same conditions, cells were washed twice with saline solution and once with modified Hank's balanced salt solution (mHBSS, same as HBSS but without glucose) following the protocol previously described (Gaddy et al., 2009). Then, the multiplicity of infection (MOI) of 10 was used; in each well 10^5 A549 cells were infected with 10^6 bacteria and incubated for 3 h in mHBSS at 37°C. To determine bacterial adhesion, the infected monolayers were washed three times with saline solution and then lysed in 500 µL of 0.5% sodium deoxycholate. Dilutions of the lysates were plated onto LB agar and incubated at 37°C for 24 h. Colony forming units were counted to determine the percent of bacteria that had attached to or invaded A549 cells as compared to the growth control. Six independent replicates were done. Student's *t*-test was performed to evaluate the statistical significance of the observed differences.

Electron Microscopy of Biofilms Formed on Plastic and Polarized A549 Cells

Sterile plastic coverslips were placed in sterile 50-mL conical tubes and then 5 ml of LB inoculated with each strain at a 1:100 dilution were added. Inoculated tubes were incubated for 48 h at 37°C without shaking as previously described (Gaddy et al., 2009). Coverslips were removed, washed, dehydrated in ethanol, processed with a critical point drier, and sputter coated as described previously (Tomaras et al., 2003). Biofilms formed above, at and below the liquid-air interface were viewed by scanning electron microscopy (SEM) using a Zeiss Supra Gemini Series 35 V scanning electron microscope as described previously (Tomaras et al., 2003).

A549 cells were polarized on the surface of Transwell 24-well permeable inserts as recently described (Álvarez-Fraga et al., 2016). Bacteria, previously grown in LB at 37°C for 24 h in a shaking incubator at 180 rpm, were washed and resuspended in Hank's Buffered Salt Solution (HBSS; Hyclone Laboratories, Inc.). An inoculum of 10^2 bacteria was applied to the apical surface of A549 cells by pipetting 1 µl of suspension onto the center of each membrane. The transwell plate was then incubated and maintained for 72 h at 37°C and 5% CO_2. After 72 h, the membranes were washed with HBSS to remove secretions and unattached bacteria. The membranes were then fixed for 24 h in 4% formaldehyde-HBSS at 4°C, prepared and viewed by SEM as previously described (Tomaras et al., 2003).

Caenorhabditis elegans Fertility Assay

Fertility assays were performed as previously described (Vallejo et al., 2015). Both the 17978 and Δ0114 strains were grown overnight in LB and then cultured at 37°C for 24 h in nematode growth medium (NG). The eggs of *C. elegans* N2 Bristol (a wild-type strain obtained from the CGC collection) were hatched in M9 medium (Vallejo et al., 2015), and worms in the first larval stage (L1) were arrested overnight at 20°C. Later, the L1 worms were added to the NG medium plates together with each bacterial strain selected for this study. One *C. elegans* worm in the last larval stage (L4) was placed on a peptone-glucose-sorbitol medium (PGS) plate individually seeded with each *A. baumannii* strain and incubated for 24 h at 25°C. The worms were transferred to new plates seeded with the same bacterial strain and the worm progeny was counted for 3 days to determine their viability. Six independent replicates were performed with each strain. Student's *t*-test was performed to evaluate the statistical significance of observed differences. Means of the differences between strains are reported.

Galleria mellonella Virulence Assay

A. baumannii cells previously grown for 24 h in LB broth were collected by centrifugation and resuspended in sterile phosphate-buffered saline (PBS). Appropriate bacterial inocula were estimated spectrophotometrically at OD_{600} and confirmed by plate counting using LB agar plates. To assess virulence, *G. mellonella* survival assays were performed by injecting in triplicate 10 randomly-selected healthy final instar *G. mellonella* larvae as previously described (Gaddy et al., 2012). The dose used for each infection consisted of a 5 µl-suspension containing 10^5 CFU (Gaddy et al., 2012). The control groups included larvae that either were not injected or were injected with the same volume of sterile PBS. The test groups included larvae infected with 17978 or Δ0114 bacteria. After injection, the larvae were incubated at 37°C in darkness, and death was assessed at 24-h intervals over 6 days. Caterpillars were considered dead, and were removed, if they displayed no response to probing. The resulting survival curves were plotted using the Kaplan-Meier method (Kaplan and Meier, 1958) and analyzed using the log-rank (Mantel-Cox) test. $P \leq 0.05$ were considered statistically significant (SAS Institute Inc.).

Murine Pneumonia Virulence Assay

A pneumonia model was used to evaluate the virulence of the 17978 and Δ0114 strains. BALB/c 9- to 11-week old female mice weighing 25–30 g were intratracheally infected with ~5.5 × 10^7 CFUs/mouse of exponentially grown cells of the 17978 parental or the Δ0114 mutant strains into groups of 10 mice. Briefly, mice anesthetized with an oral suspension of sevoflurane (Abbott) were suspended by their incisors on a board in a semi-vertical position. Correct intratracheal inoculation was confirmed by using an endoscope on the oral cavity. The trachea was accessed using a blunt-tipped needle for the inoculation of a 40-µl bacterial suspension made in sterile saline solution

and 10% porcine mucin (wt/vol; Sigma) mixed at a 1:1 ratio. Dead mice in the first 4 h after inoculation were not included in the final analyses. Mice were euthanized with an overdose of thiopental sodium (Sandoz) 44 h after inoculation. Lungs were aseptically extracted, weighed, and homogenized in 1.5 ml of ice-cold saline solution in a Mixer Mill dismembrator (Retsch). Lung lysates were 10-fold serially diluted and samples were plated onto LB agar to measure organ bacterial loads. The results are shown as means of the $\log_{10}$ CFU *per* gram of lung with their standard deviations. Student's *t*-test was performed to evaluate the statistical significance of the observed differences. All mice were maintained in the specific pathogen-free facility at the Technology Training Center of the Hospital of A Coruña (CHUAC, Spain). All experiments were done with the approval of and in accordance with regulatory guidelines and standards set by the Animal Ethics Committee (CHUAC, Spain).

RESULTS

Characterization of the *A. baumannii* ATCC 17978 A1S_0112-0119 Operon

Previous transcriptional analysis of 17978 planktonic and sessile bacteria showed the differential expression of the A1S_0112-A1S_0118 genes, with A1S_0114 being expressed most highly by cells attached to an abiotic surface (Rumbo-Feal et al., 2013). *In silico* analysis of the chromosomal region harboring these genes indicates that it potentially codes for a polycistronic operon that includes the A1S_0119 coding region (**Figure 1**). This prediction is based on the observation that all putative genes are transcribed in the same direction and either overlap or are separated by intergenic regions ranging from 24 to 53 nucleotides according to reported genomic data (Smith et al., 2007) and our *in silico* analysis. The polycistronic nature of this operon was confirmed by RT-PCR analysis of total RNA using primers connecting the eight predicted genes. **Figure S1** shows the detection of the predicted amplicons when total RNA was reversed transcribed and PCR amplified using the primers shown in **Figure 1** and listed in **Table S1**, with their sizes matching those detected when total DNA was used as a template. In contrast, no amplicons were detected in any of the RNA samples that were PCR amplified without previous reverse transcription.

The analysis of 17978 genomic data showed that the 15,551-nt region harboring the A1S_0112-A1S_0119 predicted coding region is separated from an upstream *luxR* ortholog (A1S_0111) and a downstream tRNA-Gly (A1S_0120) gene by a 616-nt and a 95-nt non-coding region, respectively, with the latter gene being transcribed in the opposite direction.

Inactivation of A1S_0114 Affects Bacterial Interaction with Abiotic and Biotic Surfaces

In previous work we demonstrated the involvement of the A1S_0114 gene in the ability of 17978 cells to form biofilms on abiotic surfaces using crystal violet assays (Rumbo-Feal et al., 2013). This result is further supported in the present work by the analysis of biofilms formed on glass using SEM, which showed that 17978 cells attach to the abiotic surface and form multicellular structures associated with mature biofilms at the air-liquid interface (**Figures 2A,B**). In contrast, single or small cell clumps of the mutant derivative lacking the A1S_0114 gene (Δ0114) attached to the substratum without forming dense and three-dimensional structures (**Figures 2C,D**). It is important to note that the site-directed deletion of A1S_0114 did not affect the growth of the isogenic derivate when cultured either in Luria-Bertani (LB) or swimming broth (SB) without selective pressure (**Figure S2**).

The biological effect of the A1S_0114 deletion was also tested using A549 human alveolar epithelial cells as a model since they represent a host cell that could be targeted by *A. baumannii* during the pathogenesis of respiratory infections. To test the effect of the A1S_0114 deletion on bacterial adherence, submerged A549 confluent monolayers were co-incubated with 17978 or Δ0114 bacteria for 3 h and CFU counts were determined by plating serial dilutions of tissue culture cell lysates. This approach showed that the amount of Δ0114 bacteria recovered from A549 infected cells was 60% lower than that recovered from monolayers infected with 17978 bacteria ($P = 0.0198$; **Figure 3A**). Electroporation of Δ0114 cells with pWH1266-Km-0114, which harbors the A1S_0114 wild type allele expressed from the tetracycline resistance gene promoter, partially restored the adherence phenotype in the Δ0114.C derivative, a phenomenon that was not observed with Δ0114.E, a Δ0114 derivative transformed with the pWH1266-Km empty vector ($P = 0.0067$; **Figure 3A**).

The results obtained with submerged A549 monolayers were further confirmed by infecting A549 polarized cells with either the 17978 parental or the Δ0114 mutant strains. SEM analysis of A549 samples infected with 17978 bacteria revealed extensive damage to the surfactant layer that covers the polarized cells as well as to the epithelial cells themselves when compared with non-infected polarized cell samples (**Figure 3B**). Furthermore, micrograph 17978 shows the presence of numerous bacteria attached to the surface of cells or cell debris clearly seen after the surfactant layer was destroyed by bacterial action. Although the infection of A549 polarized samples by Δ0114 bacteria also resulted in destruction of the surfactant layer and cell damage, it appears that the deletion of A1S_0114 results in a readily detectable reduction of bacteria attached to the polarized samples (**Figure 3B**).

Role of A1S_0114 in the Expression of Genes Involved in Adherence and Biofilm Biogenesis

The observation that the deletion of A1S_0114 significantly affects bacterial adherence and biofilm biogenesis, prompted us to examine the differential expression of genes known or predicted to be involved in these *A. baumannii* cellular functions, including *ompA* and the *csuA/B* gene of the *csuA/BABCDE* pili assembly system (Tomaras et al., 2003; Gaddy et al., 2009; Cabral et al., 2011), and the genes A1S_0690, A1S_1510, and A1S_2091 that could be involved in bacteria-surface interactions (Rumbo-Feal et al., 2013; Eijkelkamp et al., 2014; Nait Chabane

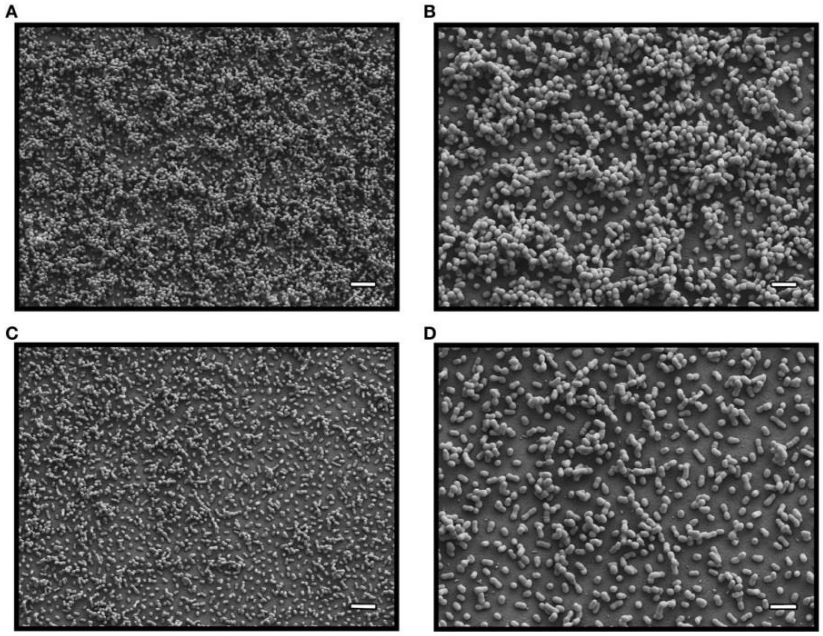

FIGURE 2 | SEM analysis of bacterial biofilms. Biofilms formed by 17978 (micrographs **A** and **B**) and Δ0114 (micrographs **C** and **D**) cells at the liquid-air interface. Micrographs A and C were recorded at a 5,000x magnification, while micrographs **B** and **D** were collected at a 10,000x magnification. White bars: 4 μm for **A**,**C**; 2 μm for **B**,**D**.

et al., 2014; Álvarez-Fraga et al., 2016). The comparative qRT-PCR analysis of total RNA isolated from 17978 and Δ0114 bacterial cells showed that the transcription of *csuA/B* is 5-fold increased in the Δ0114 mutant when compared with the parental wild-type strain (**Table 2**). In contrast, the transcriptional expression of the A1S_0690, A1S_1510, and A1S_2091 genes were significantly reduced, with A1S_2091 and A1S_1510 showing the highest (12-fold) and lowest (1.5-fold) changes, respectively. Deletion of A1S_0114 also caused a small (1.6-fold) but significant reduction in the transcription of *ompA*.

A1S_0114 Plays a Role in Virulence

The role of the A1S_0114 gene in the virulence of 17978 was assessed with a fertility assay using the *C. elegans* model, a survival assay using the caterpillar *G. mellonella* and a mouse pneumonia model, all of which have been previously used to examine the virulence of *A. baumannii*, particularly that of the 17978 strain (McConnell et al., 2013). *C. elegans* fertility assays showed that the total number of viable eggs was almost twice as high when worms were infected with Δ0114 when compared with 17978 (**Figure 4A**), with the difference between these two isogenic strains being statistically significant ($P < 0.0001$). Similarly, infection of *G. mellonella* larvae showed that *ca.* 50% of them died 5 days after being injected with 17978 (**Figure 4B**). This value was significantly different ($P = 0.024$) from that obtained with non-injected animals or animals injected with sterile PBS, which were used as negative controls. Although the infection of caterpillars with Δ0114 showed a killing rate that was significantly higher than the negative controls, the virulence of the mutant was significantly attenuated when compared with the parental strain ($P = 0.025$). Finally, the ability of the Δ0114 mutant to establish infection in an experimental murine model was evaluated. This model showed that lungs from mice infected with Δ0114 displayed a significantly lower bacterial burden than those from animals infected with 17978 ($P = 0.0165$) after 44 h of intratracheal infection (**Figure 4C**).

Identification and Characterization of the Secondary Metabolite Ac-505

The observation that the A1S_0114 gene plays a critical role in the pathobiology of *A. baumannii* prompted us to initiate a search for compounds that were produced by cells of the 17978 parental but not by cells of the Δ0114 mutant derivative. Liquid chromatography/mass spectrometry (LC-MS) analysis in positive ion mode of swimming broth (SB) static culture supernatants harvested at 48 h post inoculation showed the presence of a single peak with a retention time of ∼21 min and a molecular weight of 505.28 Da (m/z 506.29 $[M+H]^+$), which was consistently present in 17978 samples but not detected in samples from the

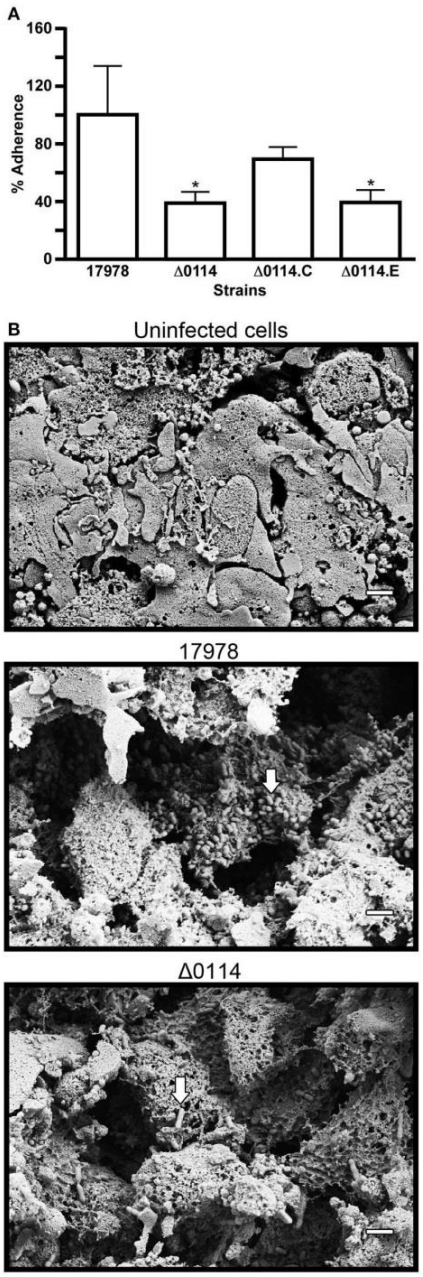

FIGURE 3 | Analysis of bacteria-human cell interactions. (A) Quantification of the adherence of 17978 and Δ0114 bacteria either not transformed or transformed with pWH1266-Km-0114, which harbors the A1S_0114 parental allele (Δ0114.C), or the empty shuttle vector pWH1266.Km (Δ0114.E) to polarized A549 cells. Bacterial adherence is *(Continued)*

FIGURE 3 | Continued
reported as the % of recovered bacteria compared to data collected with 17978, which was considered 100%. Data represent three independent replicates. Student's *t*-test was used to validate the experimental data, values are means and bars indicate standard deviation (*$P < 0.05$). **(B)** SEM analysis of A549 polarized cells either not infected (Uninfected cells) or infected with 17978 (17978) or with Δ0114 bacteria (Δ0114). All micrographs were taken at 10,000x magnification. White bars indicate the scale marks (2 μm). White arrows indicate bacteria attached to A549 cells or cell debris.

Δ0114 deletion derivative (**Figure 5A**). Based on this finding, we have named this compound acinetin 505 (Ac-505). Time course studies of SB cultures harvested every 24 h for 5 days (data not shown) showed a maximum amount of Ac-505 at 48 h post inoculation that leveled off. Further analysis of Ac-505 isolated and purified from 2 L of static SB culture supernatant showed that its molecular formula is $C_{23}H_{43}N_3O_7S$ based on ultra high-resolution 15T FT-ICR MS (*m/z* 506.28945 $[M+H]^+$, calculated 506.28945) as shown in **Figure 5B**. The number of predicted nitrogen and carbon atoms was further confirmed by growing 17978 cultures in unlabeled, and ^{15}N- and $^{15}N/^{13}C$-labeled media. LC-MS analysis of these culture supernatants confirmed the incorporation of 3 nitrogen and 23 carbon atoms in Ac-505 (*m/z* 506.3, 509.3, and 532.3 $[M+H]^+$, respectively). Additional high-resolution mass spectrometry (HRMS) analyses of FT-ICR ECD and MaXis QTOF MS/MS data are reported in **Tables S2**, **S3** and **Figures S3**, **S4C,D**, **S5**, **S6**. Nominal mass LC-MS^n fragmentation data from the Bruker amaZon were also used in the structural interpretation and two of the 40 spectra are shown in **Figures S4A,B**. Positive ion mode data were used to confirm neutral losses of water, two C-terminal glycines, cysteine, and cysteine-glycine as seen in **Figures S4A,C**. The MS/MS fragmentation data in both positive and negative ion mode correspond to data for other Cys-Gly conjugated compounds (Levsen et al., 2005). Specifically, the negative ion with 143.05 *m/z* is characteristic for detection of Cys-Gly conjugates (Dieckhaus et al., 2005). By analysis of the MS^n and FT-ICR fragmentation data, there is a neutral loss of water followed by loss of Cys-Gly and a second water leaving the remaining ion with the formula of $C_{18}H_{32}NO3^+$ (310.2 *m/z*) as seen in **Table S2** and **Figure S4C**. Further neutral loss of $C_2H_5NO_2$ from the 310.2 *m/z* ion matches a terminal glycine loss and the remaining 235.2 *m/z* ion has the formula $C_{16}H_{27}O^+$ (**Table S2**). The fragmentation patterns in the positive ion mode MS^n data for 310.2 and 235.2 *m/z* (shown in **Figure S4B**) had characteristic neutral losses of at least six 14 *m/z* corresponding to CH_2 losses, indicating a hydrocarbon chain of at least C_6. A neutral loss of $C_{14}H_{28}O$ from the 504.3 *m/z* parent ion was observed by MS/MS in negative ion mode as is shown in **Figure S3C**. Taken together, the MS data indicate that the structure of Ac-505 contains a Cys-Gly that is connected to the rest of the molecule via a sulfur linkage through the Cys side chain in a non-standard peptide linkage as shown in **Figure 5C**. The attachment of the Cys-Gly to the α-carbon of a Gly-containing moiety results in a so-called α-thio linkage. The Gly-containing moiety is connected to a hydroxylated acyl moiety of 15 carbons with a linkage that is neither *N*- or *O*-linked. The MS data supporting the

TABLE 2 | Expression level of genes *csuA/B*, A1S_2091, A1S_1510, A1S_0690, and *ompA* in *A. baumannii* ATCC 17978 and its mutant derivative 17978 Δ0114.

Gene[a]	ATCC 17978	17978 Δ0114	*P*-value
csuA/B	0.36 ± 0.14	1.81 ± 0.40	0.004
A1S_2091	2.64 ± 0.41	0.22 ± 0.04	0.0005
A1S_1510	0.86 ± 0.10	0.56 ± 0.04	0.0085
A1S_0690	8.48 ± 0.92	3.07 ± 0.38	0.0007
ompA	1.23 ± 0.14	0.76 ± 0.08	0.0071

[a] *The expression level of each gene was determined with respect to the expression level of recA, which was defined as 1.*
Values are means ± standard deviation. P-values indicate significant differences as determined by a Student's t-test.

structure shown in **Figure 5C** are detailed in **Tables S2**, **S3** and **Figures S4 –S6**.

DISCUSSION

A. baumannii has emerged as a pathogen with a remarkable ability to adapt and persist in response to a wide range of extracellular stimuli (Fiester and Actis, 2013). Such capacity relates to its genetic plasticity and the acquisition of genes coding for virulence-associated functions by horizontal gene transfer mechanisms. While some of these genes code for functions clearly related to bacterial pathogenicity (McConnell et al., 2013), there are other genes that code for potential virulence-associated traits whose mechanisms of action and biological roles are not fully understood. Among these genes, there is a gene cluster that was identified by our previous work as well as by other investigators using different *Acinetobacter* clinical isolates.

Study of the *A. baumannii* ATCC 17978, *A. baumannii* ATCC 17978hm and *A. nosocomialis* M2 strains resulted in the identification of the A1S_0112-A1S_0118 gene cluster while the analysis of the *A. baumannii* AB307-0294 isolate identified the ABBFA_003406-ABBFA_003399 cluster (Clemmer et al., 2011; Rumbo-Feal et al., 2013; Allen and Gulick, 2014; Giles et al., 2015). Importantly, these gene clusters code for cognate proteins that are highly related. Based on the nucleotide structure and the coding nature of the genes contained within this cluster of orthologous genes, it was hypothesized that it is either a 7- or 8-gene operon (Clemmer et al., 2011; Rumbo-Feal et al., 2013; Allen and Gulick, 2014; Giles et al., 2015). Transcriptional data presented in this report proved that this cluster is indeed a single 8-gene polycistronic transcriptional unit that encompasses the A1S_0112-A1S_0119 coding regions. This genetic structure explains the differential transcription reported for the A1S_0112-A1S_0118 genes in planktonic cells as compared to sessile cells (Rumbo-Feal et al., 2013). Our previous transcriptomic and mutagenesis studies also showed that site-directed deletion of A1S_0114, one of the highest transcribed genes in sessile cells when compared to exponential- and stationary-phase planktonic bacteria, caused a drastic reduction in biofilm biogenesis (Rumbo-Feal et al., 2013), a result that has been further confirmed in this work. The Δ0114 mutant not only forms less biofilms on plastic (**Figure 2**), but also shows

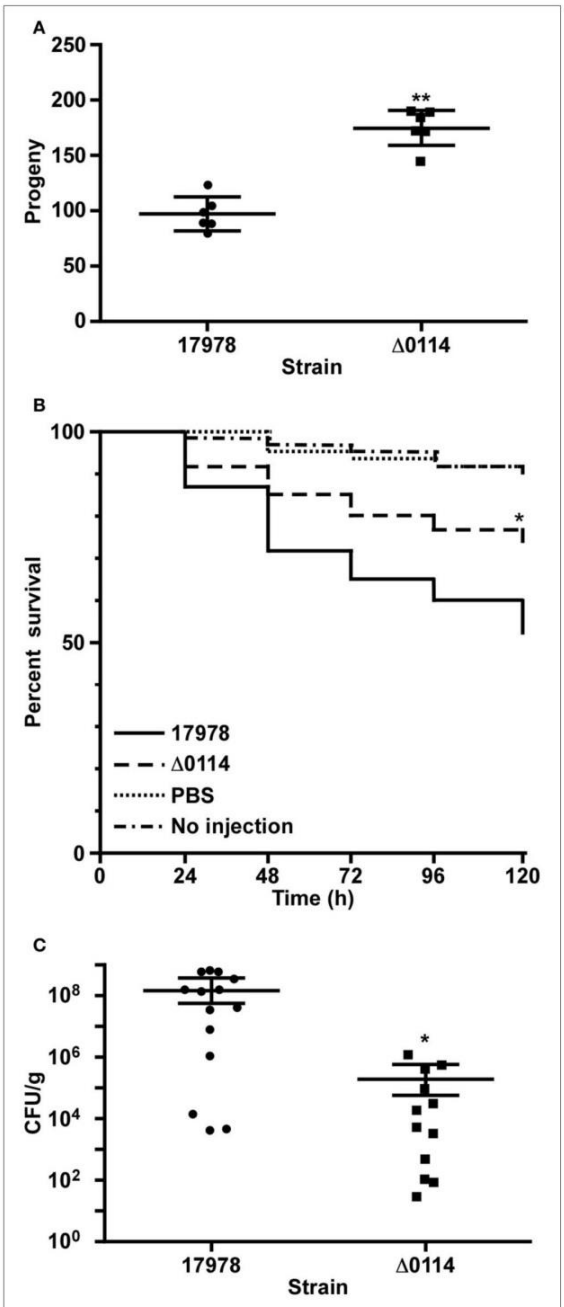

FIGURE 4 | Involvement of A1S_0114 in *A. baumannii* virulence. The virulence of the 17978 and Δ0114 strains was tested using *C. elegans* fertility and *G. mellonella* killing assays and a mouse pneumonia model. **(A)** *C. elegans* strain N2 was fed with 17978 or Δ0114 bacteria for 3 days counting progenies daily using six independent biological replicates.

(Continued)

FIGURE 4 | Continued
Each dot represents the worm progeny counted during 3 days. **(B)** *G. mellonella* caterpillars ($n = 60$ *per* group) were injected with 17978 or Δ0114 bacteria and death was determined daily for 5 days while being incubated 37°C in darkness. Caterpillars not injected or injected with the same volume of sterile PBS were used as negative controls. Log-rank (Mantel-Cox) tests were done to statistically validate experimental data. The asterisk in panel **(B)** indicates a $P < 0.05$ when the mutant and the wild type strains were compared. **(C)** Mice ($n = 10$ *per* group) were infected with $\sim 5.5 \times 10^7$ exponentially growing cells of the 17978 parental strain or the Δ0114 mutant *via* intratracheal intubation. The number of bacterial cells in lung homogenates was determined 24 h post infection. Student's *t*-test was used to validate experimental data shown in panels **(A,C)**. Values in panels **(A,C)** represent means and bars indicate the standard deviation ($^*P < 0.05$; $^{**}P < 0.001$).

less adherence when incubated with submerged or polarized human alveolar epithelial cells (**Figures 3A,B**). Interestingly, although the infection of polarized A549 cells with the Δ0114 mutant resulted in less biofilm formation on the surface of polarized cells, it did not result in an appreciable difference in the damage of the eukaryotic cells and the mucin layer that covers them when compared with samples incubated with the 17978 parent strain under the same experimental conditions (**Figure 3**). This observation suggests that A1S_0114 differentially affects distinct host-pathogen interactions that ultimately lead to the pathogenesis of respiratory infections caused by this pathogen.

If the phenotypes described above were to be pili mediated, as is the case of many bacteria, we would expect a correlation between the presence of an active A1S_0114 gene and the expression of genetic traits potentially involved in pili production and assembly. Accordingly, deletion of A1S_0114 significantly reduced the transcription of the A1S_0690, A1S_1510, and A1S_2091 genes (**Table 2**). A1S_0690 is a component of the A1S_0690-A1S_0695 operon, which codes for a predicted FilF-like Type III pili highly produced by pellicle cells (Marti et al., 2011; Nait Chabane et al., 2014); A1S_1510 is a component of the A1S_1507-A1S_1510 operon that codes for a predicted Type I pili, the production of which is controlled by iron and H-NS (Eijkelkamp et al., 2011a); and A1S_2091 is a component of the A1S_2088-A1S_2091 operon, which codes for an uncharacterized Type I pili also detected in pellicle cells (Nait Chabane et al., 2014; Álvarez-Fraga et al., 2016). Also, changes in biofilm biogenesis and adherence may reflect the fact that these responses could be mediated by the products of the *ompA* and *csuA/B* genes as described before (Tomaras et al., 2003; Gaddy et al., 2009). The expression of OmpA was reduced in the decreased biofilm former Δ0114 mutant, an observation that is in agreement with previous work (Gaddy et al., 2009). However, the biological relevance of the differential transcription of the *csuA/B* gene belonging to the *csuA/BABCDE* operon is not clear at the moment. It has been reported that this chaperone-usher pili assembly system may not be active in the 17978 strain due to a single base-pair insertion in *csuB* (Eijkelkamp et al., 2011b), although the CsuC and CsuD proteins have been detected as overproduced proteins in 17978 pellicle cells (Marti et al., 2011).

This report also provides the first experimental evidence that the A1S_0114 gene contributes to the virulence of *A. baumannii* when tested with three different experimental infection models already used to study *A. baumannii*'s virulence. It is apparent from our data that the marker-less deletion of A1S_0114, caused a significant reduction in virulence independently of the experimental infection model used to test this phenotype (**Figure 4**), without affecting the overall growth of this derivative in rich medium when compared to the 17978 parental strain (**Figure S2**).

It is interesting to note that a genome wide analysis using a random transposon mutant library containing 150,000 unique insertion derivatives, which included insertions in all components of the A1S_0112-A1S_0119 operon, showed that, in contrast to our observations, mutations within this operon did not affect the persistence of 17978 in the lungs of infected animals (Wang et al., 2014). Collection of samples at different times after infection (24 vs. 44 h) and the use of different mice strains (BALB/c vs. C57BL/6) could explain the disagreement between our data, collected using mutants generated by a site-directed approach, and that recorded with random transposon insertion derivatives. Similarly, different infection routes (pneumonia vs. bloodstream infection) and mice strains (BALB/c vs. leukopenic CBA/J) used to identified 17978 genes required for bacterial survival in the bloodstream of infected mice could explain the observation that mutants with random insertions in all A1S_0112-A1S_0119 genes did not meet the threshold to be considered critical for bacterial survival and no insertion within A1S_0114 was reported (Subashchandrabose et al., 2016). Genetic differences between the 17978 and AB5075 *A. baumannii* strains that resulted in apparent virulence differences using the *G. mellonella* as a host (Gebhardt et al., 2015) could explain the observation that none of the A1S_0112-A1S_0119 genes were identified as virulence genes when tested using this experimental infection model, which proved the critical role iron acquisition plays in *A. baumannii*'s virulence (Gaddy et al., 2012).

The putative functions for proteins encoded by this quorum-sensing regulated operon have been previously described (Clemmer et al., 2011) and include the acyl carrier protein (ACP) A1S_0114 and the predicted four-domain nonribosomal protein synthetase (NRPS) protein A1S_0115 (Allen and Gulick, 2014; Drake et al., 2016). The crystal structures of the *A. baumannii* AB307-0294 A1S_0115 orthologs, which shares 97.6% identity with the cognate 17978 gene product showed that glycine and AMP ligands were bound to the adenylation domain of this protein (PDB ID 4ZXI; Drake et al., 2016). Furthermore, glycine had the largest substrate specificity of the 20 proteinogenic amino acids tested, suggesting it could be the natural substrate incorporated into the biosynthetic product (Drake et al., 2016). A1S_0112 encodes an acyl-CoA synthase/AMP-acid ligase, which resembles fatty acid ACP ligases that activate and transfer fatty acids to ACPs *via* acyl AMP intermediates. A1S_0114, which encodes a free-standing ACP that is likely to contribute a tethered intermediate to the NRPS system involved in the biosynthesis of a secondary metabolite (Allen and Gulick, 2014), is the likely target for modification by A1S_0112, possibly yielding an acyl-ACP intermediate. A1S_0113, which encodes an acyl-CoA dehydrogenase (DH), is predicted to modify the intermediate product carried by the ACP or peptidyl carrier

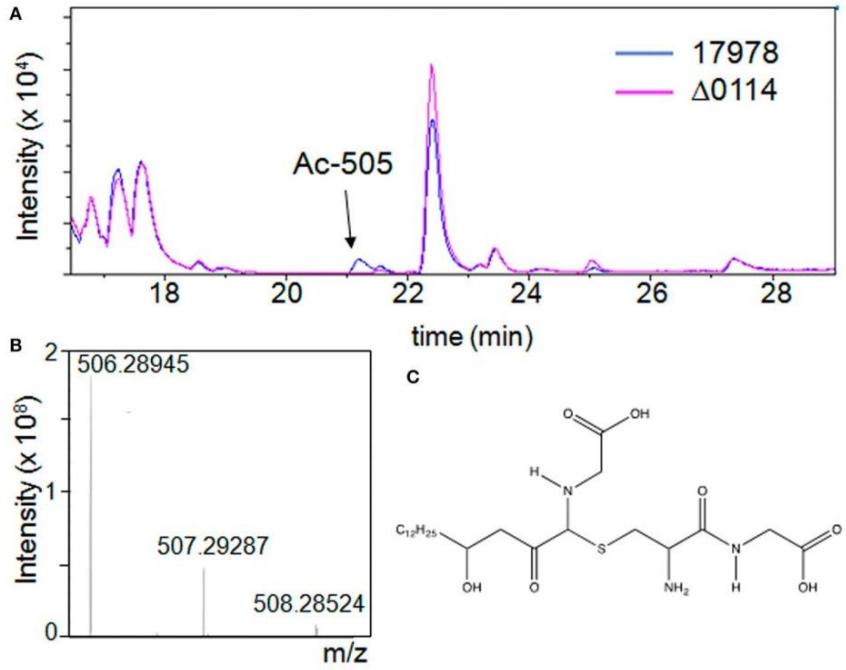

FIGURE 5 | Detection of Ac-505 in bacterial culture supernatants. (A) LC-MS chromatogram of culture supernatants from cells of the parental strain (17978) in blue and the A1S_0114 deletion derivative (Δ0114) in pink grown in static SB. The arrow indicates a compound (peak Ac-505) not present in the mutant profile. **(B)** Ultra high-resolution mass spectrum of HPLC purified Ac-505 by ESI FT-ICR showing the isotopic distribution of the $[M+H]^+$ ion. **(C)** Proposed structure of Ac-505.

protein (PCP) domain of A1S_0115. The thioesterase domain (TE) of A1S_0115 is most likely responsible for the release of acyl/peptide chains from their covalent attachment to the ACP/PCP domains. A1S_0117 and A1S_0118 encode 424- and 621-amino acid proteins, respectively, that were annotated as hypothetical proteins of unknown function (Smith et al., 2007). A more detailed *in silico* analysis showed that A1S_0117 could be related to porins while A1S_0118 harbors two domains that resemble proteins in the epimerase/dehydratase and α/β hydrolase family, respectively. However, since proteins in these families have diverse functions, the function of A1S_0118 still could not be predicted. The A1S_0119 gene, the last component of this operon, encodes a predicted 254-amino acid phosphopantetheine transferase that is expected to perform this enzymatic conversion, which is the first step in making the ACP active and therefore the likely first step in NRPS biosynthesis by this operon. Finally, the A1S_0116 gene encodes a protein that belongs to the superfamily of resistance-nodulation-cell division (RND) transporters. The function of these pumps may include efflux of signaling molecules, as is the case for the *Pseudomonas* quinolone signal called PQS (Lamarche and Deziel, 2011), and thus A1S_0116 is potentially involved in the secretion of the secondary metabolite coded for by the A1S_0112-A1S_0119 polycistronic operon. All these predictions are in accordance with our experimental observation that the expression of A1S_0114 gene not only is associated with the virulence of 17978, but also required for the production of the Ac-505 secondary metabolite.

Our mass spectrometric analyses of a purified compound present in 17978 culture supernatants but absent from those obtained from the Δ0114 mutant showed that Ac-505 resembles a three-amino acid lipopeptide, but with non-standard linkages between the amino acids as well as to the hydrocarbon moiety. Typically NRPS-produced secondary metabolites have hydroxylated or non-hydroxylated acyl moieties attached by amide or ester (*N*- or *O*-linked) linkages, which was not observed with Ac-505. We propose that the acyl-chain moiety or the neighboring Gly-containing component of Ac-505 could have been modified by other NRPS enzymes. Since it is possible for adenylation domains to be used iteratively to add sequential amino acids, it is conceivable that two or more Gly residues were incorporated via the A1S_0115 adenylation domain into this part of Ac-505. The Ac-505 second and third amino acids are Cys and Gly residues linked via a standard peptide linkage although they are connected to the Gly-containing moiety through the sulfur group of the Cys side chain forming a thioether bridge. Since we saw no evidence for unsaturation of the acyl chain, it is possible that the A1S_0113 DH acts in *cis* to modify the growing product on the glycine-containing moiety bound to the PCP of A1S_0115 rather than acting on an acyl-moiety of an acyl-ACP. The precursor to the α-thio bond could be an epoxide or a cyclic

compound, like a lactone. There are other NRPS-synthesized compounds that contain the so-called sactibiotic α-thio linkages such as the bacteriocins. These antimicrobial peptides contain a linkage between a cysteine thiol and the α-carbon of another amino acid residue. In the case of Ac-505, it is not clear how the α-thiol bond would be formed.

It is of note that the Ac-505 Cys-Gly moiety is potentially derived from glutathione, a tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) that is found in high concentrations intracellularly in *A. baumannii* and is linked with increased resistance to antibiotics (Kwon et al., 2013). Glutathione is known to deactivate xenobiotics *via* conjugation in order to make them less toxic. Glutathione conjugation most commonly occurs via a nucleophilic attack by the glutathione cysteinyl thiol on an electrophilic carbon such as a lactone or epoxide (Wang and Ballatori, 1998). The γ-glutamyl residue can then be cleaved by a γ-glutamyl transpeptidase localized outside the plasma membrane, leaving the conjugated Cys-Gly, as occurs in the case of the glutathione-modified microcystin (Schmidt et al., 2014), a cyanobacteria toxin and intra- and extra-cellular signaling molecule (Makower et al., 2015).

The Ac-505 that we detected both inside and outside 17978 cells, may be the result of the glutathione-mediated de-activation of a secondary metabolite. This potential precursor to Ac-505 would contain the hydrocarbon-chain and modified glycine-moiety and should have an electrophilic carbon, but the exact structure is unknown. Modification of this precursor by glutathione could explain our observation that no active fraction of the spent media would restore the wild type phenotype to the Δ0114 mutant, even with highly purified Ac-505 (data not shown). A similar outcome was obtained during analysis of the *A. nosocomialis* M2 strain and the isogenic derivatives M2-2 and M2-11, which harbor transposon insertions within the A1S_0113 and A1S_0115 orthologs, respectively, and display reduced surface motility (Clemmer et al., 2011). It is also possible that a different precursor to Ac-505 with a conjugated tri-peptide glutathione (prior to cleavage of the γ-glutamyl moiety), could be the active compound. We saw no evidence in the LC-MS analysis of spent media for the presence of a glutathione-conjugated derivative and were not able to identify any Ac-505 lipophilic precursors.

In summary, our work provides novel evidence that the A1S_0114 gene positively affects *A. baumannii* biofilm biogenesis, adherence and virulence responses. The molecular and cellular mechanism by which these responses are achieved and whether Ac-505 is the active effector responsible for these responses are issues that will remain unknown until this small secondary metabolite and its biosynthetic pathway are fully understood. Such knowledge would provide novel insights into the pathobiology of *A. baumannii* and potentially facilitate the development of alternative tools needed for the treatment of infections caused by MDR isolates.

AUTHOR CONTRIBUTIONS

SR: obtained the isogenic derivative and complemented strain and performed the qRT-PCR and attachment assays. AP: performed the scanning microscope assays and analyzed data of all work. TR: performed the LC-MS and high resolution MS and MS^n analysis. LÁ: performed the attachment assays. JV: performed the *C. elegans* and the mice virulence assays. AB: performed the mice virulence assays. EO: performed the scanning microscope experiments. BA: performed the *G. mellonella* virulence assay. MM: performed the attachment assays. SF: performed the complemented mutant strain analysis. MK: analyzed and supervised the work done in the Department of Chemistry and Biochemistry, Miami University, USA. LA: analyzed and supervised the work done in Department of Microbiology, Miami University, USA, interpreted all data and wrote the manuscript. GB: analyzed and supervised the work done in Spain. MP: coordinated and analyzed all the experiments of the work, interpreted data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00108/full#supplementary-material

Figure S1 | Confirmation of the polycistronic nature of the *A. baumannii* ATCC 17978 A1S_0112-A1S_0119 operon. Total DNA (lanes 2–7), cDNA (lanes 8–13) and total RNA (lanes 14–19) samples were used as templates in PCR

reactions using primers annealing to the A1S_0112-A1S 0113 (lanes 2, 8, and 14), A1S_0113-A1S_0115 (lanes 3, 9, and 15), A1S_0115-A1S_0116 (lanes 4, 10, and 16), A1S_0116-A1S_0117 (lanes 5, 11, and 17), A1S_0117-A1S_0118 (lanes 6, 12, and 18), and A1S_0118- A1S_0119 (lanes 7, 13, and 19) intergenic regions. Lanes 1 and 20 show the molecular weight standard Gene Ruler 1-Kb plus (Thermofisher Scientific). Molecular weight of each amplicon is indicated at the bottom of the figure.

Figure S2 | Growth of the 17978 parental and the Δ0114 isogenic deletion derivative strains. The OD_{600}values of each strain grown in LB or SB at 37°C for 24 h with shaking were determined hourly. Error bars represent the standard error (SE) of the mean.

Figure S3 | Ac-505 MS/MS spectra. (A) MS data collected on the FT-ICR with ECD fragmentation in positive ion mode. **(B)** MS/MS collected on a MaXis QTOF in positive ion mode (LC-MS) and **(C)** negative ion mode (direct injection).

Figure S4 | MS/MS—based fragmentation predictions (A,B) are two of the 40 total MS^n spectra from MS(3) to MS(6) recorded on the OSU Bruker amaZon in positive ion mode using direct injection of a HPLC purified Ac-505. MS(2) for 488.3 and MS(4) for 235.2 *m/z* fragment ions are shown in **(A,B)**, respectively. Neutral losses are shown in black. Panel **(C)** is a schematic diagram of MS/MS fragmentation of Ac-505 in positive ion mode and **(D)** is for negative ion mode.

Figure S5 | Predicted product ions of Ac-505 based on accurate mass and typical bond cleavage patterns under electrospray conditions in positive ion mode.

Figure S6 | Predicted product ions of Ac-505 based on accurate mass and typical bond cleavage patterns under electrospray conditions in negative ion mode.

Table S1 | Oligonucleotides used in the present study.

Table S2 | HRMS data of Ac-505. Measured m/z-values are reported for MS fragment ions measured on ESI MaXis QTOF (LC-MS2) and 15T FT-ICR ECD (direct infusion) instruments in positive ion mode, respectively. The molecular formulas of the product ions are based on accurate mass and isotopic pattern matching of the ions as well as typical bond cleavage patterns for electrospray ionization.

Table S3 | HRMS data of Ac-505 and MS2 fragments measured on a ESI MaXis QTOF (direct infusion) in negative ion mode.

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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.

3.3. CHAPTER III: Global assessment of small RNAs reveals a non-coding transcript involved in biofilm formation and attachment in *Acinetobacter baumannii* ATCC 17978

Small non-coding RNAs regulate a wide range of functions and modulate many aspects of cellular metabolisms in bacteria. RNA regulators, acting at the post-transcriptional level, are less costly and faster to produce. However, little is known about the involvement of sRNAs in controlling biofilm formation and only a few have been described in *A. baumannii*.

The objective of this chapter is to characterize the small RNA transcriptome of *A. baumannii* ATCC 17978 and to compare the expression of these sRNAs in both planktonic and biofilm-associated cells with the aim of finding possible candidates involved in biofilm formation. For this purpose, high throughput RNA sequencing was performed for exponential and stationary growth phase cells as well as for biofilm associated cells.

A total of 255 putative sRNAs were selected as differently expressed in some of the three growing conditions. All detected sRNAs were located in the chromosome except sRNA 29, which was located on plasmid pAB2. From them, a total of 185 putative sRNAs were differentially expressed in biofilm or stationary phase compared to exponential phase. In biofilm cells, 28 putative sRNAs were up-regulated and 32 down-regulated with respect to planktonic cells. One of these sRNAs, the 13573 sRNA, showed a 120-fold over-expression in biofilm cells with respect to planktonic cells. Moreover, 9 sRNAs were exclusively detected in biofilm cells and 21 were only present in planktonic cells. Deep sequencing results were corroborated using RT-qPCR procedures for six sRNAs only expressed in biofilm and the 13573 sRNA.

The high level of expression of the 13573 sRNA in biofilm-associated cells suggested its potential role in biofilm regulation. A knockout mutant strain (Δ13573), as well as a strain over-expressing the 13573 sRNA (13573), were constructed in order to elucidate its role in pathogenesis. Repression and over-expression of the 13573 sRNA in their respective strains were confirmed by RT-qPCR using Taqman probes.

Biofilm assays showed that the lack of the 13573 sRNA caused a slight but non-significant reduction on biofilm production. However, the over-expression of 13573 resulted in a 2-fold increase of biofilm formation compared to the wild type strain. SEM imaging revealed that the 13573 sRNA over-expressing cells develop tridimensional biofilm structures unlike the ATCC 17978 strain, which was not able to build such structures. The knockout strain cells showed a more disorganized pattern than cells from the parental strain.

Adhesion to eukaryotic cells experiments indicated that the 13573 over-expressing strain attached to A549 alveolar cells 30 fold more than the wild type strain. Adhesion ability decreased in the 13573 knockout strain, but this effect was restored in the complemented strain, which reached higher values than the ATCC 17978 strain. SEM analysis of polarized human cells infected with the different strains revealed that the over-expression of the 13573 sRNA allowed bacteria to form tridimensional biofilm over the epithelial cells. As expected, the knockout strain showed a reduced adherence to eukaryotic cells compared to the wild type strain.

Overall data indicated that the analysis of the sRNA transcriptome showed different patterns in biofilm-associated cells compared to both exponential and stationary phase cells. This analysis highlighted the presence of a remarkably over-expressed sRNA, named as 13573, in biofilm-associated cells. Phenotypic characterization of the 13573 sRNA revealed its role in biofilm formation and attachment to abiotic and biotic surfaces.

PLOS ONE

RESEARCH ARTICLE

Global assessment of small RNAs reveals a non-coding transcript involved in biofilm formation and attachment in *Acinetobacter baumannii* ATCC 17978

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Abstract

Many strains of *Acinetobacter baumannii* have been described as being able to form biofilm. Small non-coding RNAs (sRNAs) control gene expression in many regulatory circuits in bacteria. The aim of the present work was to provide a global description of the sRNAs produced both by planktonic and biofilm-associated (sessile) cells of *A. baumannii* ATCC 17978, and to compare the corresponding gene expression profiles to identify sRNAs molecules associated to biofilm formation and virulence. sRNA was extracted from both planktonic and sessile cells and reverse transcribed. cDNA was subjected to 454-pyrosequencing using the GS-FLX Titanium chemistry. The global analysis of the small RNA transcriptome revealed different sRNA expression patterns in planktonic and biofilm associated cells, with some of the transcripts only expressed or repressed in sessile bacteria. A total of 255 sRNAs were detected, with 185 of them differentially expressed in the different types of cells. A total of 9 sRNAs were expressed only in biofilm cells, while the expression of other 21 coding regions were repressed only in biofilm cells. Strikingly, the expression level of the sRNA 13573 was 120 times higher in biofilms than in planktonic cells, an observation that prompted us to further investigate the biological role of this non-coding transcript. Analyses of an isogenic mutant and over-expressing strains revealed that the sRNA 13573 gene is involved in biofilm formation and attachment to A549 human alveolar epithelial cells. The present work serves as a basis for future studies examining the complex regulatory network that regulate biofilm biogenesis and attachment to eukaryotic cells in *A. baumannii* ATCC 17978.

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Introduction

Acinetobacter baumannii is a non-fermentative, oxidase negative and non-flagellated Gram-negative bacillus. Although it is a normal inhabitant of the human skin flora, the intestinal tract, and the respiratory system, it has been recently described as a dangerous opportunistic pathogen [1]. *A. baumannii* exhibits a remarkable ability to develop antibiotic resistance that may lead to multiresistant patterns in a short time [2]. Its high genetic versatility facilitates the rapid adaptation to stressing or unfavourable conditions. In the last years, outbreaks caused by resistant strains of *A. baumannii* have emerged, causing serious health problems [3–11]. Many bacterial species can grow as biofilms: sessile lifestyle forms that increase protection against antimicrobial agents and host defences [12–17]. The growth of *A. baumannii* on mucous surfaces and medical devices, such as intravascular catheters or endotracheal intubation devices, may result in biofilm formation, increasing the risk of bloodstream and respiratory infections [16]. Biofilms are difficult to eliminate and may cause persistent and recurrent infections. The ability of *A. baumannii* to adhere and persist as biofilm structures onto surfaces may be the key to understanding its pathogenic mechanisms. In general, processes involved in adhesion and the subsequent formation and development of biofilms are controlled by complex regulatory networks that coordinate the expression of multiple genes [18–24]. Since biofilm formation on abiotic surfaces contributes to the unique survival pattern of *A. baumannii* in hospital settings, several studies have focused on the description of the proteins and genes involved in the adoption of that lifestyle. However, little is known about the involvement of small non-coding RNA transcripts (sRNAs) in controlling biofilm formation. sRNA molecules serve a wide range of regulatory functions in bacteria and modulate almost every aspect of cell metabolism [25–32]. RNA regulators have several advantages over protein regulators. They are less costly and can be produced faster than proteins, as they are shorter than most mRNAs and do not require the extra step of translation. Moreover, RNA regulators usually act at a level complementary to protein regulators, most often functioning at the post-transcriptional level as opposed to transcription factors that act before sRNAs or enzymes such as kinases or proteases that act after sRNAs. Different combinations of these proteins and RNA regulators can provide a variety of regulatory outcomes, such as extremely tight repression, an expansion in the genes regulated in response to a single signal or, conversely, an increase in the number of signals sensed by a given gene [33]. sRNAs, usually ranging from 50 to 500 nt in length, are typically encoded within intergenic regions and are independently transcribed, using their own promoter and terminator regions [30]. Although regulatory RNAs may control gene expression by a variety of mechanisms, including the modulation of transcription, translation, mRNA stability and DNA maintenance or silencing, most of the studied small RNA regulators act through base pairing with particular RNA targets, usually affecting the translation and stability of mRNAs. Moreover, many sRNAs have been described as involved in the regulation of important bacterial survival responses [30]. Although sRNAs were first discovered in bacteria many years ago, recent technical advances such as multilayered computational searches, deep sequencing or tiled microarrays, have facilitated the discovery of hundreds of potential regulatory sRNA genes [25, 34–40]. In fact, new high throughput sequencing approaches allowed the characterization of complete bacterial transcriptomes and the description of sRNAs in different microorganisms [29, 32, 41–46]. Although studies of sRNAs involved in bacterial infection are still in the early stages, certain sRNAs are related to virulence and pathogenicity in *Salmonella*, *Erwinia*, *Yersinia*, *Vibrio*, *Listeria*, *Pseudomonas*, *Shigella* and *Staphylococcus* [47–54]. Schilling *et al.* [28] detected a small RNA gene (Aar) in *A. baylyi* involved in the regulation of amino acid metabolism. Iron-regulated sRNAs have been associated to biofilm formation in *Aggregatibacter actinomycetemcomitans* [55]. Recently, Sharma *et al.* [56] identified novel

regulatory sRNAs in *A. baumannii* by bioinformatic approaches and described three of them as differentially expressed in different phases of the bacterial growth curve. One of them, sRNA AbsR25, was suggested to be involved in regulating the expression of a transporter.

The aim of the present study was to use deep sequencing technologies to characterize the small RNA transcriptome of *A. baumannii* and to gain insight into the mechanisms behind the ability of this organism to form biofilms. The small noncoding RNA transcriptomes of both planktonic and biofilm-associated cells of *A. baumannii* strain ATCC 17978 were then compared to identify differences in sRNA gene expression profiles. One of these sRNA molecules was described as involved in biofilm formation and in attachment to eukaryotic cells.

Materials and methods

Bacterial strains and culture conditions

A. baumannii ATCC 17978 was routinely grown in Mueller-Hinton (MH) or Luria-Bertani (LB) broth or agar. *Escherichia coli* TG1 was grown in LB broth and used for cloning procedures. *E. coli* OP50 (*Caenorhabditis* Genetics Center) was grown in LB and used for *Caenorhabditis briggsae* virulence assays. All strains were grown at 37˚C and stored at -80˚C in LB containing 10% glycerol. The concentration of antibiotic used for selection of transformants was 50 μg/mL of kanamycin (Sigma-Aldrich, St. Louis, MO). For obtaining planktonic cells, a single colony of *A. baumannii* ATCC 17978 was isolated on LB agar and grown in 5 mL of LB broth overnight at 37˚C in an orbital shaker at 180 rpm. The resulting culture was diluted 100-fold in 500 mL of LB broth in 1L-flasks and incubated under the same conditions. Optical density (OD) was evaluated at 600 nm each 30 min. Cells were harvested at exponential (OD_{600nm} = 0.4) and late stationary (OD_{600nm} = 2.0) phases of growth after inoculation. Finally, exponential and stationary cells were resuspended in RNA later reagent (Sigma-Aldrich, St. Louis, MO), frozen using liquid nitrogen and stored at -80˚C until RNA extraction.

Biofilm generation in microfermentors

Biofilm formation under flow conditions was performed as previously describe by Tormo *et al.* with some modifications [57]. Briefly, *A. baumannii* ATCC 17978 biofilms were obtained in the Fermentation Laboratory of the Agrobiotechnology Institute (Navarra, Spain) under a continuous flow culture system in LB broth medium, consisting of 60-mL microfermentors (Institute Pasteur, Paris, France) with a continuous aeration flow of 40 mL/h of compressed sterile air. Submerged glass slides served as growing substratum. A sample from an overnight culture of *A. baumannii* ATCC 17978 grown in LB broth was used to inoculate the microfermentors, which were then maintained at 37˚C for 24 h. Biofilms formed on the slides were removed with a cell scraper and immediately frozen in liquid nitrogen and stored at -80˚C.

Isolation of small RNA molecules

Three independent samples of planktonic bacteria collected at the exponential and stationary growth phases and the biofilm-associated bacteria generated in the microfermentor, all grown in LB media, were reduced to powder using mortar and pestle in the presence liquid nitrogen. RNA isolation was achieved using the *mirVana miRNA Isolation kit* (Ambion) following the manufacturer's protocols. This kit allowed the separation of a fraction enriched in small RNA molecules (sRNA). Ribosomal RNA species were removed using the Microbexpress kit (Ambion). sRNA samples were treated with DNAse I (Invitrogen), purified using phenol-chloroform and concentrated using standard ethanol precipitation. The concentration and the

purity grade of the RNA samples were evaluated using a NanoDrop ND-1000 (Thermo Fisher Scientific) and the integrity and size using a Bioanalyzer 2100 and a Small RNA Analysis Kit (Agilent Technologies Inc., Germany).

Construction of cDNA libraries

The *SOLID small RNA expression kit* (SREK, Ambion) was employed for obtaining double stranded cDNA from sRNA following the manufacturer's instructions and using the primers listed in S1 Table. Briefly, sRNA molecules were hybridized and ligated to adaptors, reverse transcribed and treated with RNase H. Then, the second strand was obtained through amplification. Quantification integrity and size determination of samples were done using a Bioanalyzer 2100 as described above.

Deep sequencing procedures

Three double-stranded cDNA libraries, derived from sRNA samples obtained from liquid cultures in the exponential and stationary phases of growth, as well as biofilm cells were pyrosequenced in the Roche 454 Sequencing Center (Connecticut, USA) using the GS FLX Titanium chemistry and following the manufacturer's protocols. Raw data obtained from pyrosequencing have been deposited in the Short Read Archive database (SRA, NCBI) under accession numbers SRX591862, SRX591861 and SRX591774.

454 read processing and gene expression profile comparison

DNA sequences were pre-processed with cross-match (Phred-Pharp-Consed package) and TrimSeq (Emboss package), to mask and trim the linkers added by the SREK kit (S1 Table). The sequences were then aligned with cross-match against the *A. baumannii* ATCC 17978 chromosome and plasmids [Genbank: NC_009085, NC_009083 and NC_009084]. Alignments were combined and processed with *ad-hoc* Perl scripts to identify read-covered regions and to exclude those overlapping with known genes. The average coverage for the remaining read-covered regions was calculated for each of the growing conditions, and normalized according to the number of mapped reads for each sample, to obtain relative abundance values (RA). RA for known sRNAs (tRNAs and 5S rRNAs) were also obtained, and the lowest (that corresponding to an arginine tRNA expressed in the exponential growth sample) was used as the upper threshold to filter out candidate transcribed regions. Remaining chromosomal regions were considered to be truly expressed. To identify those that could be differentially expressed in the different growth conditions, a 2-fold change was used as threshold. Additional details about 454 read data processing can be found in Supplementary material (S1 Text).

Real time PCR assays

To analyse the expression levels of a set of differentially expressed sRNA molecules *Taqman Micro RNA Reverse Transcription Kit* and *Taqman Universal PCR Master Mix* as well as TaqMan probes and primers were ordered from Applied Biosystems (Life Technologies). Total RNA from planktonic and biofilm cells obtained as described above was isolated using the *RNeasy mini kit* (Qiagen). 200 ng of this RNA were reverse transcribed and 2 μL of the RT mixture were used for real time PCR assays. The expression of *gyrB* was used as a constitutively expressed control. The program followed for reverse transcription consisted in 30 min at 16˚C, 30 min at 42˚C and 5 min at 85˚C. The program followed for real-time PCR was 1 cycle of 10 min at 95˚C, 45 cycles of 95˚C for 15 s, 60˚C for 1 min and final cycle at 40˚C for 20 s.

Three biological replicates were tested for each sample. Student's t-test was performed to evaluate the statistical significance of observed differences.

Cloning of sRNA gene in an expression vector

The gene coding for sRNA 13573, which is over-expressed in biofilm cells, was PCR-amplified using ATCC 17978 genomic DNA as template using specific primers that were designed using the complete genome sequence [Genbank: NC_009085] and included *Xba*I and/or *Nco*I restriction sites (S1 Table). The amplicon was then ligated into the pETRA plasmid containing the CTXM promoter [58] and a kanamycin resistance cassette previously introduced into the *Pst*I restriction site using primers listed in S1 Table. Cloning procedures were performed in *E. coli* TG1. Finally, an insert-containing pETRA derivative was used to transform *A. baumannii* ATCC 17978 as described by Aranda *et al.* [58].

Constructions of knock-out strain

Plasmid pMO130 [Genbank: EU862243], a suicide vector containing *xylE*, *sacB* and a kanamycin resistance marker, was used as described by Hamad *et al.* [59]. The sRNA 13573 gene was targeted for deletion. Briefly, 900–1,000 bp upstream and downstream fragments flanking the sRNA 13573 gene were cloned into the pMO130 vector using primers listed in S1 Table. The resulting plasmid was used to transform *A. baumannii* ATCC 17978 by electroporation. Recombinant colonies representing the first crossover event were obtained using a combination of kanamycin selection and visual detection of XylE activity following the catechol-based method. Bright yellow kanamycin resistant colonies were grown overnight in LB supplemented with 15% sucrose and then plated on the same agar medium. Second crossover events were then confirmed by PCR using primers listed in S1 Table.

Complementation of stable knockout mutant

To complement the isogenic Δ13573 mutant derivative, the cognate wild type allele was amplified from *A. baumannii* ATCC 17978 genomic DNA using the primers listed in S1 Table and then cloned into the pETRA plasmid as described above [58]. The construction was used to transform the mutant strain. Transformants were selected on kanamycin-containing plates and confirmed by PCR using primers listed in S1 Table. A strain containing the empty pETRA vector was used as control.

Adhesion to and invasion of A549 human alveolar epithelial cells

Adhesion and invasion abilities were determined following the procedure described by Gaddy *et al.* with some modifications [22]. Briefly, A549 human alveolar epithelial cells were grown in 5% CO_2 at 37°C in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% of penicillin-streptomycin (Gibco). Confluent monolayers were washed twice with saline solution and once with modified Hank's balanced salt solution (mHBSS, same as HBSS but without glucose). Then, A549 cells were infected with 10^5 bacteria *per* well and incubated for 24 h in mHBSS at 37°C. To determine bacterial adhesion, the infected monolayers were washed three times with saline solution and then lysed in 500 μL of 0.5% sodium deoxycholate. To determine bacterial invasion A549 cells were infected as described above for 24 h and each well was treated for 2 h with gentamicin (256 μg/mL) before washing. Dilutions of the cell lysates were plated onto LB agar and incubated at 37°C for 24 h. Colony forming units were counted to determine the % of bacteria that had attached to or invaded A549 cells at 24 h compared to the growth control, this

being static conditions and same medium without cells as previously described by Álvarez-Fraga *et al.* [60]. Four independent replicates were done. Student's t-test was performed to evaluate the statistical significance of the observed differences.

Scanning electron microscopy (SEM) of bacterial biofilms on plastic coverslips

Overnight cultures of *A. baumannii* were used to inoculate 5 ml of LB in 50-ml conical tubes at a 1:100 dilution. Sterile polystyrene coverslips were placed in the inoculated 50-mL conical tubes and the tubes were incubated for 48 h at 37˚C without shaking as previously described [22]. Coverslips were washed, dehydrated in ethanol, processed with a critical point drier, and sputter coated as described previously [18]. Biofilms formed above, at and below the liquid-air interface were viewed using a Zeiss Supra Gemini Series 35V scanning electron microscope as described previously [18].

Analysis of biofilms formed on polarized A549 human alveolar cells

A549 human alveolar epithelial cells were routinely maintained as previously described [61, 62]. Costar transwell permeable support polycarbonate membrane 24 well plates (Costar Transwell Polyester Supports, Corning Inc, Corning, New York) were preconditioned 24 h prior to seeding with DMEM on both sides of the membrane and incubated at 37˚C and 5% CO_2. DMEM was removed from the conditioned transwell plates and the membranes were seeded with 10^5 A549 cells per membrane. A549 cells were maintained submerged (DMEM on top and bottom) on the transwell membranes for one week. Following the initial week of submerged growth, DMEM was removed from the top of the membrane to allow the A549 cells to polarize and begin secreting surfactant. Cells were polarized for 2 weeks. One day prior to and for the duration of infection, A549 cells were fed DMEM supplemented with 10% heat inactivated FBS without penicillin-streptomycin (DMEM-). Bacteria, previously grown in LB at 37˚C for 24 h in a shaking incubator at 180 rpm, were washed and resuspended in Hank's Buffered Salt Solution (HBSS) (Hyclone Laboratories, Inc, Logan, Utah). A concentration of 10^2 bacteria was applied to the apical surface of A549 cells by pipetting 1 μL of suspension onto the center of each membrane. The transwell plate was then incubated and maintained for 72 h at 37˚C and 5% CO_2. After 72 h, the membranes were washed with HBSS to remove secretions and unattached bacterial cells. The membranes were then fixed for 24 h in 4% formaldehyde-HBSS at 4˚C. The membranes were then prepared for SEM using the previously described [18].

Quantitative biofilm assay

Biofilm formation was quantified following the procedure described by Tomaras *et al.* [18], with some modifications. *A. baumannii* was grown on LB agar 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 LB. A 1:100 dilution of each strain was incubated at 37˚C for 12, 24 and 48 h in 15 mL polyethylene tubes. Growth culture was measured at OD_{600} to estimate total cell biomass. Biofilm formation was quantified by staining with crystal violet and solubilisation 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 avoid variations due to differences in bacterial growth under different experimental conditions. Eight independent replicates were performed. Student's t-test was performed to evaluate the statistical significance of observed differences.

Results and discussion

Processing of sRNA 454 sequencing reads

sRNA fractions, obtained from *A. baumannii* ATCC 17978 planktonic cultures in the exponential and stationary phases of growth, and biofilms were pyrosequenced. The total number of reads was 689,097, 502,152 and 627,209 for the exponential, stationary and biofilm samples, respectively (S2 Table). Between 32% and 43% of pre-processed reads could be aligned for each sample (S2 Table). The total number of filtered alignments was around twice the number of aligned reads, indicating that a significant number of reads could align to more than one location with the same score. Additional information can be found in Supplementary material (S1 Text, S1 and S2 Figs, S2, S3 and S4 Tables and S1 Dataset).

Quantification of the expression level for known sRNA genes

Filtered alignments were processed to estimate the expression level for known sRNA genes described for *A. baumannii* ATCC 17978. Relative abundance (RA) values were calculated for known protein coding genes and for 16S and 23S rRNA genes (S3 Table). tRNA and 5S rRNA genes had RA average values of 1,092 to 1,338 for the three samples, which were more than 1,000-fold and about 20-fold higher than RA values calculated for protein coding genes and 16S and 23S rRNA genes, respectively (S4 Table and S1 Fig), suggesting that sRNA fractions isolated as described in Methods were significantly depleted of mRNA and rRNA. A1S_2909, coding for Leu tRNA, was the known sRNA gene expressed at the highest level, with a RA value of 18,693 in the stationary phase sample. In contrast, A1S_2764, coding for Arg tRNA, was the known sRNA gene expressed at the lowest level, with a RA value of 7.6 in the exponential phase sample. For more details see S1 Text in Supplementary files.

Identification of new sRNA gene candidates

A total of 26,956 read-covered regions (exprRegs) were detected. To identify read-covered regions overlapping with known genes, the *ad-hoc* script FindOlappingFeatures was used to compare their coordinates with the coordinates of 3,451 known protein, tRNA and rRNA coding genes. The minimal overlap required to consider two features as overlapping was set to 0.001% of the length of any of the features. A total of 21,392 exprRegs were found to overlap with already described genes. The remaining 5,564 exprRegs (S1 Dataset) were considered as new potential sRNA genes. New sRNA gene candidates were identified both in chromosome and plasmids (5,505 and 59 genes, respectively). For more details see S1 Text in Supplementary files.

Quantification of the expression level of sRNA gene candidates

The set of 5,564 exprRegs that did not overlap with known protein, tRNA and rRNA coding genes had been defined by processing the combined filtered alignments of the reads from the three samples (exponential phase, stationary phase and biofilm) against the *A. baumannii* ATCC 17978 chromosome and plasmids. They provided a common set of candidate sRNA genes whose expression could now be quantified for each of the growing conditions. To quantify the expression of the 5,564 expRegs, the script mapAlignHits was used again in "expression mode", using the filtered alignments obtained for each sample and the coordinate set for the 5,564 exprRegs as input. RA values were, in average, 500- to 1,000-fold lower than those calculated for known sRNAs (S5 Table). In fact, the distribution of RA values for the set of 5,564 expressed regions indicated that there were about 3,000 expRegs with RA values equal to zero for the biofilm and stationary phase samples and about 1,500 for the exponential phase sample

(S2 Fig). The significant abundance of exprRegs that were not expressed in some of the three growing conditions suggested that many of them could be differentially expressed under the conditions used in this study. Since the aim of this study was to identify new sRNA genes in *A. baumannii* and compare their expression levels in planktonic exponential and stationary phase cells as well as in sessile bacteria, we decided to focus on the subset of sRNA gene candidates with RA values higher than that calculated for A1S_2764 (coding for tRNA-Arg), which was the lowest RA value previously observed for a known sRNA gene as described above. Accordingly, a total of 255 exprRegs were selected because they had RA values equal or higher than 7.6 in some of the three growing conditions (S2 Dataset). Fig 1 shows the genomic location of these 255 exprRegs. Out of them, 108 exprRegs fulfilled the 7.6 requirement in the RNA samples isolated from sessile bacteria, while this requirement was fulfilled in 140 and 121 exprRegs indentified in the exponential and stationary phase planktonic samples, respectively (S6 Table and S3 Fig). With the exception of exprReg_29, which was located on plasmid pAB2 (NC_009084), all of them were located in the chromosome.

This number of 255 exprReg selected varies significantly from those observed in a study done by Weiss *et al*., where 78 sRNAs were detected in *A. baumannii* AB5075 [63]. However, the authors evidenced that only a small number of sRNAs showed high levels of conservation amongs different *A. baumannii* strains, including ATCC 17978, and that many transcripts are only present either in a small subgroup of strains or exclusively in AB5075 [63]. This issue has been observed in other species such as *Pseudomonas aeruginosa*, where numerous sRNAs exhibit a strain-specific expression pattern [64, 65]. Furthermore, the set of sRNAs identified could depend on the library preparation strategy used, as it was demonstrated by Gómez-Lozano *et al.* using three different approaches to detect novel transcripts of *P. aeruginosa* PAO1 strain [66].

Identification of differentially expressed sRNA genes

To identify differentially expressed regions among the set of 255 exprRegs, fold changes were calculated and an induction/repression ratio of 4 was chosen arbitrarily as threshold. A total of 185 exprRegs were classified as differentially expressed in biofilm or stationary phase conditions, relative to exponential phase (Table 1). Twenty-eight and 32 exprRegs were up or down-regulated in biofilm cells with respect to both types of planktonic cells, respectively (Fig 1). It is important to remark the high over-expression level of sRNA 13573; about 120-fold higher in biofilm with respect to planktonic cells. Nine sRNA gene candidates (expression regions 1004, 8014, 9803, 11065, 14414, 15677, 18098, 21533, and 24430) were only expressed in biofilm cells. In contrast, 21 putative sRNA genes (expression regions 29, 424, 788, 2345, 2892, 4493, 4750, 5958, 9947, 10022, 12757, 13281, 15861, 17548, 19511, 19890, 23002, 25126, 25193, 25499, and 25840) were only expressed in planktonic cells and repressed in biofilm (Table 1 and Fig 1). It is important to note that the expression region 29 was located in the plasmid pAB2 as mentioned above.

In addition, qRT-PCR analysis of sRNA 13573 and a group of six sRNA only expressed in biofilm cells using proper Taqman probes (S4 Fig) validated the differential production of these non-coding transcripts detected by pyrosequencing as described above.

Sharma *et al.* [56] described 31 predicted sRNAs coding regions in the genome of *A. baumannii* ATCC 17978 using computational approaches. A group of 18 of these 31 predicted sRNAs were also detected as candidate sRNAs in the present work as showed in S1 Dataset. Furthermore, these authors analyzed 10 of the 31 predicted sRNAs by northern blotting of total RNA isolated from *A. baumannii* MTCC1425 (ATCC 15308) cells at different stages of the growth cycle. This analysis showed that, although sRNAs AbsR11 and AbsR28 had almost

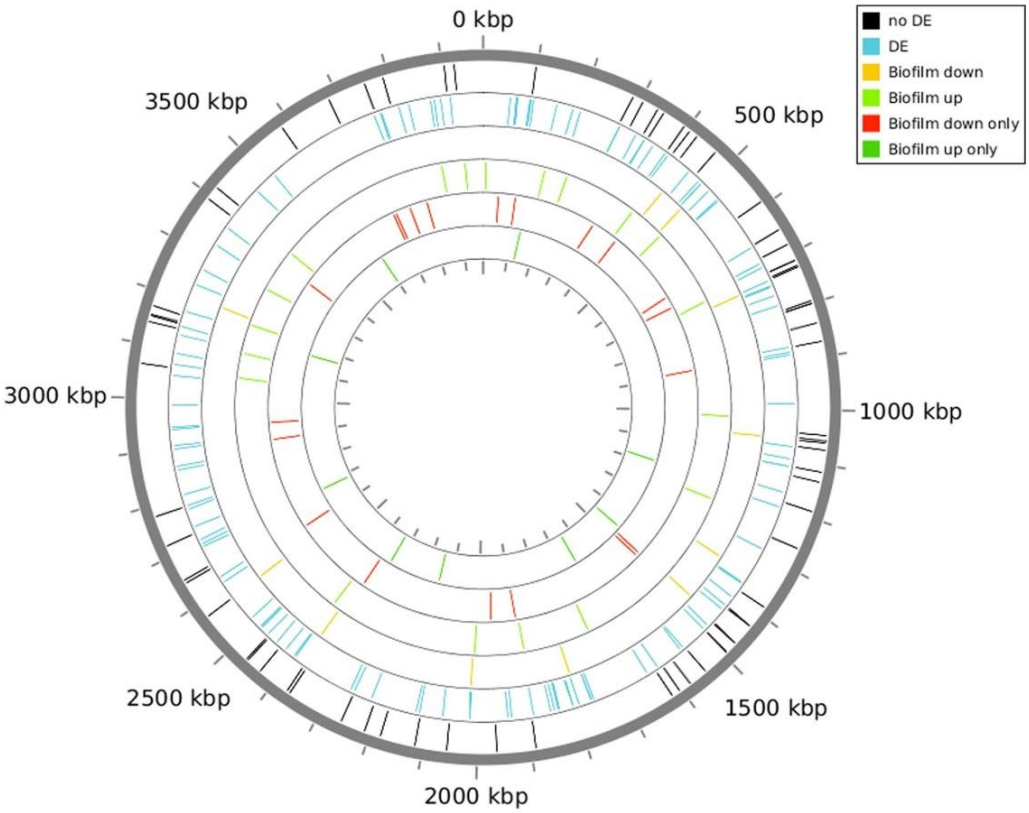

Fig 1. Representation of the 255 putative sRNA expression regions found by deep sequencing in the genome of the *A. baumannii* strain ATCC 17978. Bars indicate their location in the genome. *no DE* black bars indicate no expression differences of the sRNAs analyzed in the three growing conditions. Blue bars indicate sRNAs differentially expressed in stationary phase conditions related to exponential phase. Yellow bars indicate down regulated sRNAs in biofilm related to both types of planktonic cells. Light green bars indicate up regulated sRNAs in biofilm related to both types of planktonic cells. Red bars indicate sRNAs only expressed in planktonic cells and repressed in biofilm. Dark green bars indicate 9 sRNAs only expressed in biofilm and repressed in planktonic cells.

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the same expression level during all phases of planktonic growth, sRNA AbsR25 had no expression in the lag phase and a maximum expression in the exponential phase [56]. The AbsR25 and AbsR28 small transcripts were described in the present work as expression regions, as shown in S1 Dataset, which correspond to exprRegs 10452 and 22397. However, we did not find them as differentially expressed in the conditions analyzed. Moreover, the length of the sequences of these two regions in the ATCC 17978 strain described in the present work and in the MTCC1425 strain described by Sharma *et al.* [56] does not match completely. These variations could be due to genomic differences between these two strains or even to the different bioinformatic approaches used for prediction and analyses of these coding regions. This fact could partly explain the gene expression differences found between Sharma *et al.*'s work and the present study.

Table 1. List of the 185 differentially expressed coding regions.

Expression region No.	Biofilm	Exponential	Stationary
29**	1.00	14.20	3.39
165*	9.3	1.71	1.58
424**	1.00	1.97	20.67
497	1.00	11.91	1.00
598	4.24	16.09	1.00
601	4.25	12.83	1.00
602	6.37	12.42	1.00
611	3.86	16.89	1.00
786	13.85	1.42	25.22
788**	1.00	1.33	22.54
795	25.84	2.59	36.24
801	14.07	1.92	20.31
833	1.00	1.00	9.28
1004*	7.75	1.00	1.00
1136	7.52	7.68	1.69
1221*	7.78	6.59	1.00
1368	34.5	141.16	1.11
1435	2.53	1.00	12.49
1571*	13.75	1.54	1.73
2005	3.67	13.14	1.00
2006	1.56	9.58	1.00
2247	3.89	9.87	1.00
2345**	1.00	1.69	7.72
2350	36.03	60.87	5.13
2359	7.99	13.43	1.16
2492	1.00	7.71	1.00
2675	37.66	8.34	143.60
2711	1.22	8.06	1.00
2811*	8.3	1.00	2.58
2892**	1.00	8.52	1.69
2988**	1.65	8.89	3.41
3084	1.00	15.32	1.00
3275	1.00	1.00	21.31
3326	6.37	27.10	2.72
3333	4.00	7.90	1.00
3380**	1.74	8.66	29.98
3440*	15.43	2.94	1.00
3477	4.92	10.92	1.88
3641	4.65	10.11	1.00
3667	13.5	65.31	5.71
4493**	1.00	2.26	10.89
4505	1.00	8.20	1.00
4750**	1.00	1.50	9.92
4776	1.27	17.11	1.00
4846	1.00	1.00	15.24
4946*	9.91	1.38	1.00
5077	4.26	18.87	3.07

(*Continued*)

Table 1. (*Continued*)

Expression region No.	Biofilm	Exponential	Stationary
5098**	1.13	29.95	1.54
5148	1.00	1.00	7.84
5168	1.00	8.00	1.00
5286	1.00	20.55	1.00
5428	1.00	1.00	8.47
5881	298.42	484.14	15.87
5954	1.00	9.56	1.00
5958**	1.00	34.00	7.70
5985	8.67	1.00	10.16
6714	7.74	15.43	1.34
6904*	9.90	5.09	1.00
7203**	1.76	8.58	3.96
7297	1.00	1.00	7.93
7468	1.00	1.00	18.63
7562	3.82	10.67	1.00
7565	1.00	1.00	8.40
7923	1.00	1.00	31.41
8014*	8.72	1.00	1.00
8136	453.88	68.62	745.54
8382*	16.36	1.71	1.00
8776	1.00	7.65	1.00
9139**	1.32	8.64	1.39
9255	49.00	67.51	1.63
9276	2.07	1.00	8.95
9507	7.86	45.51	4.12
9656	11.25	9.21	43.47
9720	1.00	1.00	8.15
9803*	8.74	1.00	1.00
9878**	1.54	12.62	4.23
9947**	1.00	1.95	9.52
10021	1.37	1.00	17.98
10022**	1.00	1.79	9.67
10085	1.00	11.00	1.00
10453	5.99	16.68	3.05
10493	1.00	1.00	17.87
10859	1.00	1.00	18.08
11025	1.00	1.00	10.35
11065*	8.65	1.00	1.00
11473*	12.61	1.80	1.00
11798	1.00	12.00	1.00
11841	1.00	1.00	10.16
11854	3.78	7.81	1.16
12046	2.44	8.45	1.00
12048**	1.46	12.61	1.55
12150	3.07	15.33	1.00
12158	1.00	1.00	7.71
12361	1.00	1.00	13.55

(*Continued*)

Table 1. (*Continued*)

Expression region No.	Biofilm	Exponential	Stationary
12370	6.98	7.96	1.00
12371	4.40	10.40	1.34
12407	1.00	1.00	8.82
12460	1.00	1.00	22.09
12684*	10.17	4.62	1.00
12706	13.95	40.36	3.98
12757**	1.00	8.31	1.21
13053	5.16	16.31	1.00
13107	2.55	12.67	1.00
13281**	1.00	4.22	11.56
13573*	120.98	1.55	1.02
13621**	1.46	2.78	26.39
13625	1.00	1.00	7.90
13631	1.00	8.00	1.00
13953	1.00	1.00	8.47
14242	1.00	7.60	1.00
14286	1.00	9.00	1.00
14414*	17.52	1.00	1.00
14887	1.00	1.00	11.99
15147	1.00	1.00	10.16
15214	6.92	4.50	22.38
15677*	17.48	1.00	1.00
15861**	1.00	1.57	15.83
15955**	2.05	12.55	3.98
16106	2.52	11.71	1.00
16107*	10.66	2.50	1.00
16117	2.77	1.00	23.09
16257	1.00	1.00	13.55
16412	1.21	1.00	10.74
16574	1.00	11.68	1.00
16705	12.10	16.30	2.27
16723	12.16	19.32	3.89
16724	8.81	22.76	1.00
16912	1.00	1.00	10.16
17299**	1.13	9.73	1.31
17531	1.00	1.00	36.10
17548**	1.00	3.46	15.52
17642	1.00	1.00	37.26
18098*	9.58	1.00	1.00
18150	2.12	1.62	9.05
18205	19.44	35.13	1.00
18260	5.74	8.49	1.19
18451	6.23	11.35	1.54
18718	1.00	1.00	8.47
18763	1.00	1.00	8.31
18900	1.54	8.78	1.00
19276	1.00	1.00	11.62

(*Continued*)

Table 1. (*Continued*)

Expression region No.	Biofilm	Exponential	Stationary
19347	1.00	7.72	1.00
19511**	1.00	10.26	1.12
19604	6.55	17.70	1.00
19647	1.00	1.00	22.14
19890**	1.00	10.06	4.29
19898	6.21	22.87	1.00
19931	1.00	1.00	10.65
20262	1.00	11.00	1.00
20685	2.89	8.11	1.00
20762*	11.33	1.78	1.00
20837	1.00	1.00	10.49
21011	34.08	52.04	5.05
21223*	8.51	1.54	1.00
21275	1.00	1.00	8.47
21392	6.13	7.95	1.31
21533*	11.65	1.00	1.00
21637	4.54	10.68	1.00
21762*	8.08	7.45	1.00
21850**	3.14	20.00	3.20
22036	8.30	11.04	1.00
22275	1.00	1.00	15.24
22490*	7.75	1.88	1.00
22686	2.59	10.49	2.30
22981	1.00	1.00	11.08
23002**	1.00	1.14	8.00
23283*	12.19	9.52	1.00
23620	1.00	1.00	13.55
23869	1.00	11.00	1.00
24430*	8.30	1.00	1.00
25126**	1.00	1.21	11.90
25193**	1.00	9.98	1.18
25478	4.26	16.55	2.78
25499**	1.00	1.88	13.81
25551	18.69	3.92	46.59
25571	14.09	6.32	70.99
25811	1.96	1.00	10.16
25840**	1.00	6.00	8.36
25942	1.00	1.00	8.16
26235	1.00	1.00	8.47
26247*	17.86	16.66	3.39
26276	2.62	12.32	1.22
26388	1.88	9.77	1.38
26511	1.00	8.00	1.00

(*Continued*)

Table 1. (*Continued*)

Expression region No.	Biofilm	Exponential	Stationary
26627*	15.83	2.06	3.82

Differentially expressed coding regions assessed by 454 pyrosequencing corresponding to sRNA molecules and their normalized expression values in sessile (biofilm) and planktonic cells (in exponential and stationary phase of growth).
*sRNA molecules up-regulated in biofilmrelated to both planktonic cell types.
**sRNA molecules down-regulated in biofilm related to both planktonic cell types.
Coding regions only expressed or repressed in biofilm are shown in bold.

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Taken together, all these observations strongly indicate that the production of a relatively large number of sRNA is significantly affected when *A. baumannii* cells grow as planktonic or sessile cells. Furthermore, the type and amount of particular sRNA are significantly affected by the growth stage of planktonic bacteria. Thus, sRNA transcripts could play a critical role in the physiology of *A. baumannii* by mechanisms that are poorly understood.

Involvement of sRNA 13573 in biofilm formation

Special attention was paid to sRNA 13573 due to its high level of expression, *ca.* 120-fold in biofilm cells compared to planktonic cells as assessed by pyrosequencing. Thus, sRNA 13573 was first cloned into the pETRA vector under the control of the CTXM promoter using primers listed in S1 Table. This approach resulted in the over-expression of this coding region as confirmed by qRT-PCR (Table 2). The transformant colonies were grown and their ability to form biofilm was quantified using the wild type strain harbouring the empty pETRA vector as a control. Since the highest rate of biofilm formation was assessed at 48 h, the comparison between the different isogenic strains was perfomed at 48 h (Fig 2A, S5 Fig). Cells over-expressing sRNA 13573 (13573) showed the highest value of biofilm formation; about 2-fold compared to the strain containing the empty vector (*P* value 0.0008).

Also, the sRNA 13573 region was deleted from the ATCC 17978 genome and the biofilm formation ability was investigated for the corresponding knockout strain showing a no significant decrease (*P* value 0.22) in biofilm formation compared to the wild type strain as shown in Fig 2A. When a pETRA derivative containing sRNA 13573 was introduced into the knockout strain (Δ13573) the biofilm formation ability was higher (*P* value 0.0014) than that shown by the parental strain (17978). Expression level of the sRNA 13573 gene in the parental, the

Table 2. Expression level of the sRNA 13573 coding region.

Strain	Expression level of sRNA 13573
17978	0.86 ± 0.19
Δ13573	0
17978 with empty pETRA	0.47 ± 0.23
13573	151.45 ± 50.84
Δ13573 complemented	151.70 ± 15.98

Expression level of the sRNA 1373 was determined by qRT-PCR using Taqman probes of the strains: wild type *A. baumannii* (17978), *A. baumannii* ATCC 17978 without sRNA 13573 (Δ13573), *A. baumannii* ATCC 17978 harbouring pETRA (ATCC with empty pETRA), *A. baumannii* ATCC 17978 harbouring pETRA with over-expressed sRNA 13575 (13573) and *A. baumannii* ATCC 17978 without sRNA 13573 harbouring pETRA with sRNA 13575 (Δ13573 complemented).

https://doi.org/10.1371/journal.pone.0182084.t002

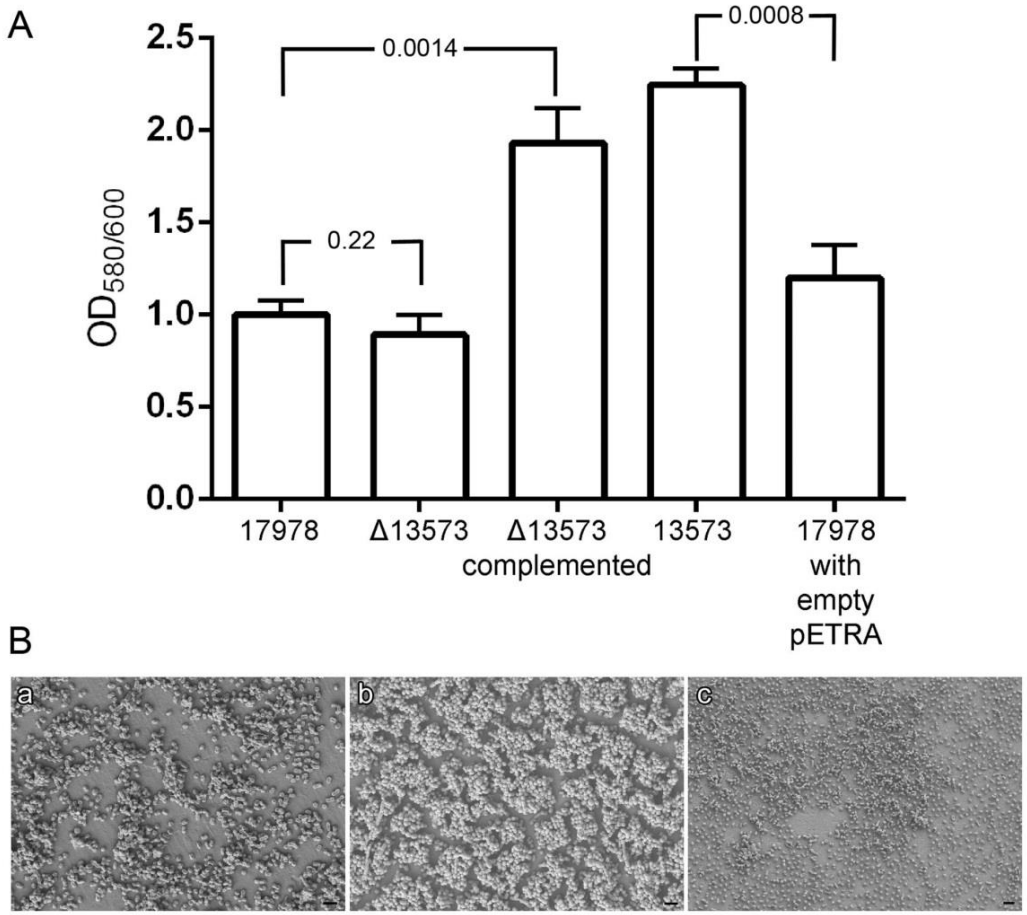

Fig 2. Biofilm formation assays. A) Quantification of biofilm formation by *A. baumannii* ATCC 17978 (17978), *A. baumannii* ATCC 17978 Δ13573 (Δ13573), *A. baumannii* ATCC 17978 harbouring pETRA (17978 with empty pETRA), *A. baumannii* ATCC 17978 harbouring pETRA with sRNA 13575 (13573), and *A. baumannii* ATCC Δ13573 harbouring pETRA with sRNA 13575 (Δ13573 complemented). B) SEM analysis of *A. baumannii* ATCC 17978 (a), *A. baumannii* sRNA 13573 over-producing strain (b) and *A. baumannii* ATCC 17978 Δ13573 (c). Micrographs were taken at 5,000x and bars indicate the scale marks (2 μm).

https://doi.org/10.1371/journal.pone.0182084.g002

Δ13573, the 13573 and the control strains was confirmed by qRT-PCR (Table 2). Growth curves performed in parallel (S6 Fig) showed the same growing profile in all cases. SEM was performed for elucidating biofilm structure in *A. baumannii* ATCC 17978 and the strain over-expressing the sRNA 13573 or the strain lacking the 13573 sRNA (Fig 2B). SEM analysis revealed that cells over-expressing sRNA 13573 were able to develop tridimensional and organized biofilm structures on the surface of plastic coverslips while cells from both the parental and Δ13573 strains showed unorganized cells. However, cells from the Δ13573 strain showed a more disorganized pattern and more dispersion than cells from the parental strain (Fig 2B).

Little is known about the involvement of sRNA molecules in the regulation of biofilm formation and maintenance. To date, some sRNA molecules have been described as regulators involved in biofilm formation in *Pseudomonas*, *Vibrio*, *Salmonella* or *Erwinia* [67, 68]. Most of the sRNAs involved in biofilm have been described in *E. coli* [69] including RprA [70], McaS [71], GcvB [72] and OmrA-B [73], being OmrA-B sRNAs involved in the biosynthesis of the biofilm matrix by controlling the expression of σ^S or CsgD, which is in turn related to biofilm formation. Thomason *et al.* [71] described that under specific conditions *E. coli* over-expressing sRNA McaS increased its biofilm formation ability while it was reduced in the corresponding McaS knockout strain. Attending to the SEM analysis described in the present work, differences in biofilm formation can be appreciated between the wild type and the Δ13573 knockout strain although the crystal violet assay did not showed significant differences between both strains. This could be due to the existence of a complex regulation network where sRNA 13573 seems to play a role in biofilm formation although its deletion causes no remarkable effects. A similar effect has been shown for sRNAs RseX, CsrC or SgrS from *E. coli* whose over-expression increased biofilm formation while their deletion did not affect biofilm formation. Bak *et al.* [74] reported that after over-expressing and deleting 99 sRNAs in *E. coli*, in only a few cases the deletion of sRNA genes had effects on biofilm formation. This observation suggests that some sRNA may not be expressed highly enough to affect this cellular process under the experimental conditions used in this study. Alternatively, it is possible that there might be redundancy of sRNAs acting on the process. Furthermore, multiple sRNAs have been indentified that modulate the activity of transcriptional regulators important for biofilm formation. CsrA is a RNA-binding protein that represses biofilm formation and its activity is repressed by sRNAs CsrB and CsrC [75, 76]. Also, the transcriptional regulator CsgG, which has been shown to be required for attachment and biofilm formation in *E. coli* is regulated at the mRNA level by several sRNAs, including McaS, RprA or OmrA/OmrB, in response to different environmental cues [71, 73]. Thus, the effect caused by the deletion of a single sRNA could be counteracted by the action of other sRNAs.

Involvement of the sRNA 13573 in attachment to human cells

Bacterial adhesion to A549 human alveolar cells was measured for the sRNA Δ13573 strain compared with the strain over-expressing sRNA 13573 from pETRA and using i) the 17978 strain, ii) the 17978 strain containing the empty pETRA and iii) the Δ13573 strain harbouring pETRA containing sRNA 13573, as controls. As shown in Fig 3A and considering the 17978 as the sample representing 100% of attached cells, the 17978 strain over-expressing 13573 (13573) attached more (30-fold) to A549 alveolar epithelial cells than the wild type strain harbouring the empty pETRA plasmid (P value < 0.0001). Once more, the 13573 strain revealed a significant phenotypic change when over-expressed, showing an increase in attachment to human cells ability. Besides, an adhesion ability decrease in the Δ13573 strain was detected when compared to the wild type strain being this difference significant (P value 0.036). The Δ13573 complemented derivative reached a higher (13-fold) value than the wild type strain (P value 0.0001). Furthermore, no colonies were detected when invasiveness was checked for all the strains, indicating that all the bacterial counts obtained after a 24 h-incubation were due to attachment (data not shown). Overall data suggest that the deletion of the sRNA 13573 produces a slight but significant change in the adhesion to A549 human epithelial cells and that its over-expression clearly increases attachment abilities.

The SEM analysis of polarized A549 alveolar epithelial cells co-incubated with bacteria revealed that bacteria over-expressing sRNA 13573 were able to form a thick and tridimensional biofilm (micrographs b and e, Fig 3B) over the A549 cell layer while the wild type

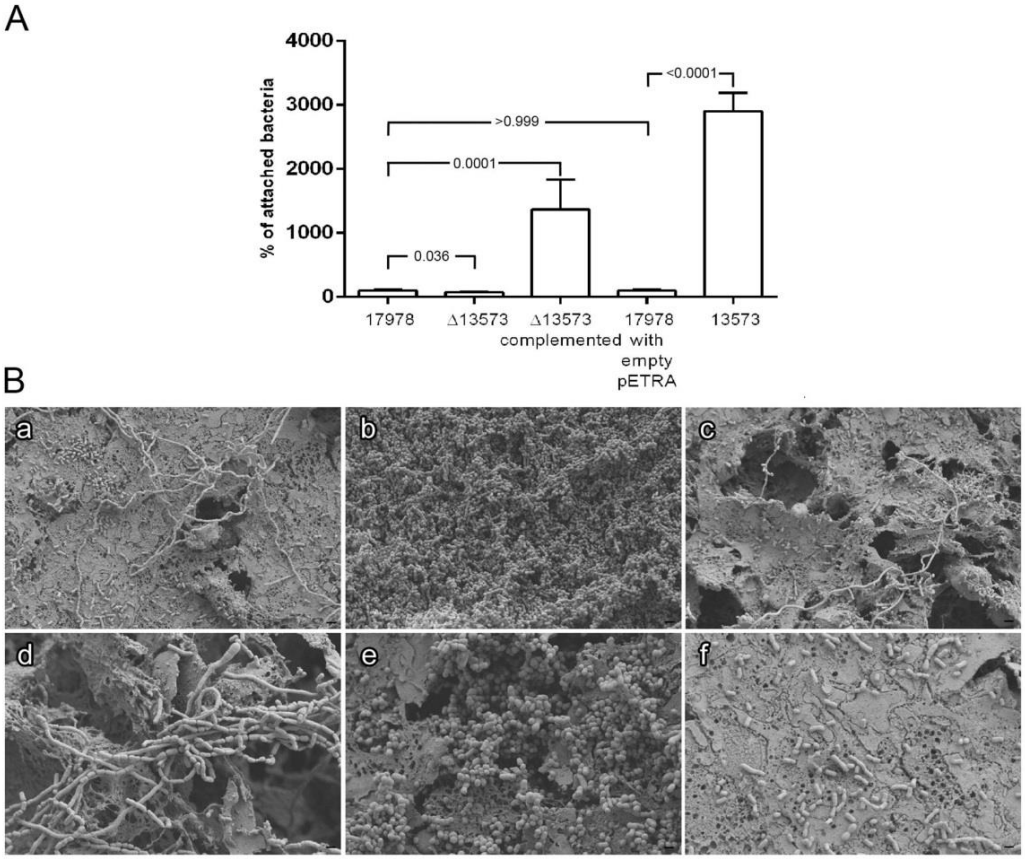

Fig 3. Adhesion assays. A) Attachment to A549 epithelial cells of *A. baumannii* wild type (17978), mutant derivative (Δ13573), mutant derivative over-expressing sRNA 13575 (Δ13573 complemented), harbouring empty pETRA vector (17978 with empty pETRA) and over-expressing sRNA 13575 (13573). The strain 17978 represents 100% of attached bacteria. B) SEM analysis of cells attached to A549 human alveolar cells of *A. baumannii* ATCC 17978 (a and d), *A. baumannii* ATCC 17978 over-expressing sRNA 13575 (b and e) and *A. baumannii* Δ13573 (c and f). Micrographs were taken at 5,000x (a, b and c) and 10,000x magnification (d, e and f). Bars indicate the scale marks (2 and 1 μm).

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showed poor biofilm formation ability (micrographs a and d, Fig 3B). Accordingly to previous results, the capacity of the Δ13573 strain to attach to eukaryotic cells was remarkably lower than the wild type strain (micrographs c and f, Fig 3B).

Little is known about the role that sRNAs can play in the adhesion to eukaryotic cells. For example, sRNA FasX from *Streptococcus* inhibited the expression of a cell surface pilus, therefore reducing the ability of the bacteria to adhere to host cells [77]. Eijkelkamp *et al.* [78] compared the ability to form biofilms and to adhere to eukaryotic cells of different strains and reported that there is not a clear relationship between these two capacities. Furthermore, previous data revealed that while the truncation of a gene as *csuE* caused a decrease in biofilm formation, the resulting mutant strain was able to adhere more to bronchial epithelial cells [79].

Taken together, these results suggest that there is no direct correlation between biofilm formation on abiotic and biotic surfaces, and that there is wide variation in the cell-surface and cell-cell interactions that result in adherence and biofilm formation by different *A. baumannii* clinical isolates [80]. However, the 13573 sRNA here described showed to be involved in both adhesion and biofilm abilities, being both functions widely related with the pathogenesis of *A. baumannii* [60].

Conclusions

The global analysis of the small RNA transcriptome of *A. baumannii* ATCC 17978 revealed different sRNA expression patterns in planktonic and biofilm associated cells, with some of the transcripts only expressed or repressed in sessile bacteria. *In vitro* and *in vivo* results demonstrated that the sRNA 13573 is involved in the control of the sessile lifestyle adoption and attachment to eukaryotic cells. The present work serves as a basis for future studies examining the complex regulatory network that regulate biofilm biogenesis and adhesion properties in *A. baumannii* ATCC 17978.

Supporting information

S1 Text. Additional details about 454 read data processing.
(DOCX)

S1 Table. Oligonucleotides and probes used in the present study.
(DOCX)

S2 Table. Number of initial reads, mapped reads and alignment hits for each sample.
(DOCX)

S3 Table. *A. baumannii* known sRNA and non-sRNA genes used as reference.
(DOCX)

S4 Table. Average, standard deviation, maximal and minimal values for the normalized expression scores calculated for protein coding genes, 16S and 23S rRNA genes and known sRNA genes, in each of the growing conditions.
(DOCX)

S5 Table. Average, standard deviation, maximal and minimal values for the normalized expression scores calculated for 5564 expressed regions not overlapping with known genes.
(DOCX)

S6 Table. Sets of expression regions not overlapping with known genes and having a normalized expression score equal or higher than 7.6 in some of the three growing conditions.
(DOCX)

S1 Dataset. Expressed regions. List of 5,564 expressed regions, their length and locations in the genome (NC_009085.1) or plasmids (NC_009083.1 and NC_009084.1) of *A. baumannii* ATCC 17978.
(DOCX)

S2 Dataset. Coding regions. List of normalized expression of 255 coding regions (expression score should be equal or above 7.6 in some growing condition: Bio, Exp or Sta), their length and locations in the genome (NC_009085.1) or plasmid (NC_009084.1) of *A. baumannii* ATCC 17978.
(DOCX)

S1 Fig. Distribution of normalized expression scores for known sRNA genes. Blue: biofilm samples. Orange: exponential phase samples. Yellow: stationary phase samples.
(DOCX)

S2 Fig. Distribution of normalized expression scores for the 5564 expressed regions not overlapping with known genes. Blue: biofilm samples. Orange: exponential phase samples. Yellow: stationary phase samples.
(DOCX)

S3 Fig. Distribution of normalized expression scores for the 255 expressed regions not overlapping with known genes and having a normalized expression score equal or higher than 7.6 in some of the growing conditions. Blue: biofilm samples. Orange: exponential phase samples. Yellow: stationary phase samples.
(DOCX)

S4 Fig. qRT-PCR assays. Expression levels of 8 sRNA regions in planktonic and sessile cells determined by qRT-PCR using Taqman probes. Y axis represents the relative expression of the genes taking the housekeeping gene *gyrB* as value 1.
(DOCX)

S5 Fig. Biofilm formation at different times. Staining at 12, 24 and 48 h of biofilm formation of *A. baumannii* ATCC 17978 (17978), *A. baumannii* ATCC 17978 Δ13573 (Δ13573), *A. baumannii* ATCC Δ13573 harbouring pETRA with sRNA 13575 (Δ13573 complemented), *A. baumannii* ATCC 17978 harbouring pETRA with sRNA 13575 (13573), and *A. baumannii* ATCC 17978 harbouring pETRA (17978 with empty pETRA).
(DOCX)

S6 Fig. Growth curves. Growth curves of wild type *A. baumannii* (17978), *A. baumannii* ATCC 17978 lacking the sRNA 13573 (Δ13573), *A. baumannii* Δ13573 over-expressing sRNA 13575 (Δ13573 complemented), *A. baumannii* ATCC 17978 harbouring pETRA over-expressing sRNA 13575 (13573) and *A. baumannii* ATCC 17978 harbouring the empty pETRA vector (17978 with empty pETRA).
(DOCX)

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4. DISCUSSION

MDR bacterial infections are becoming increasingly common in the hospital and community setting, emphasizing the need for new strategies that can effectively treat them. Over time, bacteria can develop resistance to existing drugs, making infections difficult, if not impossible, to treat. This scenario motivates prospective research towards the discovery of new antimicrobial active substances. Pioneering approaches, methodologies and technologies have promoted a new era in antimicrobial discovery.

RNA sequencing procedures are evolving technologies that allow us to discover RNA expression profiles of any organism (262, 263). The low cost of sequencing combined with an increasing data output have accelerated RNA-seq based studies. Therefore, these new tools may be very useful to find new genes and resolve the structure of transcripts (264).

RNA-seq has also become a standard method to analyse gene expression in bacterial infections since it can reveal infection-relevant RNA expression changes (265). Facilitating quantitative computational analysis, advances in RNA-seq technology have revolutionized the study of bacterial transcriptomes (266). Thus, transcriptional analyses have leaded to the identification of several *A. baumannii* genes involved in different biological processes related to pathogenesis and resistance to different compounds (267-269).

In the present study, Illumina RNA-seq was used to set up the transcriptional pattern of *A. baumannii* ATCC 17978 in biofilm-associated cells compared to planktonic cells. Several studies tried also to untangle the factors that regulate biofilm formation using different approaches such as proteomic analysis or random transposon mutagenesis (132, 270, 271). Thereby, this is the first study that uses RNA sequencing for identifying new factors involved in biofilm production and maintenance.

Our data showed notable differences in gene expression between biofilm and planktonic cells of *A. baumannii* ATCC 17978 strain, being 55 genes exclusively expressed in sessile cells. Some of them had been previously described as related to biofilm formation. One example is the type I pili encoded by *csuA/BABCDE* operon that seems to be required for attachment to plastic surfaces although is not involved in adherence of *A. baumannii* ATCC 19606^{T} to human cells (106, 107). Accordingly, our results showed that *csuA* and

csuB were exclusively expressed in biofilm cells and *csuA/B, csuC, csuE,* and *csuD* were highly expressed in biofilm cells compared to planktonic cells. However, the role of this operon in the ATCC 17978 strain remains unclear since previous *in silico* analyses revealed a deletion in the 5'-end of *csuA/B,* which could result in the abolishment of CsuA/B production, and a single bp insertion in *csuB* that truncated the open reading frame (100, 101).

Tomaras *et al*. reported that the expression of Csu usher-chaperone pili assembly system is controlled by BfmR/S, a two-component regulatory system that acts in response to environmental signals (108). As previously mentioned, both genes are involved in several biological processes related to virulence such as biofilm formation, adherence, and serum resistance (108, 109). Our data revealed an over-expression of the *bfmR* gene in biofilm-associated cells compared to stationary cells even though it did not result to be a biofilm-specific gene, which could cause the over-expression of genes belonging to the *csuA/BABCDE* operon.

Our data revealed that some other transcriptional regulators were only expressed in biofilm, but most of them are still uncharacterized and their functions remain unknown. One of these regulators is the A1S_2042 gene, related to the TetR family, a widely distributed group of transcriptional repressors that are involved in multidrug resistance, catabolic pathways, and pathogenicity (272). The disruption of this gene exclusively expressed in sessile cells caused a decrease in the biofilm formation ability of the mutant strain, suggesting that the A1S_2042 gene could participate in biofilm regulation.

Pili and fimbria are important elements for bacterial adhesion to biotic and abiotic surfaces as well as for motility (105). Genes A1S_1507, coding for a type I pili protein, and A1S_3168, coding for the TFP PilW, were selected for disruption in the present work since they were over-expressed in sessile cells compared to planktonic cells. Mutant strains showed a reduced capacity to form biofilm, confirming the role of these genes in the process. The A1S_1507 gene belongs to the A1S_1510-1507 chaperone-usher system, which was identified by Nait Chabane *et al*. as a pellicle-associated operon well conserved among different *A. baumannii* strains (111). Overall data indicated that pili and fimbria play a remarkable role in biofilm formation (105, 111).

Iron acquisition systems are important virulence factors, such as acinetobactin, which was proved to be required for *A. baumannii* ATCC 19606^T strain to persist within epithelial cells (153). Our data reflected the expression of some genes related to acinetobactin (A1S_2380 and A1S_2388) and ferredoxin (A1S_0945 and A1S_1779) only in biofilm cells and the over-expression of many genes involved in iron acquisition and transport compared to planktonic cells. These results correlate with those obtained by Marti *et al*. (271) that detected the over-expression of four siderophore iron uptake systems in cells in a late state of pellicle development. Modarresi *et al*. showed that low iron levels promoted siderophore activity, AHL production, and biofilm formation in several *A. baumannii* strains (273). Thus, iron starvation conditions in sessile cells could promote the expression of genes involved in the iron uptake process. However, the presence of this metal in the culture medium differentially affects biofilm formation depending on the strain (274), and the relationship between the iron-associated process and biofilm formation still remains unclear.

The Bap protein of *A. baumannii*, homologue to the staphylococcal biofilm-associated protein, has been widely characterized as an important factor for the development of a mature biofilm structure as well as for adherence to biotic surfaces (116, 118). This protein has been described as highly polymorphic among strains (275). The *bap* gene is disrupted in the *A. baumannii* ATCC 17978 strain genome, resulting in the A1S_2724 and A1S_2696 loci, separated by 30 kb due to chromosomal rearrangements (116, 275). In the present study those genes, homologues to *bap*, were found as over-expressed in biofilm-associated cells compared to exponential cells, suggesting that they may play a role in the biofilm formation ability of the ATCC 17978 strain.

A membrane-associated protein OmpA has been extensively related with biofilm formation (121). However, our data did not match previous reports and revealed that *ompA* gene expression was down-regulated in biofilm-associated cells. Expression level of this protein in biofilm is a controversial issue since while Cabral *et al*. found it as up-regulated in biofilm cells (270) and Marti *et al*. detected three mass isoforms of the OmpA protein under-expressed in pellicles (271). This phenomenon could be due to the utilization of different *A. baumannii* strains or to variations in the culture conditions, that could modify the structure and the amount of the biofilm formed (98, 276). OmpA was suggested to act in the initiation step of biofilm formation and iron starvation conditions

of the maturation step could decrease its expression (271). In the present study, other proteins as CarO (A1S_2538) and OprD-like (A1S_0201) were found as over-expressed in biofilm cells. Accordingly, these proteins were found to be up-regulated in previous proteomic studies (270, 271). The over-expression of these porins could complement the decrease of OmpA production, contributing to membrane permeability.

Moreover, in our work, many genes involved in amino acid metabolism and transport were up-regulated in biofilm compared to planktonic cells, corroborating the results previously obtained by Cabral *et al*. (270). Accordingly, we also detected an over-expression of genes encoding efflux system components, including a resistance-nodulation-cell division type (RND efflux pump) in biofilm cells. Efflux pumps may contribute to the inherent resistance of biofilm-forming bacteria to antibiotics.

Twelve genes, found as exclusively expressed in biofilm, encoded uncharacterized proteins. One of them, the A1S_0302 gene, was selected for disruption because of its high level of expression in biofilm cells. The corresponding mutant strain was significantly deficient in biofilm formation ability compared to the parental strain. Further studies are required in order to determine the role that this gene could play in biofilm formation and maintenance.

The gene A1S_0114, which encodes an ACP, was over-expressed in biofilm cells and its expression level was the highest detected. Surrounding genes, from A1S_0112 to A1S_0118, were also found as significantly over-expressed in biofilm compared to planktonic cells, suggesting that these genes could act as an operon. A previous transcriptome analysis revealed that the expression of this cluster was activated by quorum sensing, pointing out the relationship between these genes and the homoserine lactone synthase encoded by the A1S_0109 gene (131). In the present work it was demonstrated that the loss of the A1S_0114 gene caused a noticeable decrease in biofilm formation ability. Complementation of the knockout mutant strain with a copy of the A1S_0114 gene resulted in a partial restoration of the biofilm production ability. Expression of A1S_0109, A1S_0112 and A1S_0113 genes was conditioned by the expression of the A1S_0114 gene, since its complete deletion caused a decrease in the expression level of these surrounding genes during the stationary phase of growth. Taking together these data indicate that all these genes could act as an operon where its

disruption could have polar effects. This could explain the partial restoration of the biofilm formation ability of the mutant during complementation procedures.

Several studies of different *A. baumannii* strains suggested that the gene cluster A1S_0112-0119 could be a polycistronic operon (131, 277). Bioinformatic analysis showed that all genes from A1S_0112 to A1S_0119 were transcribed in the same direction and either overlapped or were separated by small intergenic regions, which suggests the polycistronic nature of this operon. This fact was confirmed by the RT-PCR procedures performed. Allen and Gulick proposed this cluster to act as a NRPS where the A1S_0112 gene may act as an adenylation domain and the A1S_0115 gene as a multi-domain NRPS protein containing condensation, adenylation, peptidyl carrier protein (PCP), and thioesterase domains (277). Furthermore, this operon was shown to play a role in different processes in *A. baumannii*, since disruption of genes A1S_0112 and A1S_0115 in a hyper-motile variant of *A. baumannii* ATCC 17978 abolished motility and ability to form pellicles of the mutant strains (132). Similarly, another study evidenced that insertions in genes A1S_0113 and A1S_0115 significantly reduced the migration capacity of *A. nosocomialis* M2 strain (131). Thereby, a deeper analysis of the mutant strain lacking the A1S_0114 gene was done in the present study in order to clarify how this specific gene affects biofilm, adherence and other virulence factors.

SEM analysis of bacterial biofilms revealed that the A1S_0114 gene was needed for developing multicellular structures associated with mature biofilm. When the ability to adhere to human alveolar cells of the mutant strain lacking the A1S_0114 gene was tested, the outcome showed a 60% of reduction in the number of attached bacteria compared to the wild type strain. The knockout strain complemented with the pWH1266 plasmid harbouring a copy of the A1S_0114 gene was able to restore the ATCC 17978 phenotype, verifying the involvement of this gene in the interaction of *A. baumannii* with biotic surfaces. These results were confirmed by SEM analysis of infected A549 human alveolar polarized cells. Micrographs presented extensive damage to the surfactant layer as well as to the epithelial cells caused by the ATCC 17978 strain and an important number of wild type cells attached to alveolar cells. However, although the A1S_0114 defective strain was also able to destruct the surfactant, it showed a poor adherent phenotype.

Since the A1S_0114 gene seemed to take part in the adherence to both biotic and abiotic surfaces, expression of different genes involved in these processes were checked. Results of RT-qPCR showed an increase of the expression of *csuA/B* in the knockout strain compared to the wild type strain. The CsuA/BABCDE pili assembly system plays a well-known role in attachment to abiotic surfaces and its presence in pellicles of different strains (107, 111, 271). However, the *csuA/BABCDE* operon up-regulation in the mutant strain may be irrelevant as this system may not be functional in the ATCC 17978 strain due to an insertion in *csuB* and a deletion in *csuA/B* (100, 101). Nevertheless, the lack of the A1S_0114 gene prompted an over-expression of other pili-related genes such as A1S_0690, A1S_1510, and A1S_2091. The A1S_0690 gene is part of the A1S_0690-0695 operon, a putative FilF-like type III pili over-produced in pellicle and detected in OMVs (271, 278).

The A1S_1507-1510 operon encodes a type I pili, widely related to biofilm formation, which is in accordance with our previous results that showed that the A1S_1507 gene was highly expressed in biofilm cells and its disruption caused a decrease in biofilm formation. This operon, controlled by the global regulator H-NS and repressed under iron limitation conditions (156, 188), was detected in pellicles together with the A1S_2088-2091 operon (111). Furthermore, the A1S_2091 gene was highly expressed in biofilm compared to planktonic cells and the deletion of the LH92_11085 gene of *A. baumannii* MAR002 strain, homologous to A1S_2091, reduced bacterial attachment to A549 cells and biofilm formation on plastic surfaces (110). Expression of the virulence factor OmpA was also significantly reduced in absence of the A1S_0114 gene, in agreement with previous works that identified this porin as an important factor for biofilm formation and for the interaction with eukaryotic cells (121, 279). Overall, these results suggest that the A1S_0114 gene may influence the expression of genes related to attachment and biofilm production.

In the present study, the involvement of the A1S_0114 gene in *A. baumannii* virulence has been demonstrated by using different animal models. Thus, gene deletion caused an increase in the number of viable eggs of *C. elegans* in a fertility assay, a rise in the survival rate of *G. mellonella* and a lower bacterial burden in lungs of infected mice in a pneumonia model. Furthermore, growth of the knockout strain was not affected by the lack of the acyl carrier protein encoding gene, indicating that differences between the

wild type and the knockout strain could be due to the role of the A1S_0114 gene as a virulence factor. Nevertheless, a study that combined transposon mutagenesis and next-generation sequencing (NGS) techniques identified genes from the A1S_0112-0119 operon as not necessary for persistence of *A. baumannii* ATCC 17978 in mice lungs (124). With a similar approach, Subashchandrabose *et al*. identified genes involved in survival in a leukopenic mouse model of bloodstream infection. Although no transposon insertion in the A1S_0114 gene was detected, mutants in the rest of the genes of the operon exhibited reduced fitness during growth within the host but they did not reach the threshold to be considered critical for bacterial survival (280). Variations between our results and these studies may be due to differences in the methodology, such as distinct mice strains and infection routes used. Another study showed that genes belonging to this operon were also under-expressed during bacteraemia in a mice infection model compared to *in vitro* cells (267). Murray *et al*. reported that the majority of *A. baumannii* genes previously identified as necessary for virulence were either under-expressed or their expression did not change *in vivo* (267). In fact, they noted a general down-regulation of several genes involved in biofilm and pili biosynthesis, such as *csuA/BABCDE* and A1S_2088-2091 operons, the transcriptional regulator *bfmR*, and the homoserine lactone synthase *abaI* (267). These authors suggested that the reduced expression of those biofilm-related genes may be due to the planktonic state of the bacterial cells causing blood infection, explaining that these genes could be required for virulence in later stages of septicaemia or tissues infections (267).

Due to the putative functions of the proteins encoded by the A1S_0112-0119 operon, Allen and Gulick indicated that it could be acting as a NRPS (277). The A1S_0112 gene encodes an acyl-CoA synthase/AMP-acid ligase that could activate and transfer fatty acids to ACPs *via* acyl AMP intermediates. The A1S_0113 gene, encoding an acyl-CoA dehydrogenase, is predicted to modify the intermediate product carried by an ACP or PCP domain. The acyl carrier protein encoded by the A1S_0114 gene is likely to contribute a tethered intermediate to the NRPS system involved in the biosynthesis of a secondary metabolite (277). The A1S_0115 gene is predicted to encode a four-domain (condensation, adenylation, PCP, and thioesterase) NRPS protein with a thioesterase domain most likely responsible for the release of acyl/peptide chains from their covalent attachment to the ACP/PCP domains (281). The A1S_0116 gene encodes a protein that

belongs to the superfamily of RND transporters and could be potentially involved in the secretion of the secondary metabolite synthetize by this operon. The A1S_0117 and A1S_0118 genes are annotated as hypothetical proteins (162), but *in silico* analysis showed that they could be related to porins and epimerase/dehydratase enzymes, respectively. Finally, the A1S_0119 gene encodes a phosphopantetheine transferase that is expected to perform the activation of the ACP. All these predictions are in accordance with the observation that the expression of the A1S_0114 gene is required for the production of the Ac-505 secondary metabolite.

This compound, present in culture supernatants of the ATCC 17978 strain but absent in those from the knockout strain, seems to be a three-amino acid lipopeptide. However, bonds between the amino acids as well as to the hydrocarbon moiety are non-standard. Whereas the second and third amino acids are Cys and Gly residues linked *via* standard peptide linkage, they are connected to the Gly-containing moiety through the sulfur group of the Cys side chain forming a thioether bridge. The Cys-Gly moiety could be potentially derived from glutathione (L-γ-glutamyl-L-cysteinyl-glycine), a tripeptide found in high concentrations intercellularly in *A. baumannii* and that maintains a proper cellular redox state (282). Ac-505 may be the result of the glutathione-mediated de-activation of a secondary metabolite but the structure of this potential precursor remains unknown. Modification of this precursor by glutathione could explain that Ac-505 could not be able to restore the wild type phenotype when added to the mutant strain (data not shown).

To summarize, we can conclude that the A1S_0114 gene affects *A. baumannii* biofilm formation, adherence to eukaryotic cells as well as virulence responses and that it is involved in the biosynthesis of a novel metabolite. Further analysis are required in order to determine the mechanisms by which this gene acts and if Ac-505 is responsible for these responses.

Many genes have been described to date as involved in biofilm biogenesis and maintenance. However, little is known about the sRNAs that may be involved in this process regulation. Therefore, in the present work a transcriptional analysis of the sRNAs produced by planktonic cells and biofilm-associated cells was performed to identify those small RNAs of *A. baumannii* ATCC 17978 involved in biofilm formation.

The first sRNAs were discovered in *E. coli* in biochemical or functional screens, aided by their relative abundance (283). In the latest decades numerous studies used comparative genomics, secondary structure prediction, and computational analysis searching for promoters and terminators in intergenic regions to identify potential sRNA candidates (215). Shotgun cloning or microarray analysis of cDNA added new molecules to the pool of putative sRNAs (284, 285). The development of NGS technologies for cDNA sequencing has revolutionized the identification of novel noncoding transcripts (286). Recently, novel deep sequencing techniques as global mapping of transposon insertion sites, measuring translatomes using ribosome profiling, and analysis of RNA-protein complexes have facilitated the identification and functional characterization of multiple sRNAs (287-289).

Consequently, following NGS procedures in biofilm and in exponential and stationary growth phase samples we were able to identify 255 putative sRNAs that were expressed in at least one of the conditions, being 185 of them differentially expressed. This number was significantly bigger than the data obtained by Weiss *et al*. where 78 sRNA were detected in *A. baumannii* AB5075 (259). However, conservation of sRNAs among different strains is infrequent not only in *A. baumannii*, but also in *P. aeruginosa* where multiple sRNAs exhibit strain-specific expression patterns (290, 291). Also, depending on the approach used for the library preparation or the timing for RNA extraction, different set of sRNAs could be detected (292).

Accordingly, Sharma *et al*. predicted 31 sRNAs *in silico* from *A. baumannii* ATCC 17978 (258) and only 18 of them were also detected in our study. A group of 10 of the 31 sRNAs predicted by Sharma *et al*. were analysed in *A. baumannii* MTCC1425 and 3 of them showed positive signal on Northern blot. In particular, the sRNAs AbsR25 and AbsR28 described by Sharma *et al*. corresponded to the putative sRNAs 10452 and 22397 described in the present study, respectively. Moreover, while they found variable expression level of the sRNA AbsR25 during different phases of planktonic growth (258), we did not find the sRNA 10452, its homologue, as differentially expressed under our experimental conditions. Moreover, the length of both sequences detected in the ATCC 17978 and the MTCC1425 strains did not match, probably due to genomic variations between these strains or to the distinct bioinformatics approaches used.

We found 28 of the 185 differentially expressed sRNAs as over-expressed and 32 down-regulated in biofilm compared to planktonic cells. Nine sRNAs were exclusively expressed in biofilm and 21 were only detected in planktonic cells. Gene expression of the sRNAs most over-expressed in biofilm was confirmed by RT-qPCR analysis, those being the 13573 sRNA and six sRNAs not present either in exponential or in stationary growth phase. The outcome obtained validated the results previously obtained by pyrosequencing, indicating that the profile of sRNAs produced is significantly different depending on the lifestyle.

Small RNA 13573 was selected for further characterization due to its high over-expression level in biofilm cells. The effect of the 13573 sRNA in different biological processes was investigated. Biofilm formation analysis showed that, the 13573 sRNA has an effect on the biofilm formation ability of the strain, since its over-expression prompted a 2-fold increase of biofilm formation. SEM images exposed that 13573 was able to develop a mature biofilm structures, unlike the wild type and mutant strains, which presented unorganized cells. Moreover, cells from the knockout strain showed more dispersion than the parental strain. Several sRNAs have been described as regulators implicated in biofilm formation in *Pseudomonas*, *Vibrio*, and in *E. coli* (93, 236). In previous works it was shown that over-expression of sRNAs RseX, CsrC or SgrS sRNAs from *E. coli* increased biofilm formation but its deletion did not cause the opposite effect (227). In fact, in the same study they detected that, after over-expressing and deleting 99 sRNAs in *E. coli*, only a few deletions had effects on biofilm formation (227). This correlates with the results that we have observed for the 13573 sRNA of *A. baumannii* ATCC 17978. The strain over-expressing the 13573 sRNA produced higher amount of biofilm than the wild type strain but its deletion did not affect biofilm formation. This effect could be due to several issues, such as redundancy of sRNAs acting on the same process, other sRNAs regulating the activity of transcriptional regulators involved in biofilm formation, or expression levels of sRNAs that do not reach the threshold to affect this biological process under particular experimental conditions. Therefore, we found that the over-expression of the 13573 sRNA has a remarkable effect on biofilm production but the impact of its deletion could be neutralized by other sRNAs. A more exhaustive study is required in order to untangle the mechanisms used by the 13573 sRNA to regulate this process.

Adhesion assays allowed us to elucidate the role of the 13573 sRNA on host-pathogen interaction. The ability to attach to A549 human alveolar cells decreased in the knockout strain lacking the 13573 sRNA compared to the ATCC 17978 strain. However, when the 13573 sRNA was over-expressed, a significant higher number of bacteria were attached to the biotic surface. No colonies were detected when invasiveness ability of the strains was tested. Therefore all the CFUs counted after 24 h of incubation were due to the attachment capacity of the different strains. SEM analysis of bacterial strains infecting polarized A549 cells supported these previous results, revealing that only the strain over-expressing 13579 sRNA was able to form a tridimensional biofilm over the cell layer.

There is not a clear relationship between the ability to form biofilms and the capacity to adhere to biotic surfaces as described by Eijkelkamp *et al*. in different strains of *A. baumannii* (100). Data revealed that not all the genes involved in biofilm formation play also a role in attachment to biotic surfaces. An example is the *csuE* gene, which is not implicated in the adherence to human cells even though it is in biofilm formation on abiotic surfaces (106, 107). Regarding sRNAs, little is known about the role they play in adhesion to eukaryotic cells. One example is FasX from *Streptococcus*, which inhibits the expression of a cell surface pilus, reducing the capacity of the bacteria to attach to host cells (293). Hence, we can affirm that 13573 is the first sRNA described in *A. baumannii* as involved in biological processes such as biofilm formation and attachment to biotic surfaces.

To sum up, in the present work were identified different patterns of gene expression of both mRNAs and sRNAs in biofilm-associated cells compared to planktonic cells from exponential or stationary growth stages. The analysis of these patterns allowed us to identify genes and sRNAs that may play a role in the regulation of the sessile lifestyle adoption. Therefore, we demonstrated that the A1S_0114 gene, which encodes an ACP being part of the A1S_0112-0119 operon, is involved not only in biofilm production and maintenance, but also in adherence to human alveolar cells, virulence and the biosynthesis of the metabolite Ac-505. On the other hand, this study highlighted the potential role of the 13573 sRNA in biofilm formation regulation and adherence to eukaryotic cells. This is the first work where biofilm formation and attachment have been reported as positively regulated by sRNAs in *A. baumannii*.

5. CONCLUSIONS

1. Biofilm associated cells of *A. baumannii* ATCC 17978 present a mRNA pattern that significantly differs from planktonic cells, showing a collection of differentially expressed genes where some of them are exclusively expressed in sessile cells.

2. A hypothetical protein, a fimbrial protein encoded, a pilus assembly protein, and a transcriptional regulator, encoded by the A1S_0302, the A1S_1507, the A1S_3168, and the A1S_2042 genes, respectively, are involved in biofilm formation of *A. baumannii* ATCC 17978.

3. The A1S_0114 gene of *A. baumannii* ATCC 17978, coding for an acyl-carrier protein, plays a role in biofilm formation, in attachment to biotic and abiotic surfaces, in virulence, and in the biosynthesis of the metabolite acinetin 505, this being a novel factor involved in the pathogenesis of this strain.

4. The *A. baumannii* ATCC 17978 strain expresses a remarkable number of sRNAs, being their expression level dependent on the stage of growth.

5. A total amount of 185 sRNAs are differentially expressed in biofilm associated cells of the *A. baumannii* ATCC 17978 strain compared to both exponential and stationary phase cells suggesting that they may play a role in biofilm formation regulation.

6. The 13573 sRNA regulates biofilm formation and attachment to eukaryotic cells, being these biological processes related to the pathogenesis of *A. baumannii* ATCC 17978.

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ANNEX

SUPPLEMENTARY MATERIAL
CHAPTER I

Figure S1

Gene level counts. Left: boxplot (median, first and third quartiles and standard deviation) of the number of reads per gene. Right: density functions of the number of reads *per* gene.

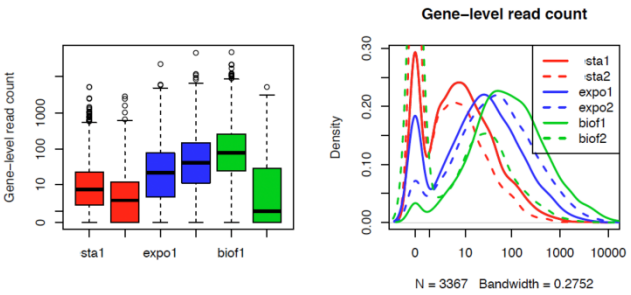


Figure S2

MD plots and correlation between samples. Upper right: MD plots showing (countsA+countsB)/2 against (countsA-countsB), with A and B being the samples shown on the diagonal.

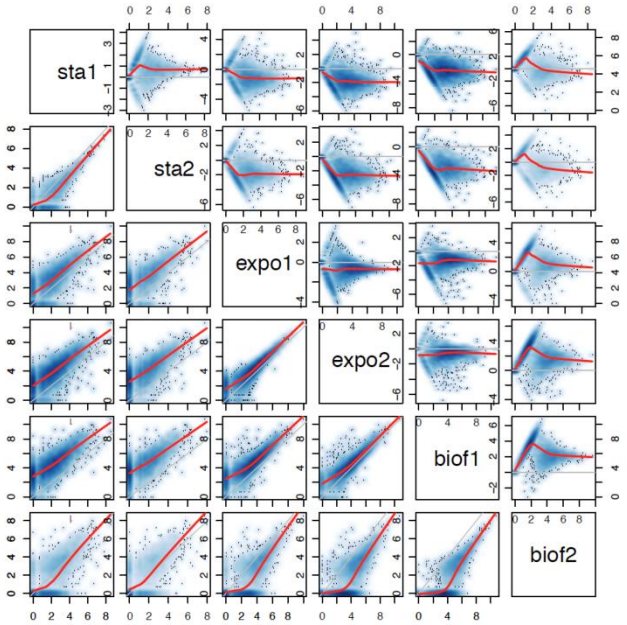


Figure S3

Sequence distribution of genes up-regulated in biofilm-associated cells. The data were filtered based on $p < 0.001$ and with respect to biological processes. A) Exponentially growing cells, filtered by the number of sequences (cutoff 6). B) Stationary phase cells, filtered by the number of sequences (cutoff 1).

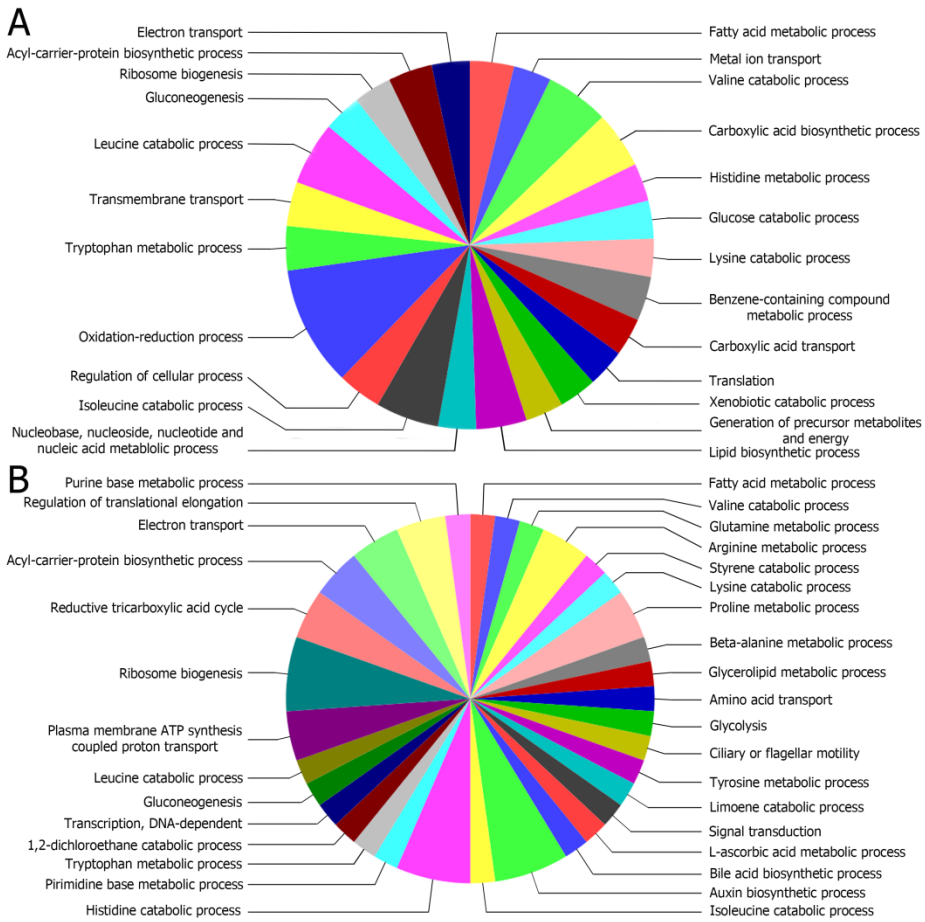


Table S1

Gene expression data from the complete transcriptome analysis of *Acinetobacter baumannii* ATCC 17978, showing gene expression levels in exponentially growing vs. stationary phase cells. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB:

normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA, log2Fold-change: $\log_2$ baseMeanB/baseMeanA, pval: *p* value, padj: *p* value adjusted for multiple testing, resVarA: variance of A, resVarB: variance of B.A: stationary phase cells. B: exponential phase cells. NA, non-applicable because of zero expression.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s004.xlsx

Table S2

Gene expression data from the complete transcriptome analysis of *Acinetobacter baumannii* ATCC 17978, showing gene expression levels in biofilm-associated cells vs. exponentially growing cells. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: exponential phase cells. B: biofilm-associated cells. NA, non-applicable because of zero expression.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s005.xlsx

Table S3

Gene expression data from the complete transcriptome analysis of *Acinetobacter baumannii* ATCC 17978, showing gene expression levels of biofilm-associated vs. stationary phase cells. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: biofilm-associated cells. NA: non-applicable because of zero expression.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s006.xlsx

Table S4

The expression levels of genes down-regulated in biofilm-associated vs. stationary phase cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: biofilm-associated cells.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s007.xlsx

Table S5

The expression levels of genes up-regulated in biofilm-associated vs. stationary phase cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: biofilm-associated cells.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s008.xlsx

Table S6

The expression levels of genes down-regulated in exponentially growing vs. stationary phase cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of

interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: exponential phase cells.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s009.xlsx

Table S7

The expression levels of genes up-regulated in exponentially growing vs. stationary phase cells, filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: stationary phase cells. B: exponential phase cells.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s010.xlsx

Table S8

The expression levels of genes down-regulated in biofilm-associated vs. exponentially growing cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value; padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: exponential phase cells. B: biofilm-associated cells.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s011.xlsx

Table S9

The expression levels of genes up-regulated in biofilm-associated vs. exponentially growing cells. The data were filtered based on $p < 0.001$. Id: name or code of the region of interest; baseMean: mean of the two next columns; baseMeanA: normalized number of counts for sample A; baseMeanB: normalized number of counts for sample B; Fold-change: baseMeanB/baseMeanA; log2Fold-change: $\log_2$ baseMeanB/baseMeanA; pval: *p* value, padj: *p* value adjusted for multiple testing; resVarA: variance of A; resVarB: variance of B.A: exponential phase cells. B: biofilm-associated cells.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3758355/bin/pone.0072968.s012.xlsx

SUPPLEMENTARY MATERIAL
CHAPTER II

Figure S1

Confirmation of the polycistronic nature of the *A. baumannii* ATCC 17978 A1S_0112-A1S_0119 operon. Total DNA (lanes 2–7), cDNA (lanes 8–13) and total RNA (lanes 14–19) samples were used as templates in PCR reactions using primers annealing to the A1S_0112-A1S 0113 (lanes 2, 8, and 14), A1S_0113-A1S_0115 (lanes 3, 9, and 15), A1S_0115-A1S_0116 (lanes 4, 10, and 16), A1S_0116-A1S_0117 (lanes 5, 11, and 17), A1S_0117-A1S_0118 (lanes 6, 12, and 18), and A1S_0118- A1S_0119 (lanes 7, 13, and 19) intergenic regions. Lanes 1 and 20 show the molecular weight standard Gene Ruler 1-Kb plus (Thermofisher Scientific). Molecular weight of each amplicon is indicated at the bottom of the figure.

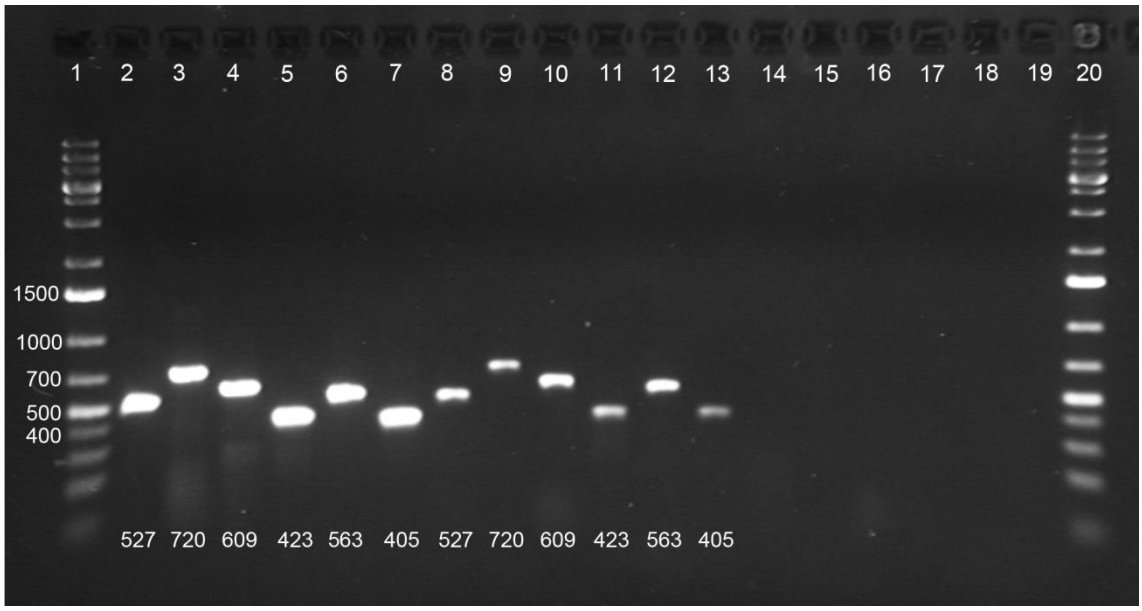


Figure S2

Growth of the 17978 parental and the Δ0114 isogenic deletion derivative strains. The OD_{600} values of each strain grown in LB or SB at 37°C for 24 h with shaking were determined hourly. Error bars represent the standard error (SE) of the mean.

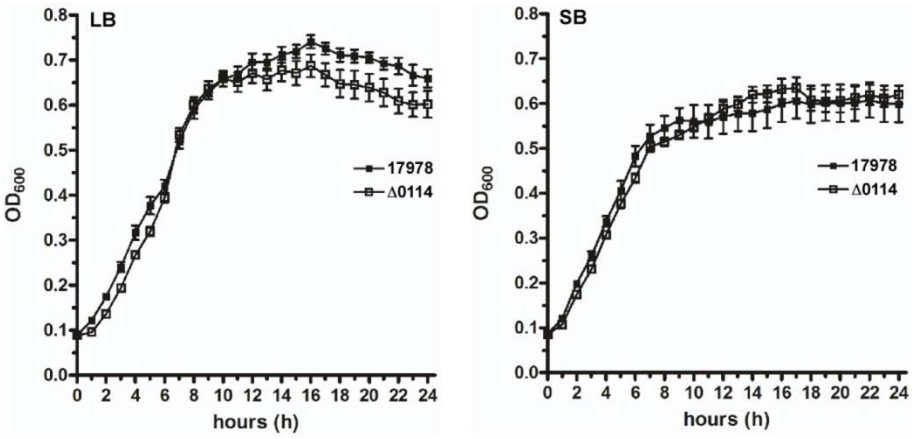


Figure S3

Ac-505 MS/MS spectra. (A) MS data collected on the FT-ICR with ECD fragmentation in positive ion mode. (B) MS/MS collected on a MaXis QTOF in positive ion mode (LC-MS) and (C) negative ion mode (direct injection).

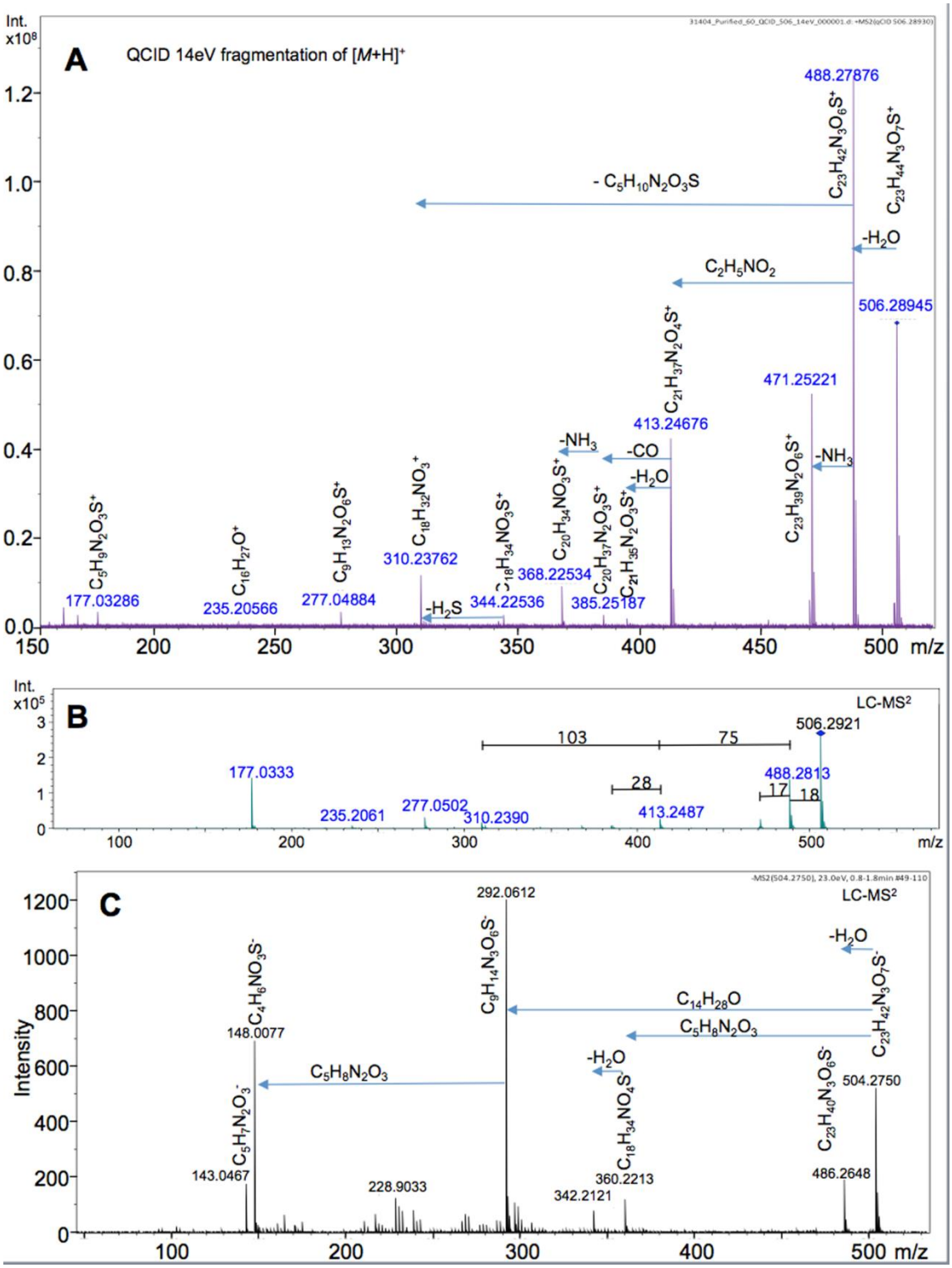

A
QCID 14eV fragmentation of [M+H]+
Int.
x10^8
488.27876
506.28945
471.25221
413.24676
310.23762
368.22534
177.03286
235.20566
277.04884
344.22536
385.25187
- C5H10N2O3S
-H2O
C2H5NO2
-NH3
-CO
-H2S
m/z
B
LC-MS2
Int.
x10^5
506.2921
103
75
28
17
18
177.0333
235.2061
277.0502
310.2390
413.2487
488.2813
m/z
C
LC-MS2
Intensity
292.0612
148.0077
143.0467
228.9033
360.2213
342.2121
504.2750
486.2648
C14H28O
C5H8N2O3
-H2O
m/z

Figure S4

MS/MS—based fragmentation predictions (A,B) are two of the 40 total MS^n spectra from MS(3) to MS(6) recorded on the OSU Bruker amaZon in positive ion mode using direct injection of a HPLC purified Ac-505. MS(2) for 488.3 and MS(4) for 235.2 *m/z* fragment ions are shown in (A,B), respectively. Neutral losses are shown in black. Panel (C) is a schematic diagram of MS/MS fragmentation of Ac-505 in positive ion mode and (D) is for negative ion mode.

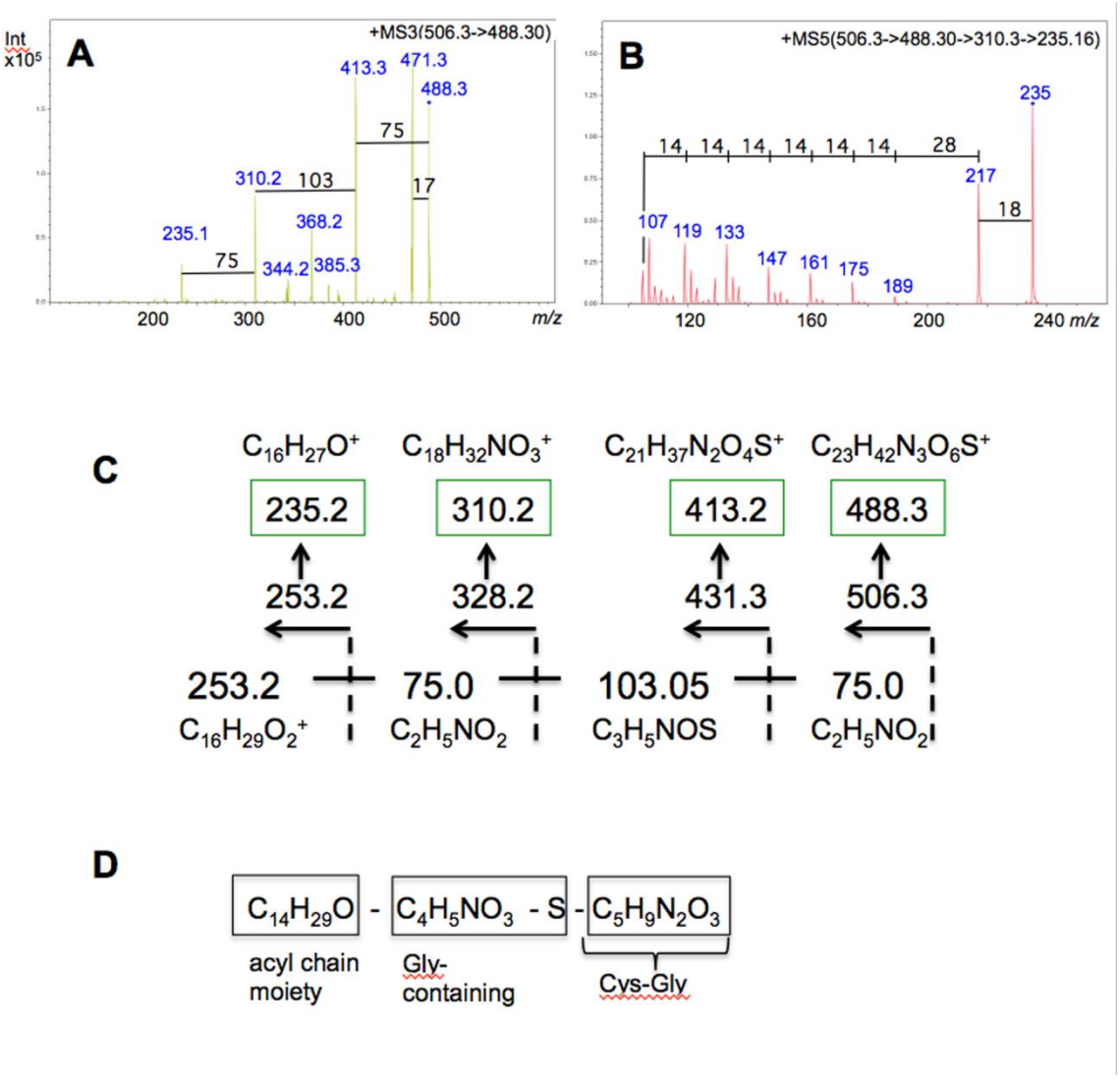


Figure S5

Predicted product ions of Ac-505 based on accurate mass and typical bond cleavage patterns under electrospray conditions in positive ion mode.

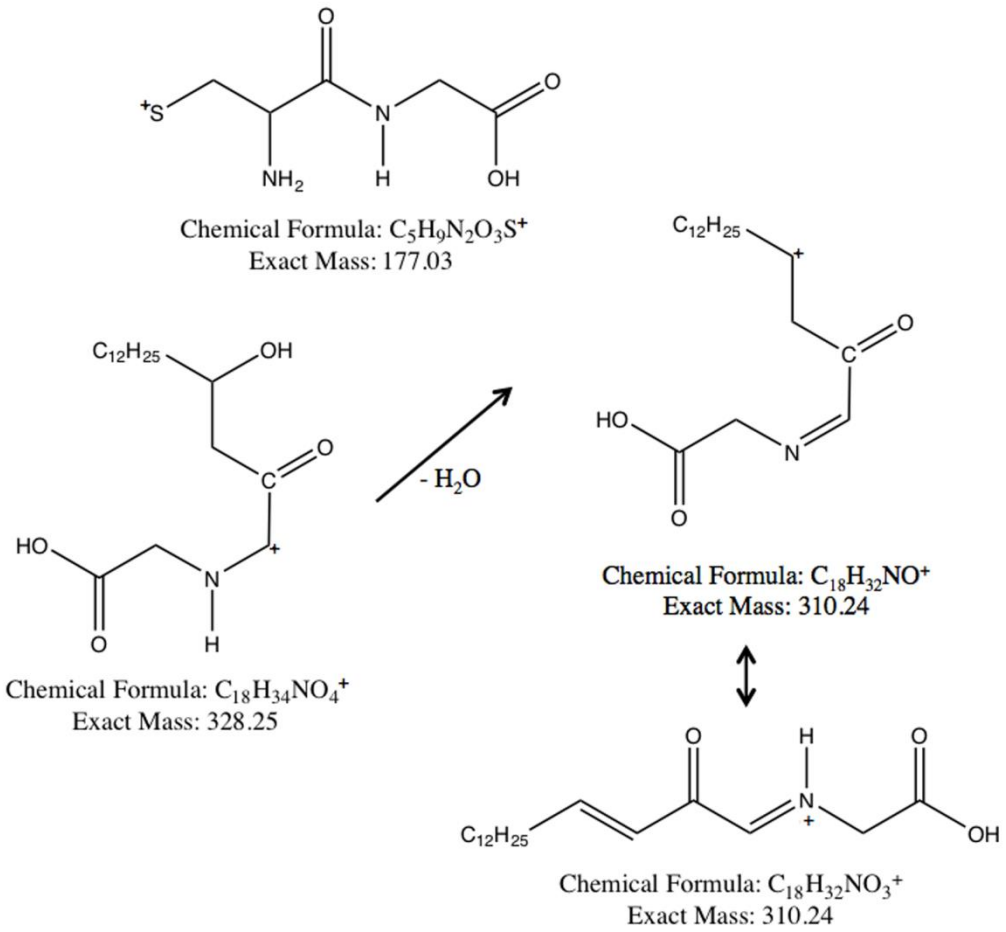
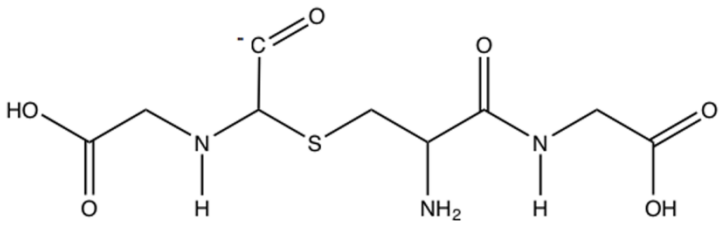
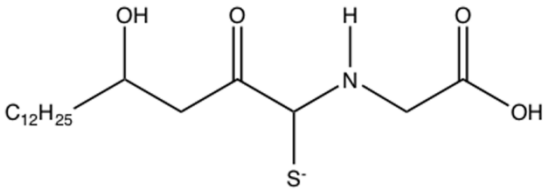


Figure S6

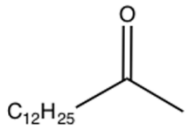
Predicted product ions of Ac-505 based on accurate mass and typical bond cleavage patterns under electrospray conditions in negative ion mode.



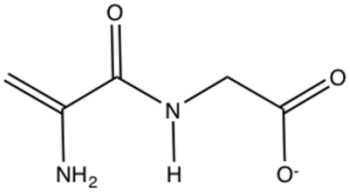
Chemical Formula: $C_9H_{14}N_3O_6S^-$
Exact Mass: 292.06



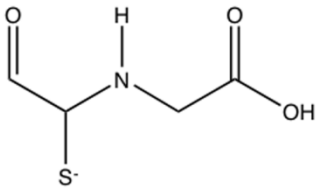
Chemical Formula: $C_{18}H_{34}NO_4S^-$
Exact Mass: 360.22



Chemical Formula: $C_{14}H_{28}O$
Exact Mass: 212.21
neutral loss of 212.21



Chemical Formula: $C_5H_7N_2O_3^-$
Exact Mass: 143.05
dehydroalanyl-glycine



Chemical Formula: $C_4H_6NO_3S^-$
Exact Mass: 148.01

Table S1

Oligonucleotides used in the present study.

Primer name	Sequence (5'-3')	Purpose
0114Up*Pst*I	CCCCTGCAGGGGTTGGTACGTGAGC AACTC	Construction of Δ0114
0114Up*Eco*RI	GGGGAATTCCCCGCGCTCCAGTAAG CTTT	Construction of knockout strain Δ0114
0114Down*Eco*RI	CCCGAATTCGGGGAGCCAACACTAT TATGGGA	Construction of Δ0114
0114Down*Bam*HI	GGGGGATCCCCCTCTCATAATCTTT CGCCAAG	Construction of Δ0114
0114extF	CAAGGAGTTTGAAACGAT	Confirmation of Δ0114
0114extR	CTCGCAGCAATAGACCAA	Confirmation of Δ0114
0114*Eco*RVF	CCCGATATCGGGACCGGTTAAAAAG GAGATTAC	Complementation of 17978 Δ114
0114*Bam*HIR	GGGGGATCCCCCCTGGTTCTAGTCG TGCAA	Complementation of 17978 Δ114
pWH1266Fw	TAGGCTTGGTTATGCCGGTA	Confirmation the complementation of the 17978Δ0114
pWH1266Rv	AAGGAGCTGACTGGGTTGAA	Confirmation the complementation of the 17978Δ0114
KanaR*Pst*IFw	CCCCTGCAGGGGCCGGAATTGCCAG CTGGGGCG	Cloning the kanamycin resistance gene into pWH1266 plasmid
KanaR*Pst*IRv	GGGCTGCAGCCCTCAGAAGAACTCG TCAAGAAG	Cloning the kanamycin resistance gene into pWH1266 plasmid
0112F	TTACTGCACCAAGGCCGAAT	Check expression of the operon
0113R	AATTTCCATGCGACCTCCGA	Check expression of the operon
0113F	GCTCGTATTGCTGTGTTGGG	Check expression

		of the operon
0115R	CTTACGCGCAGTAGCGGATA	Check expression of the operon
0115F	CCTCTCGATGCAGACCCATC	Check expression of the operon
0116R	CGTACCTTCCGGATGTGGTT	Check expression of the operon
0116F	CCGATATCCGTCCTTACGGC	Check expression of the operon
0117R	GGGTTTACAGTGTGGTCCGT	Check expression of the operon
0117F	GAGCTATGCTGCGTTATGCG	Check expression of the operon
0118R	TTGTCTGGGCGTCCGATAAT	Check expression of the operon
0118F	CTGGCGCAGGTCATAATCCA	Check expression of the operon
0119R	AATAAGGTCCGCGGAGTGAC	Check expression of the operon
csuA/B F	GCAGCTGTTACTGGTCAG	qRT-PCR
csuA/B R	GTCTGTGCGTTCACCACC	qRT-PCR
A1S_2091 F	GTCCACCATCAAATGACAAAGTCC	qRT-PCR
A1S_2091 R	CTGTGTCCTGAATACCTCAGC	qRT-PCR
A1S_1510 F	GATGTTGCTGGTCGTACACC	qRT-PCR
A1S_1510 R	GACATTGGTAGCTGCACCAG	qRT-PCR
A1S_0690 F	AAACAACCGCAACTCGTGG	qRT-PCR
A1S_0690 R	CAGCGGCGTCTTTAATACC	qRT-PCR
ompA F	CGACGCTTTATCTCTTCG	qRT-PCR
ompA R	GGAGCAGCAGGCTTGAAG	qRT-PCR
recA F	TACAGAAAGCTGGTGCATGG	qRT-PCR
recA R	TGCACCATTTGTGCCTGTAG	qRT-PCR

Table S2

HRMS data of Ac-505. Measured *m*/*z*-values are reported for MS fragment ions measured on ESI MaXis QTOF (LC-MS2) and 15T FT-ICR ECD (direct infusion) instruments in positive ion mode, respectively. The molecular formulas of the product ions are based on accurate mass and isotopic pattern matching of the ions as well as typical bond cleavage patterns for electrospray ionization.

Formula	Calc. (*m*/*z*)	Measured (*m*/*z*) MaXis QTOF	D ppm err.	Measured (*m*/*z*) 15T FT-ICR	D ppm err.
$C_{46}H_{87}N_6O_{14}S_2^+$	1011.57162	1011.5718	0.2		
$C_{23}H_{43}N_3O_7SNa^+$	528.27139	528.2718	0.8	528.27144	0.1
$C_{23}H_{44}N_3O_7S^+$	506.28945	506.2921	5.2	506.28945	0.0
$C_{23}H_{42}N_3O_6S^+$	488.27888	488.2813	5.0	488.27876	-0.2
$C_{23}H_{39}N_2O_6S^+$	471.25233			471.25221	-0.3
$C_{21}H_{37}N_2O_4S^+$	413.24686	413.2487	4.5	413.24676	-0.2
$C_{20}H_{37}N_2O_3S^+$	385.25194	385.2541	5.6	385.25187	-0.2
$C_{20}H_{34}NO_3S^+$	368.22539			368.22534	-0.1
$C_{18}H_{34}NO_3S^+$	344.22539			344.22536	-0.1
$C_{18}H_{32}NO_3^+$	310.23767	310.2390	4.3	310.23762	-0.2
$C_9H_{13}N_2O_6S^+$	277.04888	277.0502	4.8	277.04884	-0.1
$C_{16}H_{27}O^+$	235.20564	235.2064	3.2	235.20566	0.1
$C_5H_9N_2O_3S^+$	177.03284	177.0333	2.6	177.03286	0.1

Table S3

HRMS data of Ac-505 and MS2 fragments measured on a ESI MaXis QTOF (direct infusion) in negative ion mode.

Formula	Calc. (*m/z*)	Measured (*m/z*) MaXis QTOF	Δ ppm err.	Neutral Loss formula
$C_{23}H_{42}N_3O_7S^-$	504.27490	504.2750	0.2	
$C_{23}H_{40}N_3O_6S^-$	486.26433	486.2648	1.0	H_2O
$C_{18}H_{34}NO_4S^-$	360.22140	360.2213	-0.3	$C_5H_8N_2O_3$
$C_{18}H_{32}NO_3S^-$	342.21084	342.2121	3.7	
$C_9H_{14}N_3O_6S^-$	292.06088	292.0612	1.1	$C_{14}H_{28}O$
$C_4H_6NO_3S^-$	148.00739	148.0077	2.1	
$C_5H_7N_2O_3^-$	143.04622	143.0467	3.4	

SUPPLEMENTARY MATERIAL
CHAPTER III

Text S1

Additional details about 454 read data processing.

Processing of sRNA 454 sequencing reads

sRNA fractions, obtained from liquid *A. baumannii* ATCC 17978 cultures in the exponential and stationary phases of growth, plus biofilms were pyrosequenced at the Roche 454 Sequencing Center (Connecticut, USA) using GS FLX Titanium chemistry. The total number of reads was 689,097, 502,152 and 627,209 for the exponential, stationary and biofilm samples, respectively. FASTA formatted sequences and quality values extracted from SFF files were used as input for cross-match (Phred-Phrap-Consed package) to mask the sequence of the adapters used with the SREK kit (Table S1). Masked segments were then removed with Trimseq (Emboss package). No procedure to eliminate identical reads was performed, given that the pyrosequencing libraries consisted of short cDNA sequences representing, in principle, full-length sRNAs and the presence of identical reads was expected.

Pre-processed reads were aligned with cross-match against the *A. baumannii* ATCC 17978 chromosome and plasmid nucleotide sequences (Genbank: NC_009085, NC_009083 and NC_009084). Alignments were filtered with an *ad-hoc* Perl script (crossMatchParser) to recover, for each read, those alignments with the highest score and fulfilling the minimal requirements of 90% identity and 15-base pair length. Between 32 % and 43 % of pre-processed reads could be aligned for each sample (Table S2). The total number of filtered alignments was around twice the number of aligned reads, indicating that a significant number of reads could align to more than one location with the same score.

Quantification of the expression level for known sRNA genes

To estimate the expression level for known sRNA genes described for *A. baumannii* ATCC 17978, which included 74 genes coding for 5S rRNAs and tRNA, filtered alignments were processed with the *ad-hoc* script mapAlignHits, which in “expression mode” calculated the average coverage for sets of target sequence segments defined by gene coordinates. Relative abundance (RA) values were also calculated for known protein

coding genes and for 16S and 23S rRNA genes (Table S3). Reads aligning to multiple locations with identical score contributed to the coverage of each region with an amount that was inversely proportional to the number of locations. The resulting average coverage values were normalized to obtain relative abundance (RA) values, by dividing them by the total number of mapped reads for each sample, and multiplying them by the number of mapped reads for the exponential phase sample, which was defined as the reference condition. tRNA and 5S rRNA genes had RA values of 1,092 to 1,338, in average, for the three samples, which were more than 1,000-fold and about 20-fold higher than RA values calculated for protein coding genes and 16S and 23S rRNA genes, respectively (Table S4 and Figure S1), suggesting that sRNA fractions isolated as described in Methods were significantly depleted of mRNA and rRNA. A1S_2909, coding for Leu tRNA, was the known sRNA gene expressed at the highest level, with a RA value of 18,693 in the stationary phase sample. In contrast, A1S_2764, coding for Arg tRNA, was the known sRNA gene expressed at the lowest level, with a RA value of 7.6 in the exponential phase sample.

Identification of new sRNA gene candidates

To identify new sRNA gene candidates, filtered alignments were combined into a single file and processed with script mapAlignHits, which, in “detection mode” identified continuous read-covered regions, with a minimal average coverage of 1, in target sequences (chromosome and plasmids). A total of 26,956 read-covered regions (exprRegs) were detected. To identify read-covered regions overlapping with known genes, the *ad-hoc* script FindOlappingFeatures was used to compare their coordinates with the coordinates of 3,451 known protein, tRNA and rRNA coding genes. The minimal overlap required to consider two features as overlapping was set to 0.001 % of the length of any of the features. A total of 21,392 exprRegs were found to overlap with already described genes. The remaining 5,564 exprRegs (Dataset S1) were considered as new potential sRNA genes. However, since the coordinate set used to define known protein coding genes referred exclusively to coding regions, some of the 5,564 exprRegs could correspond to 5' or 3' UTRs of genes that had not been covered by a single, continuous alignment, spanning the whole length of the transcribed region. New sRNA gene candidates were identified both in chromosome and plasmids (5,505 and 59 genes, respectively). Their average length was 63.39 base pairs (Figure S2).

Figure S1

Distribution of normalized expression scores for known sRNA genes. Blue: biofilm samples. Orange: exponential phase samples. Yellow: stationary phase samples.

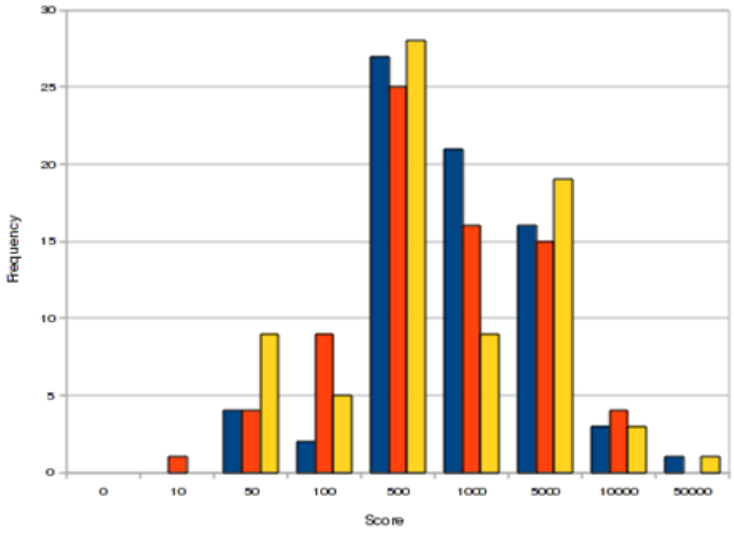


Figure S2

Distribution of normalized expression scores for the 5564 expressed regions not overlapping with known genes. Blue: biofilm samples. Orange: exponential phase samples. Yellow: stationary phase samples.

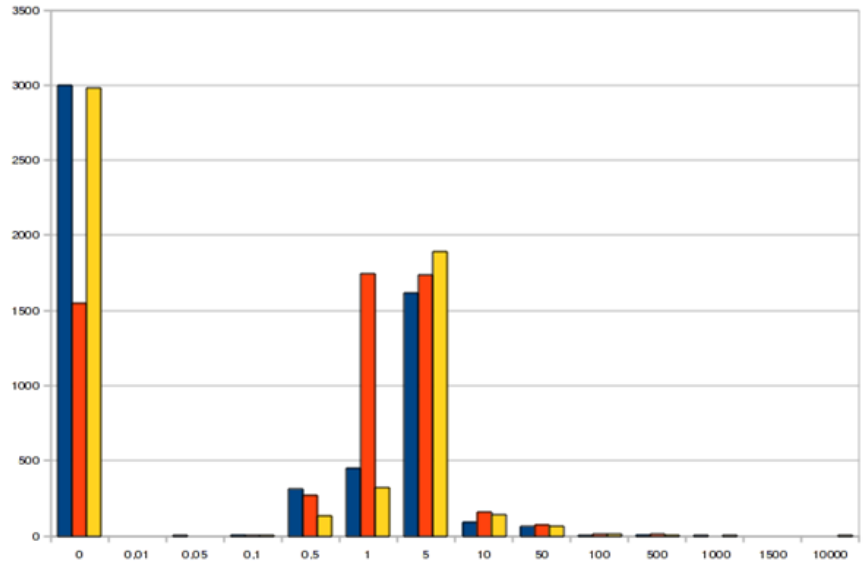


Figure S3

Distribution of normalized expression scores for the 255 expressed regions not overlapping with known genes and having a normalized expression score equal or higher than 7.6 in some of the growing conditions. Blue: biofilm samples. Orange: exponential phase samples. Yellow: stationary phase samples.

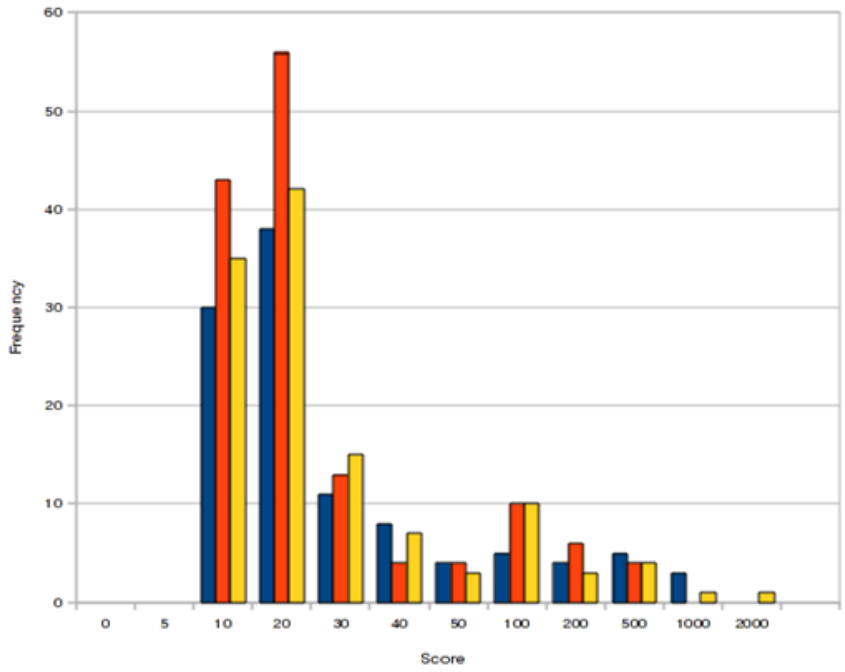


Figure S4

qRT-PCR assays. Expression levels of 8 sRNA regions in planktonic and sessile cells determined by qRT-PCR using Taqman probes. Y axis represents the relative expression of the genes taking the housekeeping gene *gyrB* as value 1.

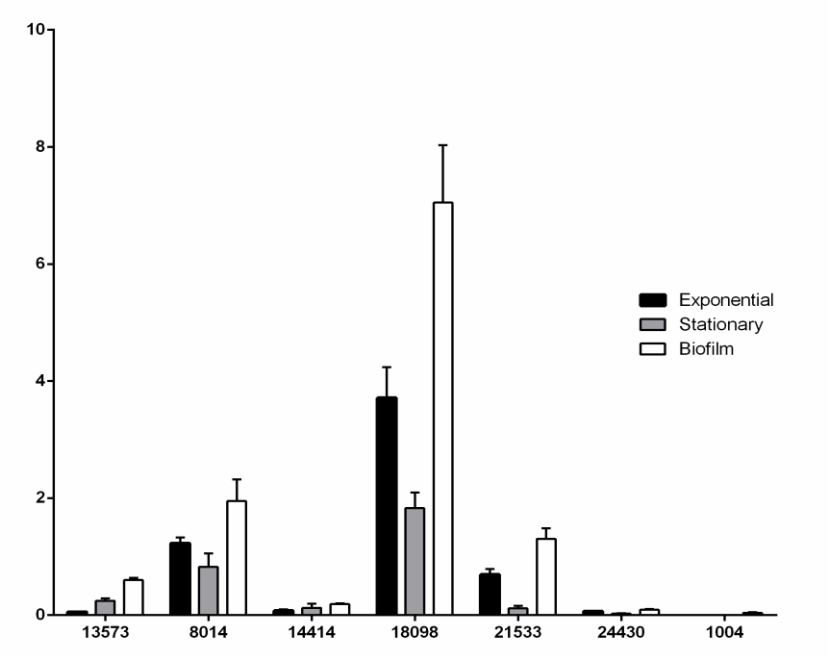


Figure S5

Biofilm formation at different times. Staining at 12, 24 and 48 h of biofilm formation of *A. baumannii* ATCC 17978 (17978), *A. baumannii* ATCC 17978 Δ13573 (Δ13573), *A. baumannii* ATCC Δ13573 harbouring pETRA with sRNA 13575 (Δ13573 complemented), *A. baumannii* ATCC 17978 harbouring pETRA with sRNA 13575 (13573), and *A. baumannii* ATCC 17978 harbouring pETRA (17978 with empty pETRA).

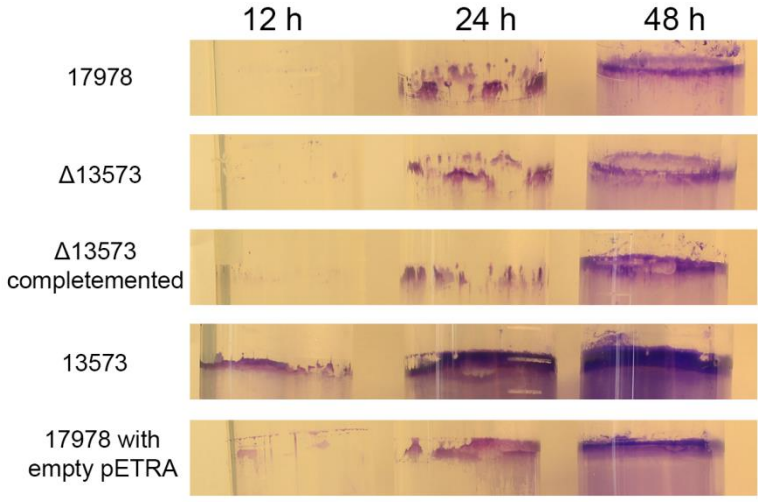


Figure S6

Growth curves. Growth curves of wild type *A. baumannii* (17978), *A. baumannii* ATCC 17978 lacking the sRNA 13573 (Δ13573), *A. baumannii* Δ13573 over-expressing sRNA 13575 (Δ13573 complemented), *A. baumannii* ATCC 17978 harbouring pETRA over-expressing sRNA 13575 (13573) and *A. baumannii* ATCC 17978 harbouring the empty pETRA vector (17978 with empty pETRA).

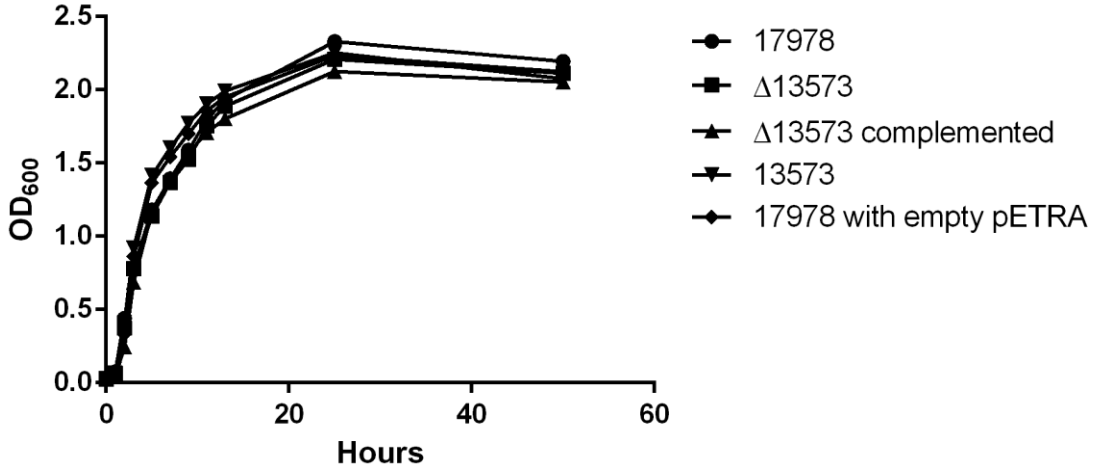


Table S1

Oligonucleotides and probes used in the present study.

Oligonucleotide name	Oligonucleotide sequence	Use of the oligonucleotides
13573 F	ccctctagagggattattatgactgcttaaatc	Over-expression of sRNA 13573 in pETRA
13573 R	gggccatggcccaataggttagtaaggtaataa	Over-expression of sRNA 13573 in pETRA
KM F	cccctgcaggggccggaattgccagctggggcg	Cloning kanamycin resistance cassette in pETRA
KM R	gggctgcagccctcagaagaactcgtcaagaag	Cloning kanamycin resistance cassette in pETRA
SREK 1	ctgccccgggttcctcattctctgcggtcctgctgta	Construction of cDNA

	cggccaaggcg	libraries SREK (Ambion)
SREK 2	ccactacgcctccgctttcctctctatgggcagtcggtgat	Construction of cDNA libraries SREK (Ambion)
13573BUpFNotI	cccgcggccgcgggttggtcaaaggtgtgaaaatgt	Construction of the knockout mutant of sRNA 13573
13573 UpR3 BamHI	gggggatcccccactaaagtatctatttgggtgtacg	Construction of the knockout mutant of sRNA 13573
13573 DownF3 BamHI	cccggatccgggaggatcggttattgaatcag	Construction of the knockout mutant of sRNA 13573
13573DownRSphI	ggggcatgcccccaagtcacaagcaccttcttt	Construction of the knockout mutant of sRNA 13573
13573extF2	tataaggtgtaagcacgctg	Checking of the knock-out mutant of sRNA 13573
13573extR2	tcacgagacaagagatgaac	Checking of the knockout mutant of sRNA 13573

Table S2

Number of initial reads, mapped reads and alignment hits for each sample.

Sample	Number of Reads	Number of Mapped Reads	Number of Hits
Exp	689,097	298,048 (43%)	691,710
Sta	502,152	176,003 (35%)	429,806
Bio	627,209	204,637 (32%)	505,760

Biofilm: Bio. Exponential phase of growth: Exp. Stationary phase of growth: Sta.

Table S3

A. baumannii known sRNA and non-sRNA genes used as reference.

Gene set	Subset	Number of genes
rRNA 5S	Known sRNA	5
tRNA	Known sRNA	69
rRNA 16S and 23 S	Known, non sRNA	10
Protein coding	Known, non sRNA	3,367

Table S4

Average, standard deviation, maximal and minimal values for the normalized expression scores calculated for protein coding genes, 16S and 23S rRNA genes and known sRNA genes, in each of the growing conditions.

	Protein Coding			16S, 23S			tRNA, 5S		
	BioN	ExpN	StaN	BioN	ExpN	StaN	BioN	ExpN	StaN
Ave	0.62	1.05	1.15	57.27	35.41	61.07	1,260.37	1,092.71	1,338.55
Std	1.03	1.27	1.32	1.72	3.09	16.6	2,719.95	1,797.35	2,689.99
Max	20.34	26.49	27.48	59.15	38.44	77.3	18,375.78	9,944.99	18,693.1
Min	0	0	0	55.65	32.08	45.3	15.9	7.6	14.93

Biofilm: BioN. Exponential phase of growth: ExpN. Stationary phase of growth: StaN. Average: Ave. Standard deviation: Std. Maximal values: Max. Minimal values: Min.

Table S5

Average, standard deviation, maximal and minimal values for the normalized expression scores calculated for 5564 expressed regions not overlapping with known genes.

	Bio	Exp	Sta
Ave	1.83	2.01	2.06
Std	20.62	12.96	27.64
Max	826.28	484.14	1,728.21
Min	0	0	0

Biofilm samples: Bio. Exponential phase of growth samples: Exp. Stationary phase of growth samples: Sta. Average: Ave. Standard deviation: Std.

Table S6

Sets of expression regions not overlapping with known genes and having a normalized expression score equal or higher than 7.6 in some of the three growing conditions.

	Bio	Exp	Sta
Number of expression regions	108	140	121
Average normalized score	57.88	35.64	54.53
Standard deviation	137.16	74.12	180.27
Maximal normalized score	826.28	484.14	1,728.21
Minimal normalized score	7.74	7.6	7.7

Dataset S1

Expressed regions. List of 5,564 expressed regions, their length and locations in the genome (NC_009085.1) or plasmids (NC_009083.1 and NC_009084.1) of ***A. baumannii*** ATCC 17978.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5538643/bin/pone.0182084.s008.docx

Dataset S2

Coding regions. List of normalized expression of 255 coding regions (expression score should be equal or above 7.6 in some growing condition: Bio, Exp or Sta), their length and locations in the genome (NC_009085.1) or plasmid (NC_009084.1) of ***A. baumannii*** ATCC 17978.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5538643/bin/pone.0182084.s009.docx

SUMMARY

Un análisis transcripcional revela nuevos factores relacionados con la capacidad de formar biopelículas de *Acinetobacter baumannii*.

Las enfermedades infecciosas se consideran un importante problema de salud pública tanto en países desarrollados como en países en vías de desarrollo. La adaptación de las bacterias a condiciones de estrés, debido en parte al mal uso de los antibióticos, ha propiciado la aparición de cepas multi-resistentes. Esto es especialmente peligroso en el ambiente hospitalario, donde los pacientes inmunocomprometidos son muy vulnerables.

Los patógenos que en la actualidad causan un mayor número de infecciones hospitalarias y que son capaces de "escapar" a la acción de los antimicrobianos se engloban dentro del término ESKAPE. En este grupo está incluido *Acinetobacter baumannii*, un cocobacilo Gram-negativo que se aísla frecuentemente a partir de equipos médicos y pacientes hospitalizados. La habilidad de esta bacteria de desarrollar factores de virulencia y resistencia constituye la clave de su éxito como patógeno humano.

A. baumannii causa diversos tipos de infecciones, siendo predominantes las de tracto respiratorio. Los pacientes con ventilación mecánica o que han sufrido cirugías son especialmente sensibles a este tipo de infecciones. Las tasas de mortalidad asociadas a infecciones producidas por *A. baumannii* son significativamente superiores cuando las cepas son resistentes a los carbapenemes.

La plasticidad genética de *A. baumannii* le permite adquirir genes de resistencia de otras especies y desarrollar mutaciones para adaptarse a condiciones de estrés. La rápida emergencia de cepas de *A. baumannii* resistentes a todos los β-lactámicos, incluyendo los carbapenemes, explica el potencial de este microorganismo para responder a la presión selectiva del ambiente. Se han detectado infecciones por cepas de esta especie resistentes a todos los antibióticos utilizados en clínica, lo que dificulta enormemente su tratamiento.

Además, el éxito de *A. baumannii* se puede atribuir a otros factores como su capacidad para formar biopelículas y resistir a la desecación en superficies abióticas. La biopelícula es una compleja estructura formada por una comunidad de células sésiles embebidas en una matriz producida por ellas mismas. Las bacterias que forman parte de las biopelículas presentan una serie de características diferentes a las células bacterianas que se encuentran en un modo de vida planctónico. Así, las bacterias asociadas a las biopelículas

poseen mayor resistencia a los antibióticos, a los desinfectantes y a componentes del sistema inmune de los hospedadores.

La alta capacidad de *A. baumannii* de adherirse a superficies abióticas y bióticas origina el primer paso para la colonización y suele ir acompañado del desarrollo de biopelículas. Esta habilidad para adherirse puede variar entre diferentes cepas y está influida por diversos factores, tales como la disponibilidad de nutrientes, los componentes de la superficie bacteriana u otros factores ambientales.

Se han identificado varios genes que participan en la adhesión y la capacidad de formar biopelículas en *A. baumannii*. Los pili tipo I, unas estructuras proteicas muy frecuentes en la superficie celular de numerosos patógenos, suelen estar involucrados en ambos procesos en bacterias Gram-negativas. En *A. baumannii* se han identificado varios operones que codifican pili tipo I, cuya inactivación causa un descenso en la producción de biopelículas y en la adherencia.

Otras muchas proteínas asociadas a la membrana bacteriana están involucradas en estos procesos. Así, la proteína autotransportadora Ata, un sistema de secreción tipo V, también es necesaria para el desarrollo de biopelículas, la adhesión bacteriana y la supervivencia de *A. baumannii* durante la infección. La adhesina Bap (*biofilm-associated protein*) es un factor importante para la formación de una biopelícula madura y para la adherencia a células epiteliales humanas. También la porina de la membrana externa OmpA está implicada en la adherencia a plástico y a células eucariotas, además de inducir la apoptosis en células epiteliales.

Los mecanismos de comunicación entre bacterias, o *quorum sensing*, también están relacionados con la capacidad de adherencia y la formación de biopelículas. Las bacterias Gram-negativas utilizan acil-homoserina-lactonas como moléculas señalizadoras. En *A. baumannii* la presencia de estas moléculas provoca un aumento en la densidad de la biopelícula y la degradación de las acil-homoserín-lactonas interrumpe su formación.

La cantidad de biopelícula formada por *A. baumannii* también se ve alterada por señales ambientales diversas como la temperatura, la luz, la concentración de hierro disponible o la superficie sobre la que se forma.

Los procesos biológicos bacterianos están regulados a distintos niveles. Existen pequeñas moléculas de RNA (sRNAs) que actúan como reguladores modulando la transcripción, la traducción y la estabilidad del RNA mensajero (mRNA). Los sRNAs ejercen su acción mediante diferentes mecanismos; provocando cambios en la conformación del RNA, uniéndose a proteínas o a otros RNAs por emparejamiento de bases o interaccionando con el DNA. Los sRNAs mejor caracterizados son los que regulan la expresión génica mediante emparejamiento de bases con el mRNA.

La formación de biopelículas es uno de los procesos biológicos más complejos que llevan a cabo las bacterias, por lo que su activación debe responder de manera precisa a cambios ambientales. Aparte de la regulación transcripcional que controla la producción de biopelículas, existe una regulación post-transcripcional mediada por sRNAs que permite a la bacteria adaptarse rápidamente a las variaciones del entorno. Se han identificado numerosos sRNAs capaces de integrar distintas señales y actuar como reguladores que controlan la formación de biopelículas, especialmente en *Escherichia coli*. Además, muchos de estos sRNAs participan también en la coordinación de diversos procesos como el metabolismo celular, la virulencia o la resistencia a antibióticos.

En la actualidad la información sobre la regulación mediada por sRNAs en *A. baumannii* es escasa. Se han identificado una serie de sRNAs, mediante métodos bioinformáticos y de secuenciación masiva, pero no se conocen los procesos biológicos en los que podrían estar implicados.

En el presente trabajo se realizó un análisis transcripcional empleando técnicas de secuenciación masiva usando como material de partida la totalidad de las moléculas de mRNA y de sRNA extraídas a partir de células de la cepa *A. baumannii* ATCC 17978 tanto asociadas a biopelículas como planctónicas (en fases de crecimiento exponencial y estacionaria). Esto ha permitido la identificación de varios nuevos factores implicados en el proceso de formación de biopelículas.

Los resultados obtenidos mostraron que, aunque muchos genes se expresaban de manera constitutiva tanto en biopelículas como en células planctónicas, muchos otros presentaban una expresión diferencial en células sésiles con respecto a las bacterias plantónicas. Un total de 1621 genes aparecieron sobre-expresados en las células asociadas a biopelícula con respecto a las células planctónicas. Además, 55 genes se detectaron exclusivamente

en células sésiles, estando totalmente inhibidos en células planctónicas. Una vez clasificados los genes sobre-expresados en las biopelículas en función de los procesos biológicos en los que participaban, se observó que el grupo mayoritario era el implicado en regulación transcripcional. Otros grupos de genes sobre-expresados resultaron estar relacionados con el metabolismo de aminoácidos, el metabolismo de ácidos grasos, el transporte de iones, la biosíntesis de carbohidratos o la respuesta al estrés.

Muchos de estos genes codifican proteínas cuya función ya se había relacionado previamente con procesos de formación y mantenimiento de las biopelículas, mientras que otros se detectaron en este trabajo por primera vez. Entre estos genes relacionados con la formación de biopelículas en *A. baumannii*, aparecieron varios genes del operón *csuA/BABCDE* y el gen *bfmR*, que forma parte de un sistema regulador de dos componentes y controla la expresión de los genes de dicho operón. Asimismo, se observó la sobre-expresión de genes relacionados con la formación de pili; como el gen A1S_1507, que codifica una proteína de una fimbria, y el gen A1S_3168, que codifica la proteína de formación de pilus PilW.

Un importante número de genes relacionados con el transporte y la captación de hierro se encontraron sobre-expresados en las células asociadas a biopelículas en comparación con las células planctónicas. Así, se encontraron, entre otros, un receptor de una enterobactina, una proteína de unión a hierro, un receptor de una acinetobactina, una proteína de síntesis de un sideróforo o una proteína de síntesis de acinetobactina. Además, algunos de estos genes, como los relacionados con la acinetobactina (A1S_2380 y A1S_2388), estaban exclusivamente expresados en biopelículas y no se detectaron en las muestras de células plantónicas.

Otro factor muy implicado en la formación de biopelículas es la proteína Bap que, en *A. baumannii* ATCC 17978, se encuentra codificada por dos loci homólogos, los genes A1S_2724 y A1S_2696. Ambos genes se encontraron sobre-expresados en células sésiles en comparación con células planctónicas, por lo que se sugiere que las proteínas codificadas por estos genes podrían estar implicadas en la formación de biopelículas en la cepa ATCC 17978.

La homoserín-lactona sintasa, codificada por el gen A1S_0109, responsable de la síntesis de la acil-homoserín-lactona, y que ha sido descrita como necesaria para la formación de

biopelículas, presentó unos niveles de expresión mayor en biopelículas. Asimismo, el grupo de genes contiguo a esta sintasa (A1S_0112-A1S_0118) resultaron estar también sobre-expresados en células sésiles, destacando entre ellos el gen A1S_0114, que codifica una proteína transportadora de grupos acilo, y que presentó los niveles de expresión más altos detectados en células asociadas a biopelículas (de orden de 120 veces más).

Se seleccionaron 5 genes entre los genes sobre-expresados en biopelículas. Estos fueron los genes A1S_1507, A1S_3168, A1S_2042, A1S_0302 y A1S_0114 que codifican una proteína de fimbria, una proteína de un pilus, un regulador transcripcional, una proteína hipotética y un transportador de grupos acilo, respectivamente. Mediante RT-qPCR se confirmaron los resultados obtenidos a partir del análisis transcripcional masivo y se elaboraron cepas mutantes por disrupción de los genes de interés con el plásmido pCR-Blunt-II-TOPO. El análisis de la formación de biopelículas de las cepas mutantes reveló un descenso en el caso de las cepas mutantes con respecto a la cepa salvaje *A. baumannii* ATCC 17978, confirmándose el papel de estos genes en el proceso de formación de biopelículas.

Se elaboró un mutante estable libre de marcadores de resistencia a antibióticos eliminando permanentemente el gen A1S_0114. El mutante estable, que carece del gen A1S_0114, mostró una capacidad para formar biopelículas unas tres veces menor a la de la cepa salvaje. Además, este mutante estable permitió analizar la relación del gen A1S_0114 con los genes próximos A1S_0109, A1S_0112 y A1S_0113, que codifican una homoserín-lactona sintasa, una acil-CoA sintasa y una acyl-CoA deshidrogenasa, respectivamente. Mediante qRT-PCR se demostró que en ausencia del gen A1S_0114, la expresión de los otros genes descendía en células en fase estacionaria de crecimiento. Estos resultados sugieren que el gen A1S_0114 podría estar involucrado en la formación de biopelículas a través de mecanismos de *quorum sensing*, aunque se postula que su actividad como donador de grupos acilo podría estar también relacionada con el metabolismo de ácidos grasos.

Con el fin de caracterizar el gen A1S_0114 y conocer su papel en la patogénesis y de establecer su relación con los genes adyacentes se realizaron una serie de análisis genotípicos y fenotípicos.

Tanto los datos del análisis transcriptómico masivo como un análisis *in silico* del grupo de genes A1S_0112-0119 sugerían que estos genes podrían estar actuando como un operón policistrónico, ya que presentaban niveles de sobre-expresión semejantes en células asociadas a biopelículas, estando localizados próximos en la misma orientación. Se confirmó la naturaleza policistrónica del operón mediante técnicas de RT-PCR utilizando oligonucleótidos que conectaban todos los genes.

Se llevaron a cabo ensayos de adherencia a células epiteliales alveolares humanas A549 para evaluar los efectos biológicos causados por la eliminación del gen A1S_0114. Estos experimentos mostraron que la cantidad de bacterias de la cepa mutante recuperadas era un 60% inferior a los recuentos de células alveolares infectadas con la cepa salvaje ATCC 17978. Cuando el mutante estable se complementaba con el plásmido pWH1266-Km portador de una copia del gen A1S_0114, la capacidad de adherencia de la cepa mutante se restauraba parcialmente.

Los resultados obtenidos en los experimentos de adherencia a células alveolares humanas se confirmaron mediante microscopía electrónica de barrido. El análisis de células A549 polarizadas infectadas corroboraron el papel del gen A1S_0114 en la adherencia a superficies bióticas. Las células A549 infectadas con la cepa salvaje *A. baumannii* ATCC 17978 presentaban importantes daños tanto a nivel celular como en la capa de surfactante que las recubre. Además, se detectó un gran número de bacterias adheridas a la superficie de las células alveolares. Por su parte, la infección causada por la cepa mutante provocó importantes daños celulares y en la capa de surfactante, pero apenas se detectaron bacterias adheridas a la superficie de las células polarizadas.

También se utilizó la microscopía electrónica de barrido para respaldar los resultados de formación de biopelículas. Tal y como se esperaba, las micrografías mostraron una mayor capacidad de las células de la cepa salvaje ATCC 17978 para adherirse a la superficie abiótica y configurar estructuras multicelulares y tridimensionales, que se asocian con la formación de biopelículas maduras. Por el contrario, sólo se detectaron pequeñas agrupaciones no tridimensionales de bacterias en el caso de la cepa mutante.

Debido a que la eliminación del gen A1S_0114 afecta a la capacidad de *A. baumannii* para adherirse y formar biopelículas, se realizó un análisis de los niveles de expresión de genes relacionados con estas funciones. Se seleccionaron el gen que codifica la proteína

de membrana OmpA y los genes asociados a sistemas de pili *csuA/B,* A1S_0690, A1S_1510 y A1S_2091. La RT-qPCR comparativa entre la cepa salvaje ATCC 17978 y el mutante estable demostró que la expresión de los genes de pili se reducía de forma estadísticamente significativa en la cepa carente del gen A1S_0114, excepto en el caso de *csuA/B,* cuya expresión aumentaba en la cepa mutante. Sin embargo, este último dato podría no ser muy relevante, ya que el gen *csuA/B* parece estar truncado en la cepa ATCC 17978. La eliminación del gen A1S_0114 también causó una pequeña reducción de la expresión del gen *ompA.*

El papel que juega el gen A1S_0114 en virulencia se evaluó mediante diferentes modelos animales: un ensayo de fertilidad en *Caenorhabditis elegans*, un experimento de supervivencia en la oruga *Galleria mellonella* y un modelo de neumonía en ratón. El estudio de fertilidad en *C. elegans* demostró que el número de huevos viables era casi dos veces superior en gusanos infectados con la cepa mutante en comparación con la cepa salvaje ATCC 17978. Un 50% de las larvas de *G. mellonella* infectadas con la cepa salvaje murieron a los 5 días de ser infectadas, mientras que el porcentaje de muerte entre las larvas infectadas con el mutante estable fue significativamente menor. El modelo de neumonía mostró que la carga bacteriana en los pulmones de ratones infectados con la cepa mutante era significativamente inferior a la de los animales infectados con la cepa salvaje ATCC 17978. Los resultados de estos experimentos evidencian que el gen A1S_0114 contribuye a la virulencia de *A. baumannii*.

Debido a la implicación del gen A1S_0114 en virulencia y a la predicción de que los genes del operón A1S_0112-0119 podrían actuar como una péptido sintasa no ribosomal, se realizó una búsqueda de compuestos que podrían estar producidos por este operón mediante cromatografía líquida/espectrometría de masas. Dicho análisis mostró la presencia de un compuesto de peso molecular de 505,28 Da presente en las muestras de la cepa salvaje ATCC 17978, no detectado en el caso de la cepa mutante. La fórmula molecular de dicho compuesto, que hemos denominado Acinetina 505, es $C_{23}H_{43}N_3O_7S$ y resultó ser semejante a un lipopéptido de tres aminoácidos.

Los datos demuestran que el gen A1S_0114 afecta a la formación de biopelículas, a la adherencia y a la virulencia de *A. baumannii*. Sin embargo, todavía no hemos conseguido desentrañar el mecanismo mediante el cual se regulan estos procesos y el papel de la

Acinetina 505 en dicha regulación. . Ahondar en estos aspectos podría aportar importantes datos sobre la patobiología de *A. baumannii* y facilitar el desarrollo de herramientas alternativas para el tratamiento de infecciones causadas por este patógeno.

A continuación, se utilizaron técnicas de secuenciación masiva para caracterizar el transcriptoma global a nivel de sRNAs en células sésiles y planctónicas de *A. baumannii* ATCC 17978. Se compararon los transcriptomas de sRNA de células planctónicas obtenidos en fases de crecimiento exponencial y estacionario y de células asociadas a biopelículas para identificar diferencias en los perfiles de expresión de sRNAs.

Se identificaron un total de 5.564 presuntos sRNAs, fragmentos de RNA no coincidentes con ningún gen previamente descrito ni con RNA transferentes ni ribosómicos. Un total de 255 de estos sRNAs se describieron como expresados diferencialmente en alguna de las tres condiciones de crecimiento. Todos ellos estaban localizados en el cromosoma bacteriano excepto uno, localizado en el plásmido pAB2. En células asociadas a biopelículas, 28 sRNAs estaban sobre-expresados mientras que 32 tenían una expresión menor que en células planctónicas. Uno de ellos, el sRNA 13573, mostró unos valores de sobre-expresión de unas 120 veces más en biopelículas con respecto a células planctónicas. Además, 9 sRNAs fueron detectados únicamente en células de biopelículas mientras que 21 se expresaron exclusivamente en células planctónicas. Los resultados de secuenciación masiva de 6 de los sRNAs únicamente expresados en biopelículas y del sRNA 13573 se corroboraron mediante RT-qPCR.

El alto nivel de expresión del sRNA 13573 en células sésiles sugería su posible papel en la regulación de las biopelículas. Se elaboró una cepa mutante estable que carecía del sRNA 13573, así como una cepa que sobre-expresaba el sRNA 13573 desde un plásmido, con el fin de dilucidar el papel de este sRNA en la patogénesis de *A. baumannii*. Los experimentos de formación de biopelículas mostraron que la falta del sRNA 13573 provocó una pequeña pero no significativa reducción de la producción de biopelícula. Sin embargo, la cepa que sobre-expresaba el sRNA 13573 duplicó la formación de biopelícula con respecto a la cepa salvaje ATCC 17978. Las imágenes de microscopía electrónica de barrido mostraron que las células que sobre-expresaban el sRNA 13573 eran capaces de desarrollar biopelículas con estructuras tridimensionales, a diferencia de

la cepa salvaje que presentaba estructuras más simples. Las células de la cepa mutante mostraron un patrón más desorganizado que las de la cepa ATCC 17978.

Los ensayos de adhesión a células eucariotas epiteliales A549 indicaron que la sobre-expresión del sRNA 13573 provocaba un aumento de la adherencia de unas 30 veces superior a la cepa salvaje. La capacidad de adherirse disminuyó en la cepa que carecía del sRNA 13573, pero este efecto se restableció en la cepa mutante complementada, portadora de una copia del sRNA 13573 en el plásmido pETRA, alcanzando valores superiores a la cepa salvaje. Un análisis por microscopía electrónica de barrido de células alveolares humanas polarizadas infectadas con las distintas cepas reveló que la sobre-expresión del sRNA 13573 confería a la bacteria una espectacular capacidad de formar biopelículas con estructura tridimensional sobre las células epiteliales. Tal y como se esperaba, la cepa mutante mostró una menor adherencia a las células eucariotas que la cepa ATCC 17978.

Los datos recogidos en este trabajo indican que el transcriptoma de sRNAs presenta un patrón de expresión diferente en células asociadas a biopelículas en comparación con células planctónicas. Además, se ha confirmado que el sRNA 13573, altamente expresado en células asociadas a biopelículas, está implicado en la formación de biopelículas así como en la adherencia a superficies tanto abióticas como bióticas.

En conclusión, en el presente trabajo se ha demostrado que el patrón de mRNA de las células asociadas a biopelículas de *A. baumannii* ATCC 17978 es significativamente diferente al de las células planctónicas. Se han identificado una serie de genes que participan en la regulación de las biopelículas, destacando entre ellos el gen A1S_0114, que codifica una proteína transportadora de grupos acilo, y que juega un importante papel en adherencia a superficies bióticas y abióticas, virulencia y biosíntesis del metabolito Acinetina 505. Además, se han descrito un importante número de sRNAs en la cepa *A. baumannii* ATCC 17978, encontrándose muchos de ellos expresados de forma diferencial en células sésiles con respecto a células en fase exponencial o estacionaria. Entre los nuevos sRNAs descritos, se encontró el sRNA 13573, demostrándose su implicación en la regulación de la formación de biopelículas y en la adherencia a células eucariotas. Este trabajo sirve como base para futuros estudios que traten de desentrañar la

compleja red que regula tanto la formación de biopelículas como otros procesos biológicos asociados a la patogénesis de *A. baumannii*.

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Participation in Research Projects

2012-2014	Genomics and transcriptomics of *Acinetobacter baumannii* biofilm. Health Institute Carlos III (ISCIII). **Margarita Poza. PI11/01034.**
2016-2019	Identification of the mechanism of action of a novel series of compounds active against *Acinetobacter baumannii*. ABAC Therapeutics. **Germán Bou. Ab-S1-004-2016.**
2018-2020	Evaluation of novel antimicrobian strategies by RNA silencing transported in nanocapsules and enzymatic inhibitors. Health Institute Carlos III (ISCIII). **Margarita Poza and Alejandro Beceiro. PI17/01482.**

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