Study of tolerance, persistence and resistance mechanisms in nosocomial pathogens: search of new treatments targets

Laura Fernández García

Tesis doctoral UDC

Mayo / 2019

UNIVERSIDADE DA CORUÑA

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Memoria presentada por la Licenciada Laura Fernández García para optar al grado de Doctor en Ciencias de la Salud

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Y para que así conste, y surta los efectos oportunos, firmamos el presente certificado en A Coruña, 14 de Mayo del 2019.

Dra. María del Mar Tomás Carmona	Dr. Germán Bou Arévalo
Directora	**Tutor**

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And for this to be recorded, and take the appropriate effects, we sign this certificate in A Coruña, May 14, 2019.

Dra. María del Mar Tomás Carmona — **Director**

Dr. Germán Bou Arévalo — **Tutor**

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ABREVIATIONS

%	Percentage
(p)ppGpp	Guanisil (penta) tetrafosfate
µg	Micrograme
µl	Microliter
2-AA	2-amino-acetophenone
3-Hidroxy-C12-HSL	3-hidroxy-dodecanoyl-homoserine lactone
3-Oxo-C12-HSL	3-oxo-dodecanoyl-homoserine lactone
Ab	*Acinetobacter baumannii*
agr	Accesory gene regulator
AHL	Acyl-homosernine lactone
AI	Autoinducer
AiiA	Autoinducer inactivator
ANI	Average nucleotide identity
ATP	Adenosine tri-phosphate
bp	Base pair
BLUF	Blue light using flaving
BVC	Bacterial volatile compounds
C	Centigrades
CA	California
CcpA	Catabolism control protein A
CDS	Coding sequence
CEFOBI	*Centro de Estudios Fotosinteticos y Bioquimicos de Rosario*
CFU	Colony formation units
CHDL	Carbapenem-hydrolycing class D β-lactamase
CHLX	Chlorhexidine
CHUAC	University Hospital Center of A Coruña
CLSI	Clinical and Laboratoy Standars Institute
CRAb	Carbapenem-resistant *Acinetobacter baumannii*
DMSO	Dimetyl sulfoxide
DNA	Desoxiribonucleic acid
DSF	Diffusible signal factor
EDF	Extracellular death factor
EE.UU	United states of America
FOW	Forward
g	Grame
GEIH	Hospital Infection Study Group
GKL	*Geobacilluskaustophilus* thermostable lactonase
GTPase	Guanosil tri-fosfatase
h	Hours
H-NS	Histone-like nucleoid-structuring
Hod	3-hidroxy-4-oxoquinaldine
HSL	Homosernine lactone
IBIS	Biomedical Institue of Sevilla
ICU	Intensive care units
IDSA	Infectious Diseases Society of America

IF2	Initiation factor 2
IL	Ilinois
IMP	Imipenem
INIBIC	Institute for Biomedical Research of A Coruña
IPTG	Isopropil-β-D-1-tiogalactopiranósido
IS	Insertion sequences
KEGG	Kyoto encyclopedia of genes and genomes
L	Litre
LN-LB	Low nutrient Luria Bernati
log	Logaritm
MALDI-TOFMS	Matrix-assisted laser desortion ionization-time of flight mass spectrometry
MBL	Class B metalo-β-lactamase
MDR	Multiresistance
Mg	Magnesio
MIC	Minimal inhibitori concentration
ml	Mililitre
MLST	Multilocus sequence typing
mM	Milimolar
MMC	Mitomycin C
ng	Nanograme
nm	Nanometres
Ntn	N-terminal nucleophile
OD600	Optical density 600
OHHL	N-(3-oxo-hexanoyl)-L-homoserine lactone
ORF	Open reading frame
PBS	Phosphate buffer saline
PCD	Programmed cell death
PDB	Protein data bank
Pn-B	Bacteremic pneumonia
Pn-NB	Non-bacteremic pneumonia
ppX	Exopolyphosphatase
PQS	*Psesudomona* quinolone signal
PTE	Phosphotriesterases
QN	Quorum network
QQ	Quorum quenching
QS	Quorum sensing
REIPI	Spanish Network for Research in Infectious Diseases
REV	Reverse
RE	Relative expresión
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell división
ROS	Reactive oxygen species
rpm	Revolutions per minute
rpoB	RNA polymerase beta subunit
rRNA	Ribosomic ribonucleic acid
RSH	RelA/spoT homologue
RTq-PCR	Real time quantitative reverse transcription polymerase chain

	reaction
SAM	s-adenosyl-metionine
SEIMC	Spanish Society of Infectious Diseases and Clinical Microbiology
SNP	Sinlge nucleotide polymorphism
SOD	Superoxide dismutase
Sp	Species
ST-2	Sequence type 2
T6SS	Type VI secretion system
TA	Toxin-antitoxin
TelR	Telurite resistence
tRNA	Transcriptomic ribonucleic acid
UDC	University of A Coruña
UK	United Kingdom
UPL	Universal probe library
WGS	Whole genome sequence
WHO	World Health Organization

RESUMEN

Una de las mayores amenazas para la salud pública es el incremento de bacterias resistentes a los antimicrobianos. Dicho incremento se debe a múltiples factores, entre los que comienzan a destacar la existencia de poblaciones bacterianas tolerantes y/o persistentes a las condiciones de estrés, incluyendo el tratamiento antimicrobiano. A lo largo de la presente tesis doctoral se han analizado diversos mecanismos moleculares de persistencia y/o tolerancia bacteriana, destacando la red del quorum (*sensing/quenching*) y los sistemas toxina-antitoxina (TA) en aislamientos ambientales y clínicos.

En relación con la red del quorum, llevamos a cabo estudios genómicos y de expresión ante condiciones de estrés en la cepa aislada de ambiente hospitalario aéreo *Acinetobacter sp*. 5-2AC02 (cercano a la especie *A. towneri*) destacando el cluster del metabolismo de la acetoína y caracterizando el regulador negativo, AcoN. Posteriormente, estudiamos la importancia de dicha red del quorum en el desarrollo de bacteriemia secundaria a neumonía en aislamientos clínicos de *Acinetobacter baumannii*.

Finalmente, se realizaron estudios de caracterización funcional y análisis bacteriano de los sistemas toxina-antitoxina (TA) (como el módulo AbkB/AbkA y otros) en aislamientos de *A. baumannii* y *Escherichia coli*.

RESUMO

Unha das maiores ameazas para a saúde pública é o incremento de bacterias resistentes aos antimicrobianos. Devandito incremento débese a múltiples factores, entre os que comezan a destacar a existencia de poboacións bacterianas tolerantes e/ou persistentes ás condicións de estrés, incluíndo o tratamento antimicrobiano. Ao longo da presente tese doutoral analizáronse diversos mecanismos moleculares de persistencia e/ou tolerancia bacteriana, destacando a rede do quorum (*sensing/quenching*) e os sistemas toxina-antitoxina (TA) en illamentos ambientais e clínicos.

En relación coa rede do Quorum, levamos a cabo estudos xenómicos e de expresión ante condicións de estrés na cepa illada de ambiente hospitalario aéreo *Acinetobacter sp*. 5-2AC02 (próximo á especie *A. towneri*) destacando o clúster do metabolismo da acetoína e caracterizando o regulador negativo, AcoN. Posteriormente, estudamos a importancia da devandita rede do quorum no desenvolvemento de bacteriemia secundaria a pneumonía en illamentos clínicos de *Acinetobacter baumannii*.

Finalmente, realizáronse estudos de caracterización funcional e análise bacteriano dos sistemas toxina-antitoxina (TA) (como o módulo AbkB/AbkA e outros) en illamentos de *A. baumannii* e *Escherichia coli*.

ABSTRACT

One of the greatest current threats to public health is the rapid increase in the emergence of antibiotic resistant strains of bacteria. This increase is due to multiple factors, including the development of tolerant and/or persistent populations under stress conditions (e.g. antimicrobial treatment). The present thesis reports research investigating several molecular mechanisms of bacterial persistence and/or tolerance, particularly the quorum network (sensing/quenching) and toxin-antitoxin systems (TA), in both clinical and environmental isolates.

In relation to the quorum network, we carried out genomic and expression studies with an airborne strain isolated from a hospital environment, *Acinetobacter sp*. 5-2AC02 (closely related to *A. towneri*), and subjected to different stress conditions. We identified the acetoin metabolism cluster and characterized the negative regulator, AcoN protein. We then investigated the importance of the quorum network in clinical isolates of *Acinetobacter baumannii* in the development of secondary bacteraemia following pneumonia.

Finally, we carried out functional characterization studies and examined toxin-antitoxin (TA) systems (such as AbkB/AbkA among others) in strains of *A. baumannii* and *Escherichia coli*.

INTRODUCTION

1. *Genus Acinetobacter*

Acinetobacter is a Gram-negative, aerobics, non-fermentative, catalase positive, oxidase negative, with guanine/cytosine content between 39-47%, cocobacillus gender (*1*). Members of this genus can be found in a wide variety of environments from water to air, passing by human skin (*2*). *Acinetobacter sp*. has developed the ability to stay in hospitals environments, thanks to their capacity to survive to desiccation, disinfectants and antibiotics, and their incredible capacity to acquire genes and modify their genome (genome plasticity). These characteristics cause a high rate of nosocomial infections of this opportunistic pathogen, which generates a wide spectrum of infections such as pneumonia, urinary tract infections, bacteraemia, secondary meningitis and infections in burned tissues (*3*). Among all the members of this genus mentioned above, there are four highly-related and difficult to differentiate, which are: *A. baumannii, A. pitti, A. nosocomiales* and *A. calcoaceticus*; these species are usually included in *A. calcoaceticus-baumannii* complex being the first three the most common *Acinetobacter* in hospitals (*4*). That is why in 2006 the Infectious Diseases Society of America (IDSA) included *A. baumannii* into ESKAPE group, which include the most important nosocomial pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomona aeruginosa* and *Enterobacter sp*.) (*5*). Nowadays, *A. baumannii* cause more than 20% of the intensive care units (ICUs) patients' infections (*6*).

2. *Genus Escherichia*

The members of this genus are Gram-negative, rod-shaped, facultative anaerobic, and belonging to the *Enterobacteriaceae* family (*7*). The most known species from this genus is *Escherichia coli*, which is one of the most prevalent bacterium in the gastrointestinal tract of animals, as a commensal. Besides, *E. coli* has been deeply analysed, and is usually used as bacteria model because of the fact that it has a rapid growth rate, is easy to culture *in vitro*, can growth under several growth conditions, there are very harmless strains and its genome has been deeply

analysed and is easy to manipulate (*8*). Nevertheless, *E. coli* produces a wide range of disease both diarrheagenic and extraintestinal pathogenic, being the most common pathogen not only in humans but also in animals (*9*). The classification of the pathogenic *E. coli* strains is based on the presence of some specific virulence genes, being classifty in: enteropathogenic, enterotoxigenic, enteroinvasive, enteroaggregative, Shiga toxin-producers, diffusely adherent and adherent invasive (associated with Crohn's disease) (*10*).

Highlighting, for the first time in its history the world health organization published in 2017 the list of antibiotic-resistant priority pathogens (*11*). The main point of this list is highlight the threat of the bacteria included in this list, which are *Acinetobacter*, *Pseudomonas* and various *Enterobacteriaceae* (such as *Klebsiella*, *Escherichia*, *Serratia* and *Proteus*).

3. Multi-Drug Resistance (MDR), tolerance and persistence

Since the discovery of pathogen bacteria, scientists tried to fight against them without much success until 1928 with the discovery of penicillin (*12*), when humanity began to take advantage from this "silent" threat. However, as early as 1945 Sir Alexander Fleming warned about the antibiotic resistant phenomenon. In the last few decades, the wide range of antibiotic resistance in pathogenic bacteria has been exponentially improved, becoming one of the most important worldwide health problems nowadays. Furthermore, resistant cells are highly related to tolerant and persister cells, these two concepts being relatively new in the general scientific community. It was in 1942 that Hobby *et al*. described for the first time non metabolically active cells, which were called persister cells by Joseph Bigger in 1944 (*13, 14*).

These three concepts are sometimes confused due to their subtle differences. Below there are some clues to distinguish them. Resistant bacteria are characterized by: i) using active mechanisms in their defence, usually associated to mutations; ii) growing under drug pressure; and iii) their phenotypes are inherited. Tolerant bacteria are those that can survive to antibiotic exposure without modifications in its

minimal inhibitory concentration (MIC) reducing its biological processes. Nevertheless, persister bacteria are characterized by: being a subpopulation of the culture, tolerant to antibiotics or other stress conditions remaining dormant in their presence by stopping their cellular activity, do not inheriting the persister phenotype, and being able to turn into wild-type as soon as the antibiotic is eliminated from the environment (*15, 16*).

In the last decades bacteria have developed resistance not only to antibiotics, but also to biocides (common used antiseptics to clean and sterilize hospitals surface) (*17*). The acquisition of resistance usually comes from particular mutations or foreign DNA (*18*), but these processes need time. The presence of tolerant or persister cells contributes to the appearance of resistance (*19, 20*). These findings can be explained by the fact that those tolerant cells, which can survive under high concentrations of antimicrobial, are the ones that eventually generate the resistance to those treatments (*21*). Therefore, the study of the relationship between tolerance and persistence mechanisms in multi-drug resistant pathogens (MDR) is clue to analyse new anti-infectious treatments (Figure 1) (*22*). Among them we highlight:

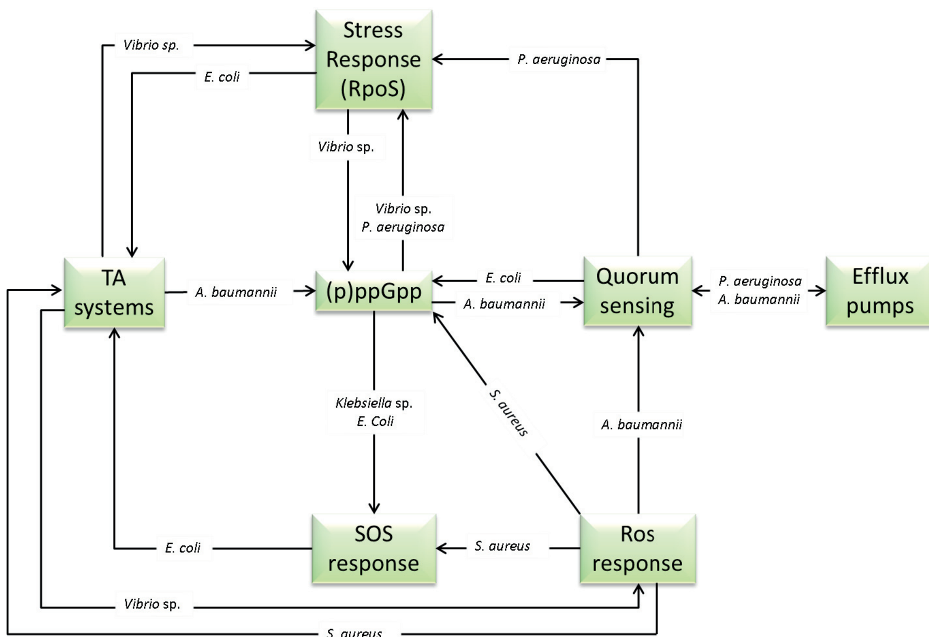

Figure 1. Links between the different mechanisms of tolerance/persistence. (Figure adapted from Trastoy *et al*. 2018).

- **General stress response:** These mechanisms as well as the RpoS response contribute to bacterial survival under stress conditions (*23*). The RpoS response has been related to toxin antitoxin (TA) systems in E. coli, in which MqsR/MqsA system and YafQ/DinJ system modify *rpoS* gene transcription and translation under stress situations (*24, 25*).

- **Reactive oxygen species (ROS) response:** ROS are chemical species with oxygen, which are highly reactive. They are produced under normal conditions as part of bacterial metabolism and are used as cell signals. Under certain situations, ROS levels increase reaching dangerous concentrations, with the result that they can produce damage in lipids, proteins and even DNA causing cell death (*26*). However, bacteria present a superoxide dismutase (SOD) and catalase enzymes as well as some other antioxidants that eliminate ROS from bacterial cytoplasm (*27*).

- **Energy metabolism:** In this area there are two mechanisms principally related to tolerant cells. Firstly, cytochrome bd, a quinol:O_2 oxidoreductase from respiratory metabolism in prokaryotes under oxygen limitation conditions, which contributes to improve the ROS response and the tolerance to nitrosative stress (*28*). Secondly, Tau metabolism, active under cystine or sulfate deficiency for sulfite production in bacteria in order to use it as a substrate (*29*).

- **Efflux pumps:** They are used to eliminate any possible toxic element in the bacterial cytoplasm or to maintain the molecular balance between inside and outside of cell (*30*). It has been described that efflux pumps are essential for maintenance of low antibiotic concentration in the bacteria, especially in persister cells (*15, 31*). Efflux pumps are known to be regulated by ROS response, secretion systems such as type III (T3SS) or type VI (T6SS), QS among others (*32, 33*).

- **SOS response:** This response is activated under DNA damage conditions to repair the bacterial genetic material. The SOS system regulates genes involved in DNA repair and recombination, as well as biofilm production and antimicrobial resistance (*34*). Some researchers have also associated the

SOS system with the generation of persister cells in *E. coli* through TisB/TisA TA system (*35*).

- **Guanosyl (penta)tetra-phosphate ((p)ppGpp):** This molecule is regarded as the alarmone signal, being activated under environmental pressure such as amino acid starvation (*36*). The (p)ppGpp is highly related to RelA/SpoT homolog (RSH) protein, which are responsible for synthetic and/or hydrolytic functions. This molecule is involved in all the DNA-RNA process, under a nutrient-deficient environment, being highly related to persister cell formation (*37*).

- **Bacterial volatile compounds (BVC):** BVCs are molecules of low molecular weight that can rapidly evaporate. They are produced by bacteria to improve their competence, and have been related to antibiotic resistance and persister cell production (*38*).

- **Quorum network (sensing/quenching) and secretion systems:** QN, bacterial communication mechanisms, has been related to virulence mechanisms such as toxins production, motility chemotaxis, biofilm production and secretion systems T3SS and T6SS in bacterial competition (*39*). However, Quorum Quenching (QQ) is known as the capacity of bacteria to interrupt the Quorum sensing (QS), it has been described a wide number of QQ molecules, not only in bacteria but also algae, invertebrates and plants (*40*).

- **Toxin-antitoxin systems:** TA systems are one of the widely studied mechanisms related to persister cell formation. There are seven different types of TA systems that can be found in plasmids, bacterial chromosomes and bacteriophages in almost all bacteria (*41*).

4. Quorum network

4.1 Quorum sensing

It was in 1970 that Nealson *et al*. found that the luciferase operon, of *Vibrio fischeri*, suffered an autoinduction phenomenon (*42*). However, it was not until 1994 that the

term quorum sensing (QS) appeared with reference to the mechanism by which bacteria can detect the population density (*43*), and until 2001 that it was identified as a mechanism of bacterial communication (*44*).

The QS has been identified in both Gram-positive and Gram-negative bacteria. In Gram-positive bacteria these systems worked with secreted oligopeptides and two component systems (cytoplasmic transcription factors and a membrane-bound sensor kinase receptor) (*45*). Gram-negative bacteria usually have various autoinducers (AIs), which are detected by receptors that discern between closely related molecules (Figure 2) (*46*). The QS is characterized by: i) diffusible molecules, both acyl-homoserine lactones (AHLs) and molecules synthesized from S-adenosyl-L-methionine (SAM) in Gram-negative bacteria; ii) specific receptors for the autoinducers, either cytoplasmic or inter-membrane; iii) these molecules have effect over several bacteria mechanisms; iv) the molecules can produce its own activation (*47*).

Some authors speculate about the fact that the heterogeneity of the QS systems can be essential for those non-related cells that share environment (*48*). It is known that autoinducers are produced by prokaryotic and eukaryotic organisms, which implied that they could act as one, two or multi-way communication. Moreover, some autoinducers can be used as probiotics, due to the microbiotic alteration that they produce, their interaction with the expression of virulence factors, and their ability to stimulate pathogens to abandon biofilms (*49*).

Gram-positive autoinducers are principally **autoinducing peptides (AIPs)**, being the best-known the accessory gene regulator (agr) locus of the QS system of *Staphylococcus aureus*. This locus is composed by two different transcripts, RNAII, which codifies the Agr cluster and RNAIII, represses the synthesis of cell wall-associated proteins and increase the production of exoproteins in response to high cell density (*50*). AIPs are recognized by a histidine kinase in the cell membrane, which produces a phosphorylation cascade that ultimately modify the transcriptional process of the cell (*51*).

On the contrary, in Gram-negative bacteria there are identified two classical types of signals: the N-acyl-homoserine lactones (AHL) and the quinolones. The **AHLs** are homologues to Lux signal from *V. fischeri* and are considered the most extensive

ones. These systems are composed by a transcriptional regulator LuxR-like and a synthase LuxI-like. AHLs diffusible molecules, synthesize from the combination of S-adenosyl methionine (SAM) and a particular fatty acyl chain (*50, 52*); the length of the acyl chain can be from 4 to 18 carbons and the length of it might influence their stability, affecting signalling dynamics (*53, 54*). Most bacteria have more than one type of signal, as is the case of *P. aeruginosa* that not only has AHLs (which has two, known as *las* and *rhl*) but also quinolones. **Quinolone** system also has two components: the MvfR, a transcriptional regulator; and the PqsH, which transform 3,4-dihydroxy-2-heptylquinolone in 4-hydroxy-2-alkylquinolone (HAQ), commonly known as Pseudomonas quinolone signal (PQS) (*55*).

Moreover, in the last few years new autoinducers have been described such as: the **diffusible signal factor (DSF)** these molecules have been found in clinical pathogens, and the most surprising fact about them is that one RpfF can synthesize several DSFs in the same bacterium (*56*); the **autoinducer 2 (AI-2)**, which is present in more than 500 bacterial species, is a furanosyl borate diester synthesized from S-4,5-dihydroxy-2,3-pentanedione by a LuxS-like synthase in *Escherichia coli* (*57, 58*), although is also produced by Gram-positive bacteria being considered an inter-species communication signal (*59, 60*); the **indole** identified as AI in 2007 in *E. coli* and regarded as an interkingdom signal in the gastrointestinal tract (*61-63*); the pentapeptide **extracellular death factor (EDF)** signal that is highly related to the MazF/MazE TA system in the programmed cell death (PCD) and the production of ROS (*64, 65*).

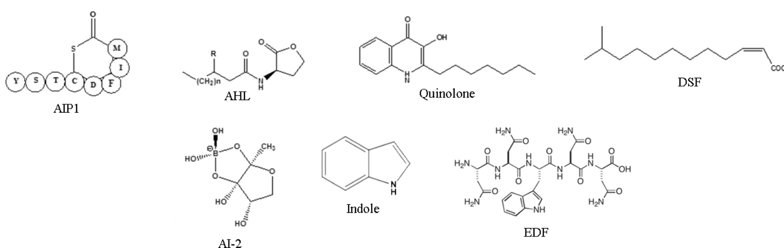

Figure 2. Different autoinducers. AIP (autoinducing peptides), AHL (acil-homoserine-lactone), AI-(autoinducer 2), EDF (extracellular death factor).

Quorum sensing model of *Vibrio fischeri*

Each bacterium produces autoinducers (AIs) that are exported and accumulated in the medium or environment. When the population is numerous enough and the amount of AIs accumulate in the environment passes the threshold concentration, the receptors of every bacterium in the medium are activated and begin a transduction cascade that activates several bacterial mechanisms that are only effective with the proper amount of population (*66*).

The *V. fischeri* system is used as model to explain most of the systems from Gram-negative bacteria, due to the fact that almost all of them use at least one AHL as QS signal (Figure 3a). *V. fischeri* used N-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL) as principal AI in the bioluminescence mechanism. This mechanism is codified by *lux* operon (*lux*CDABE), the LuxI synthase and the LuxR receptor. Under low bacteria density, the lux operon is repressed and LuxI produces basal levels of OHHL, which spread to the medium through the cell membrane. Once the population has reached enough density and the amount of OHHL accumulated in the environment exceeds certain concentration, the OHHL diffuses across the membrane to the cytoplasm. The cytoplasmic OHHL binds to the LuxR receptor creating a complex that can interact and activate the *LuxBox* (located in the promoter region of *luxI*). The *LuxBox* begins the transcription not only of *luxI* but also of lux operon, producing luciferase (protein required for light generating) and LuxI, which transforms SAM in OHHL (creating a positive feedback loop) (*67*).

Surprisingly, it was found that around 76% of the bacterial species studied only present a LuxR receptor but not a synthetase gene as LuxI (*68*). These findings imply that these bacteria employ another molecules as AIs and that they employ the LuxR receptor to detect the QS signals from another species (*69*).

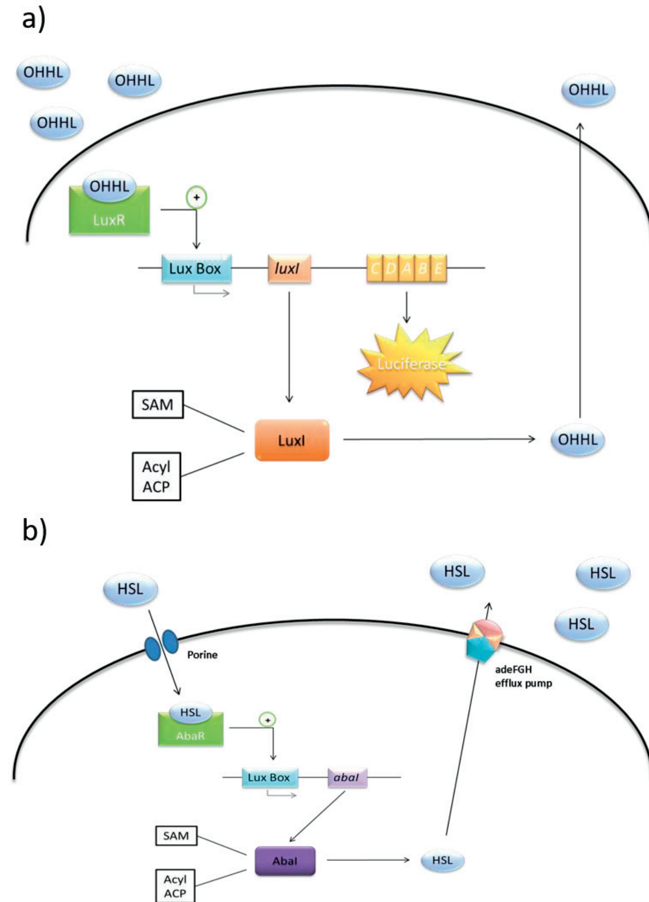

Figure 3. a) Quorum sensing model in *V. fischery*, previously described. **b)** Quorum sensing model in *A. baumannii*, descrived bellow.

Quorum sensing in *Acinetobacter sp*.

QS system in *Acinetobacter* has been described as homologous to the *Vibrio fischeri*; having a LuxR-like receptor (AbaR), LuxI-like synthase (AbaI) (*2*) and AHLs as autoinducers. *Acinetobacter* synthesize different long-chain AHLs, such as C12, C10, C14, C13 and C16 in *A. baumannii*. Nevertheless, these AHLs are not specific of this species, but they can be found in others (*70*). It is known that only small molecules can diffuse across the cell membrane, the limit has been stipulated in less than 8 carbons, which is why long chain AHLs are transported outside the cell by efflux pumps (*2*). It has been observed that when *Acinetobacter* is cultured in

shaking, there are no AHL in the medium. Although, when strains from this gender are cultured in static conditions the AHL signal can be detected. These findings suggest that the activation of AbaI in the *Acinetobacter* AHL-mediated QS is dependent on cell-to-cell contact (*71*).

Like in *V. fischeri*, the synthesis of 3-hydroxy-dodecanoyl-(L)-homoserine lactone (3-Hidroxy-C12-HSL) is produced by AbaI in a basal level until the population reaches the proper density. Once the concentration of 3-Hidroxy-C12-HSL is high enough outside the cell, its molecules enter the cell (using porines, activated by the concentration difference) and bind its receptor AbaR. The complex AbaR-AHL joins with the Lux-box-like (promoter) that is 67 bp upstream abaI, and activate its transcription. AbaI synthase begins the production of new 3-Hidroxy-C12-HSL molecules, which are exported to the medium by AdeFGH efflux pump (*72*), feeding the positive feedback loop (Figure 3b). Nowadays, it is not completely clear which are the synthases of the rest of the AHLs in *Acinetobacter* gender (*73*).

In the last few years, it has been found that many bacteria can be influenced by light/dark exposure, modifying their Quorum Network or affecting its virulence. Mussi *et al*. found that *Acinetobacter baumannii* moves by switching under dark condition at 24ºC, but not at 37ºC, meanwhile they do not move under light exposure. They noticed that it was blue light the one which produce motility inhibition (*74*). Researchers analyzed other members of the *Acinetobacter calcoaceticus-baumannii* complex (ACB), showing that not all of the members of the group have the same capacity to respond to dark/light conditions. Surprisingly, meanwhile *A. baumannii* showed an inhibition of its biofilm formation ability under blue light, the rest of the members of the group, which responds to light, showed a stimulation of biofilm formation in the presence of blue light and an inhibition in darkness, and not only at 24ºC, but also at 37ºC (*75*). Mussi *et al*. found a photoreceptor known as BlsA (for blue light sensing A) in *A. baumannii*, which is overexpressed under blue light at 24ºC (*74*). They hypothesized that BlsA play a role in the pathogenesis of surface-exposed wounds, due to its light exposure and the low temperature, but no in systemic infections (*76*). Furthermore, it has been analyzed the relation between light and the resistance to some antibiotics, such as Minocycline and Tigecycline. These experiments showed an increase of the halo produced by the presence of each antibiotic in the dark. They found that in the presence of $FeCl_3$ the effect of light

was reduced in resistance. The next step was the search of the photoreceptor implied in these mechanisms, as it was demonstrated that BlsA has no effect on resistance. After several experiments, they found that the regulation was produced by $1O_2$, which is a single oxygen produced by photosensitizer MB under red light (which showed a production of smaller halos than blue light) (*77*). In addition, these researchers observed that light produce an increment of surfactant and the tolerance to fluoroquinolones, these findings could be related to the persistence ability under stress condition of *A. baumannii*. Furthermore, light is also implied in the regulation of the expression of type VI secretion system genes, lipid metabolism cluster, efflux pumps genes and acetoin pathway (*78*).

4.2 Quorum quenching

Quorum quenching (QQ) is defined as the mechanisms by which quorum sensing is inhibited. The inhibition of the QS can be produced by degradation of the AIs, inhibiting the signal efflux, avoiding AIs accumulation or competing with the AI for the receptor (*79, 80*). Antagonists of QS can be found in a great variety of species such as bacteria, algae, invertebrates, plants and even mammalian (*40*). QQ mechanism has been found in every bacterium, indicating the importance of these mechanisms in bacterial fitness. Different roles of bacterial QQ enzymes has been described such as recycling QS signals (*81*), interrupting the QS from other bacteria (*82*), and detoxification. Mostly of the current research about this subject have been focused on the degradation of AHL due to its wide distribution in clinical pathogens.

Bacterial AHL degradation molecules can be classified in three types, depending on their mechanism of action against AHL (Figure 4):

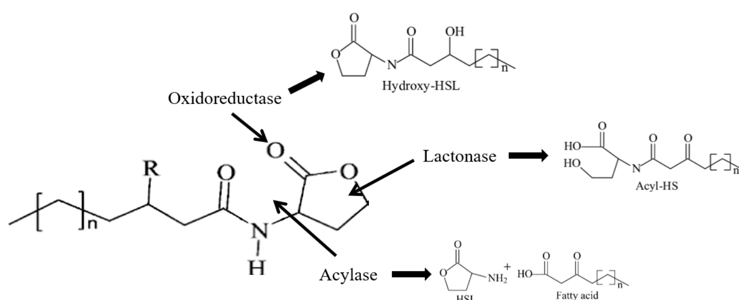

Figure 4. Action mechanism of different QQ enzymes

- **Lactonases**: Enzymes from this group are mostly metallo-β-proteins that hydrolyze the ester bond of the HSL ring. The first enzyme of this type was discovered in *Bacillus* sp. 240B1, AiiA (autoinducer inactivator) (*83*). However, there are other less abundant types of lactonases: i) phosphotriesterases (PTE) that can inactivate AHLs with chains between 6 and 14 carbons (*84*); ii) the *Geobacillus kaustophilus* thermostable lactonase (GKL), which is a PTE metalloprotein with preference for AHL with 8 or less carbons (*85*); iii) α/β hydrolases, which include the AidH from *Ochrobactrum* anthropic ATCC 49188 (*86*), the AiiM from *Microbacterium testaceum* StLB037 that use the α/β hydrolase domain for the AHL degradation (*87*) and AidA from *A. baumannii* a dienelactone that inactivate AHLs at least with chains of 6 and 12 carbons (*88*); iv) QsdH from *Pseudoalteromonas byunsanensis* strain 1A01261, a GDSL-like hydrolase located in the N-terminus of a resistance-nodulation-cell division (RND) efflux pump (*89*).

- **Acylases**: These types of enzymes were discovered in *Variovorax paradoxus* (a betaproteobacterium) and are suspected to break the amide bond between the HSL ring and the acyl side chain (*90*). Acylases can inactivate AHLs completely unlike the lactonases, whose products can revert to active, depending on the pH (*83*). These enzymes were found in several bacteria, which showed their similarity to Ntn (N-terminal nucleophile) hydrolases, and their action against long chain AHLs (*91*).

- **Oxidoreductases**: These enzymes do not degrade AHLs but modify them by interfering with its specificity and union with the receptor. The first discover oxidoreductases where the ones from *Rhodococcus erythropolis* W2. These enzymes change the oxo group of the C3 to a hidroxy group in AHLs ranging from C8 to C14 (*92*).

- **Halogenated furanones**: These compounds are not produced by bacteria, instead they are produced by the macroalga *Delisea pulchra*; these furanones act as substrate of LuxR receptor blocking AHL union to it (*93*).

There are different types of AIs, with the exception of AHLs, whose inhibition has not been deeply studied. Some research about the DSF have shown that *E. coli* DH10β

can degrade DSF when it comes into contact with it for 24h or more (*94*). Besides, Llamas *et al*. have found out that *carA* and *carB*, two genes from the operon for the synthesis of carbamoyl phosphate (precursor of arginine and pyridine biosynthesis), in *Pseudomonas sp*., can inactivate DSF by an unknown mechanism (*95*). This operon has been found needed for DSF modification in different bacteria with various inactivation rates (*94*). Quinolone, AI of *Pseudomonas* (PQS) is quenched by the dioxygenase Hod (1H-3-hydroxy-4-oxoquinaldine) from *Arthrobacter nitroguajacolicus* due to its similarity with Hod substrate, being transform into N-octanoylanthranilic acid and CO (*96*). Finally, two possible interspecies mechanisms that quench AI-2 depending on QS has been analysed in enteric bacteria, based on the Lsr system. Roy *et al*. have showed that LsrK (kinase that phosphorylate AI-2) can quench AI-2 responses in cultures of *E. coli, V. harveyi* and *Salmonella typhimurium* alone or in combination (*97*). Furthermore, LsrG (enzyme that catalyse the modification of P-AI-2 into 3,4,4-trihydroxy-2-pentanone-5-phosphate in *E. coli*) can be a valuable target for AI-2 based QQ therapies (*98*).

Quorum Quenching in *Acinetobacter sp.*

In *Acinetobacter* different QQ enzymes had been found in the last few years, such as AmiE, an acylase from *Acinetobacter sp*. Ooi24 (*99*); or AidE, a lactonase from *Acinetobacter sp*. 77 (*100*). The expression of QQ enzymes in this genus was found during the stationary phase, when the concentration of AHL became low due to the activity of these enzymes, showing an endogenous regulation of the QS system (*101*).

Recently, AidA protein was discovered, a dienelactone (*102*). It was found during a study of clinical isolates of *A. baumannii*, in which it was found that the majority of the non-motile strains were isolated from respiratory tract and presented the *aidA* gene in their genomes. These strains are subject to an extremely stressful environment with high concentrations of ROS. Some authors have related the response to ROS and QS in *Acinetobacter* (*103, 104*). Lopez *et al*. also related the overexpression of the *aidA* gene with the presence of 3-oxo-dodecanoyl-HSL (3-Oxo-C12-HSL) from *P. aeruginosa*. 3-Oxo-C12-HSL is quite similar to *Acinetobacter* AHL (3-Hidroxy-C12-HSL). This similarity could imply an interaction between AbaR and LasR (receptors from *Acinetobacter* and *P. aeruginosa* respectively) with the

other AI, suggesting an inter-species communication (*103*), mediated by the activation of QQ enzyme AidA (*102*).

Subsequently, Mayer *et al*. have found that AidA is upregulated in early stages of AHL-producing static cultures and its expression is lower under low-salt conditions producing a higher 3-Hidroxy-C12-HSL concentration. These authors also found high number of lactonase sequences belonging to metallo-β-lactonases family in *A. baumannii* ATCC 17978, selecting three of them to be analyzed. One of the QQ enzymes studied showed activation in the presence of 3-Hidroxy-C12-HSL, which indicates that this enzyme might be under QS regulation. The presence of different QQ enzymes in *Acinetobacter* and the fact that they have not only different regulation, but also small differences in their substrates, highlights the importance of the AHL-mediated QN in this species. Besides, the four QQ enzymes described in *A. baumannii* may regulate the concentration of exogenous and endogenous AHLs (*88*).

4.3 Bacterial volatile compounds (BVCs)

Bacterial volatile compounds are small molecules produced as secondary metabolites that are naturally secreted by bacteria as antibacterial "weapons" in order to control their competitors, as well as a help to environmental adaptation (*105, 106*). These molecules have a low molecular weight, which contributes to their rapid evaporation, letting them pass through solids, liquids and gasses (*107*). BVCs are principally organic compounds, but there are also inorganic ones, such as nitric oxide, hydrogen cyanide, also known as ammonia and hydrogen sulfide. Among organic compounds can be found: i) hydrocarbons, usually derived from fatty acid biosynthesis (*108*); ii) ketones/alcohols, generated during the decarboxylation of fatty acids, the most remarkable ones being acetoin and its oxidized form 2,3-butanedione (*109*), this molecules are commonly associated with *Enterobacteriaceae*; iii) acids, organic acids are less common than the previous ones, although several short-chain fatty acids have been described to be related to this subject, such as acetic, butyric and propionic acids (*107*); iv) sulfur compounds, which are related to fermentations specially used in food industry (*110*); v) nitrogen-containing compounds, trimethylamine and trimethylamine oxide are two abundant compounds in animal and human intestines, being more abundant in fish, in which produce the smell of spoiling fish; 2-amino-acetophenone (2-AA) is the responsible

for the odor of *P. aeruginosa* (*106*); in this category indole, a QS molecule can also be included (*111*); vi) terpenes, only monoterpenes, sesquiterpenes and their derivatives are volatile compounds, these products derived from mevalonato or deoxyxylulose pathway (*107*).

BVCs have been related with bacterial competition, stress resistance and virulence, among others.

- **Competence**: It has been observed that certain bacteria from animal intestinal tract such as *Veillonella sp.* produce BVCs that inhibit enteropathogens growth such as *Salmonella enteritidis, Salmonella typhimurium, E. coli* and *P. aeruginosa* (*112*). It was reported that 1-butanol, 2-butanone, indole or acetoin can affect motility in *E. coli* and *P. aeruginosa* (*113*). Besides, *Pseudomonas fluorescens* and *Serratia plymuthica* produce dimethyl disulfide, which has bacteriostatic effects against *Agrobacterium tumefaciens* and *A. vitis* (*114*). Moreover, *P. aeruginosa* is influenced by BVCs from other bacteria to produce BVCs against *Bacillus sp*. or pyocyanin, which is enhanced by 2,3-butanediol of *Serratia marcescens* (*115*); in adition, 2,3-butanediol has been found in cystic fibrosis patients (*116*).
- **Stress resistance**: BVCs are involved in an increase of tolerance to environmental stress, including antibiotic resistance (*117*). Bernier *et al*. demonstrated that under high population density, the production of volatile ammonia by bacteria increases tetracycline and ampicillin resistance as well as the decrease of resistance to aminoglycosides, in Gram-negative and Gram-positive bacteria (*118*). Trimethylamine, produced by *Enterobacteriaceae*, increase resistance to tetracycline and aminoglycosides but decrease the resistance to chloramphenicol and oxidative stress (*113*). 2,3-butanedione and glyoxylic acid, both produced by *Bacillus subtilis*, have been related to modifications in *E. coli* resistance profile through *hipA* toxin activation (*38*). Indole, a QS molecule, can increase acid and drug resistance in *P. aeruginosa, S. enterica* and *E. coli* (*119*). 2-AA alter antibiotic tolerance by producing persister cells in *P. aeruginosa, B. thailandensis* (both productors of it) and *A. baumannii* (non productor of 2-AA), suggesting the role of 2-AA in Gram-negative antibiotic tolerance during polymicrobial

infections (*106, 120*). Que *et al*. postulated that BVCs used as sub-MIC concentrations can produce bacterial persistence helping some cells to survive unpredictable stress (*106*).

- **Bacterial virulence**: 2,3-butanediol and acetoin, volatile molecules from glucose fermentation metabolism, increase virulence factors in *Enterobacteriaceae* pathogens (*121*). The genes implied in its production are organized in an operon, which is regulated by pH, QS and oxygen (*122*). Furthermore, in *P. aeruginosa* the QS genes *lasR/lasI* are activated under 2,3-butanedione presence, increasing the biofilm and phenazine production (*115*).

One of the most studied mechanisms related to the BVCs is the acetoin/butanediol metabolism. Under high cell density, the medium is acidified (low pH) as consequence of glucose metabolism, is at this moment that acetoin cluster is activated reducing the acetoin levels and increasing the pH of the environment, and has been related to bacterial pollution resistance as well as to an increase capacity of environmental adaptation (*122, 123*). Acetoin/butanediol cluster, usually named as *aco* operon, is present in a broad spectrum of bacteria species such as: *Bacillus subtilis, Pleobacter carbinolicus, Vibrio cholerae, Pseudomonas putida, Klebsiella sp.* among others (*121, 122, 124-126*). The *aco* operon is regulated, in *B. subtilis*, by AcoR as positive regulator, found downstream of the operon in contrast to other bacteria (*127*); and is composed by two acetoin oxidoreductases encoded by *acoA* and *acoB*, a acetyltransferase encoded by *acoC*, and a dehydrogenase encoded by *acoD*, although this enzyme has also been named as *acoL* (*124*). Furthermore, in some bacteria such as *Pseudomonas putida* it has been found a butanediol dehydrogenase being also part of the operon (*125*). The regulation of this operon by the QS has been shown in *V. cholerae* (Figure 5), in which, depending on the cell density, the QS regulator LuxO controls both the repressor AphA and the activator HapR of the operon regulatory gene *alsR*, (*128*). Under high cell density and therefore elevated AI levels, LuxO is inactivated by dephosphorylation, preventing the activation of *aphA* and stabilizing *hapR*, activating *alsR* of the cluster (*122, 129*). Hawver *et al.* demonstrated that *V. cholerae* change pyruvate flux to acetoin and 2,3-butanediol production, reducing the ATP produced, in order to maintain appropriate environment to prevent population collapse at high cell density, demonstrating that

QS is essential to the survival of the *V. cholerae* population to low pH (*128*). Furthermore, in *Bacillus subtilis* under glucose presence the CcpA (catabolite control protein A) repress the *aco* operon by binding itself to both a *cre* site from the AcoR regulator and *cre* site in the *sigL*, which has been related to an RNA polymerase that recognize the sequence of the *aco* operon promoter (*127*); however, in the presence of acetoin CcpA is repressed and AcoR and SigL induce operon expression (*124*).

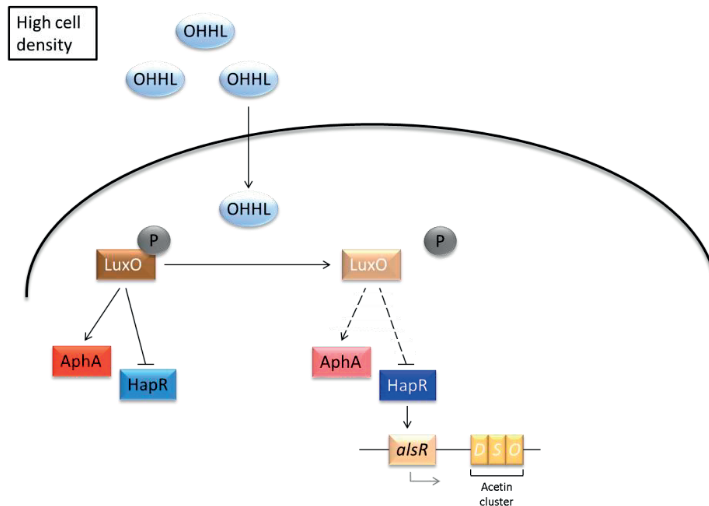

Figure 5. Regulation of acetoin cluster by QS molecules. The presence of homoserine-lactones produces the inactivation of LuxO, eliminating the inhibition of HapR, which produced the activation of *alsR* and the acetoin cluster.

5. TA systems

Toxin-antitoxin systems are usually two component systems composed by a stable protein, the toxin, and an unstable protein, the antitoxin (*130*). TA systems can be found not only in bacteria, but also in archaea and even some fungi (Doc toxin homologues identified) (*131*). These systems were originally found in bacterial plasmids (*132*) being associated with plasmid stabilization (*133*), although nowadays it is known that they can be found in bacterial chromosome and in bacteriophages. In contrast to what their name could imply, the toxins of these systems are not a weapon against other bacteria, but they affect their own host metabolism; that is why a more proper name would be "growth inhibitors" (*134, 135*). Nowadays, seven types

of TA systems have been described, depending on how the antitoxin interact with the toxin (Figure 6). i) Type I (e.g., RalR/RalA), the antitoxin is an antisense RNA of the toxin; ii) type II (e.g., MqsR/MqsA), the antitoxin protein binds the active center of the toxin; iii) type III (e.g.,ToxN/ToxI), the same mechanism than the type II, but in this case is the antitoxin RNA, which binds the protein (*136*); iv) type IV (e.g., CbtA/CbeA), the antitoxin produces a competitive inhibition with the toxin for its target (*137*); v) type V (GhoT/GhoS), the antitoxin cleaved the toxin mRNA (*138*); type VI (e.g., SocB/SocA), the antitoxin binds the toxin producing its degradation by cellular proteases (*139*); vi) type VII (Hha/TomB), the antitoxin oxidase a cysteine residue of the toxin inactivating it (*140*). Surprisingly, Georgiades *et al*. found that there is a correlation between the number of TA systems and the virulence of bacteria (*141*), corroborating the importance of these systems to bacteria and explaining their wide distribution and even their redundancy in bacteria genomes.

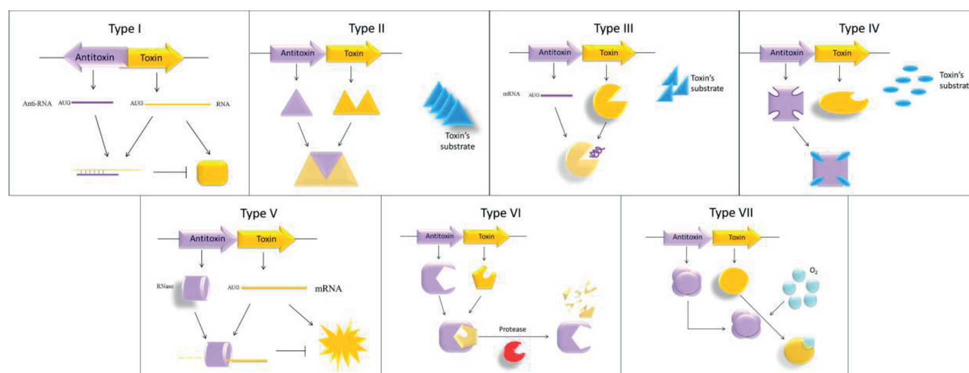

Figure 6. Types of toxin-antitoxin systems. Type I: The antitoxin mRNA binding to toxin mRNA which prevent toxin protein formation; type II: it has been formed a TA complex by the union of toxin and antitoxin proteins; type III: it has been formed a TA complex by the union of toxin protein with antitoxin mRNA; type IV: the antitoxin protein binds to toxin target blocking its action; type V: the antitoxin mRNA encodes an RNase which degrades the toxin mRNA; **t**ype VI: a TA complex is formed by the union of toxin and antitoxin proteins, producing decomposition of the toxin by a cellular protease; type VII: the antitoxin attenuated toxin activity by forming a complex with it.

In the last decade, some secreted toxins from TA systems have been found. i) EsaD, from the EsaD/EsaG TA system, is secreted by type VII secretion system in *S. aureus* with the object of using it as a competitive weapon (*142*); ii) AvRxo1, from the AvRxo1/Arc1 TA system, is secreted by type III secretion system in *Xanthomonas oryzae* modificating nicotinamide adenine dinucleotide in plants (*143*); iii) MqsA the

antitoxin from the MqsR/MqsA TA system, is secreted in membrane vesicles by *Xylella fastidiosa* (*144*).

TA systems are usually inactive, but they are activated under stress situations such as nutrition starvation, oxidative stress, antibiotic exposure and phage infection among others, they are activated (*145*). TA systems have been related to the generation of persistence and tolerance, but also to programming cell-death (PCD) or biofilm formation, demonstrating that the TA systems are integral part of the bacterial stress response network and generate tolerance to the stress.

Furthermore, it has been shown that QS signals can regulate TA system expression, such as AI-2 that uses MqsR toxin from the MqsR/MqsA TA system to produce motility and biofilm formation. The researchers proposed that AI-2 induces MqsR expression, which stimulates several motility related genes expression, activating the cell motility and biofilm formation in *E. coli* (*146*). MqsR toxin has also been related to persister cell formation (*147*) by its effect over ClpXP, a protease that degrades RpoS and DNA binding-protein; Lon proteases, which degrade antitoxins such as MazE and RelB; Hha, the toxin from the Hha/TomB type VII TA system that has been related to biofilm formation (*148*). Apart from MqsR, other toxins from different TA systems have been related to persister cell formation, for example: i) HipA, from HipA/HipB type II system, the first toxin related to persister cells (*149*); HipA, which has been shown to activate (p)ppGpp production via RelA (*150*); ii) HokB toxin, from HokB/SokB type I system, which is regulated by (p)ppGpp (*151*); iii) YafQ toxin, from YafQ/DinJ type II system, which increases persistence by reducing indole signal (*152, 153*); iv) TisAB, from TisAB/IstR-1 type I system, in which TisB is the toxin that acts as an ion channel reducing the proton motive force, and IstR-1 is the antitoxin that binds the untranslated ORF of *tisA*, this TA system showed that persistence can appear due to the SOS response and DNA repair helping to understand why persister cells appear during exponentially growing phases (*151, 154*).

Biofilm formation is also influenced by cryptic-prophages (bacteriophages whose genome is integrated in bacteria chromosome, which have lost its lysis capacity) TA systems, such as YpjF/YfjZ type IV system from *E. coli* CP4-57 cryptic-prophage, which reduce biofilm formation; or RelE/RelB type II system from Qin cryptic-prophage, which increase biofilm formation (*155-157*). Nevertheless, TA system, as

it mentioned above, can also been found in bacteriophages as is the case of Doc/Phd type II system is codified in bacteriophage P1 (*158*).

The MazE/MazF, a type II TA system, has been found to mediate PCD as a population phenomenon, requiring the involvement of QS extracellular death factor (*159*). When the bacteria is under rifampicin or chloramphenicol presence, or other antibiotic that inhibits transcription or translation, the EDF initiate ROS production, which activates the MazF/MazE system in order to produce PCD. However, under other type of antibiotics that cause DNA damage the activation of the TA system is not through the ROS pathway (*65*). Besides, it has been observed that RpoS produce a protection against MazF/MazE mediated PCD during stationary growth (*160*). Furthermore, MazF/MazE is also implied in the inhibition of the phage P1 by PCD mechanism (*161*). Besides, in the last few years, TA systems have been found to generate resistance against bacteriophage infection. Hok/Sok type I system, which is found in the plasmid R1, blocks T4 phage propagation (*162*); RnlA/RnlB and IsoA/IsoB type II systems from *E. coli* suppress T4 propagation (*163, 164*); ToxN/ToxI type III system has activity against several phages, being the most studied system in relation to bacteriophages (*165*); and AbiEii/AbiEi TA type IV TA system from *Lactobacillus lactis* has also been reported to relate to phage inhibition (*166*).

A. baumannii has several TA systems described in its genome, such as MazF/MazE in the chromosome of a wide range of clinical strains, although most of them are located in plasmids. The most common plasmid in clinical isolates in p3ABAYE, which encode RelE/RelB, two HigB/HigA, SplT/SplA and CheT/CheA systems (*167*). Interestingly, the SplT/SplA system, also known as AbkB/AbkA system, has been usually found in a resistance plasmid that also carries a β-lactamase $bla_{OXA\text{-}24/40}$ gene (*168*). This system found in *E. coli* too, can prevent translation by AbkB endoribonuclease activity through the scission of *lpp* mRNA.

OBJECTIVES

Multiresistance is one of the most serious health problems nowadays. However, the problem cannot be considered in isolation as it is closely related to, and in some cases dependent on, tolerance and persistence mechanisms. Understanding these mechanisms will help in the fight against pathogenic bacteria. The research reposted in this thesis focuses on some of these mechanisms, mainly the quorum network and toxin-antitoxin systems in clinical strains such as *Acinetobacter sp*. and *E. coli*.

The specific objectives of this doctoral research are as follows:

Chapter 1

- To determine the complete genome sequence of airborne *Acinetobacter sp*. strain 5-2Ac02 isolated from air samples collected in an ICU in a hospital in Rio de Janeiro (Brazil).
- To determine the complete sequence of the airborne *Acinetobacter sp*. strain 5-2Ac02 in order to investigate the genomic characteristics of the persistome, mobilome and tolerome.

Chapter 2

- To study the global gene expression of airborne *Acinetobacter sp*. strain 5-2Ac02 under stress conditions such as the SOS response (mitomycin C) and quorum network (AHLs).
- To characterize the AcoN protein as a negative regulator of the acetoin/butanediol operon.
- To investigate the relationship between acetoin/butanediol cluster and the quorum network and also photoregulation of the relationship.

Chapter 3

- To study the relationship between QS (*abaR* gene) and QQ (*aidA* gene) mechanisms in clinical strains of *A. baumannii* in connection with development of pneumonia and bacteraemia.

- To investigate the mortality of a QS deficient (lacking *abaI*) *A. baumannii* strain in comparision with the wild type strain (*A. baumannii* ATCC 17978) in an animal model.

Chapter 4

- To study gene expression in a chlorhexidine-tolerant clinical strain of *A. baumannii* belonging to clone ST-2, which carries a β-lactamase $bla_{OXA\text{-}24/40}$ (resistance to carbapenems) and AbkB/AbkA toxin-antitoxin system in a plasmid.

- To examine the relationship between the AbkB/AbkA toxin-antitoxin system and persister cell formation in the presence of imipenem in this chlorhexidine-tolerant clinical strain of *A. baumannii*.

Chapter 5

- To investigate phenotypic *E. coli* reaction under an overexpression of a toxin cloned in an expression plasmid, from different types of toxin-antitoxin systems.

- To investigate genetic modifications in the bacterial plasmid and genome that lead to inactivation of external toxins.

CHAPTERS

<u>Chapter I.</u> Genome sequence of airborne *Acinetobacter* sp. strain 5-2Ac02 in the hospital environment, close to the species of *Acinetobacter towneri*

Although the atmosphere does not have its own microbiota, it is a means for the dispersion of many types of microorganisms (spores, bacteria, viruses and fungi) from other environments. Some microbes have specialized adaptations that favour their survival and permanence. Aerially dispersed microorganisms are of a great biological and economic importance. They produce diseases in plants, animals and humans, cause alteration of food and organic materials and contribute to the deterioration and corrosion of monuments and metals. Air microbiology was introduced in the nineteenth century by Pasteur and Miquel, who designed methods of studying microorganisms in the air and discovered the cause of some diseases. Since then, numerous researchers have worked in this field focusing both on outdoor air and enclosed spaces. Airborne diseases caused by bacteria, viruses and fungi include respiratory (pneumonia, whooping cough, tuberculosis, legionellosis, cold, flu), systemic (meningitis, measles, chicken pox, mycosis) and allergic diseases (*169*).

Several authors have studied the survival of different microorganisms in aerosols: *Bacillus (170)*, *Escherichia coli* and *Pseudomonas* (*171*) *Corynebacterium*, *Micrococcus*, *Serratia* and *Mycobacterium*, *Staphylococcus*, as well as fungi (*172*), and the influenza virus (*173*).

Information about of the mechanisms of bacterial persistence and tolerance will help to identify the factors that favour the survival of airborne pathogens with the aim of developing infection control strategies with public health and biodefence applications. Several tolerance and persistence mechanisms have been described to date, including the general stress response (RpoS mediated), reactive oxygen species (ROS), energy metabolism, drug efflux pumps, the SOS response, the quorum network (QS/QQ) and TA systems (*22*).

Acinetobacter species are found in almost all environments. In the past decade, researchers began to speculate about the capacity of members of the *Acinetobacter* genus to be transmitted by air (*174*). However, it remains unclear whether the presence of these bacteria in the air occurs as a result of contamination due to the presence of sick patients or merely as a result of the airborne mode of transmission.

Genome sequencing techniques facilitate both phylogenetic and epidemiologic analysis. This type of analysis provides genomic data, thus helping to identify the proteins, involved in the development of tolerance, persistence and resistance.

In the research reported in chapter I, we sequenced and analysed the whole genome of *Acinetobacter* sp. strain 5-2Ac02, which was isolated from air samples from an Intensive Care Unit (ICU) in a hospital in Brazil.

The genome of this strain includes a small chromosome of 2,951,447 base pairs (bp) (the chromosome of *A. baumannii* ATCC 17978 has 4,004,792 bp), with 40.9% G+C content and 2,795 predicted coding sequences (CDSs). Analysis of the resistome revealed the presence of TerC family proteins belonging to the *ter* operon, *klaA* and *klaB* genes from the *kil* operon, arsenic operon genes (this operon distribution has only been described in *Pseudomona stutzeri* TS44) (*175*), and a *bla*$_{OXA-58}$ with an ISAba3-like transposase upstream that blocks its function.

Four different toxin-antitoxin systems have been identified in the persistome: RelE-RelB, ParD-ParE, HipB/HipA and HigB/HigA. Remarkably, analysis of the mobilome revealed that insertion sequences (IS) accounted for 5% of the genome, i.e. a higher level than in clinical strains of *Acinetobacter sp*. Finally, phylogenetic analysis revealed that this strain belongs to a new *Acinetobacter* species closely related to *Acinetobacter towneri*.

Attached is the corresponding paper at Genome Announcements journal:

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Genome Sequence of Airborne *Acinetobacter* sp. Strain 5-2Ac02 in the Hospital Environment, Close to the Species of *Acinetobacter towneri*

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***Acinetobacter* spp. are found in 53% of air colonization samples from the hospital environment. In this work, we sequenced all the genome of airborne *Acinetobacter* sp. strain 5-2Ac02. We found important features at the genomic level in regards to the rhizome. By phylogenetic analysis, *A. towneri* was the species most closely related to *Acinetobacter* sp. 5-2Ac02.**

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Acinetobacter species can be found in any environment: water, soil, body surface, etc., and is one of the most dangerous nosocomial pathogens in the world (1), being found principally in intensive care units (ICUs) (2). In the last few years, it was discovered that members of the genus *Acinetobacter* can be transmitted by air (3), although it is true that air contamination when there are any patients infected is rare. However, some researchers refer to intense *Acinetobacter* species air contamination when there are infected patients around (4, 5). Nowadays, little is known about this method of transmission; therefore, many researchers are studying this transmission method.

In this study, we report the genome sequence of airborne *Acinetobacter* sp. strain 5-2Ac02, which was collected from the air in an ICU of a hospital in Rio de Janeiro, Brazil. Genomic DNA was isolated using the Wizard genomic DNA kit (Promega). Genome sequencing was determined using an Ilumina MiSeq system. The final draft genome was annotated using the RAST server to identify the protein-coding genes, rRNA, and tRNA genes, and to assign functions to these genes. The predicted open reading frames (ORFs) were confirmed using BlastP in the Protein Data Bank (PDB) and COG databases from NCBI and InterProScan. Using the Antibiotic Resistance Database, we could predict the antibiotic resistance genes. The insertion sequences (IS) were analyzed by IS Finder software. Antimicrobial susceptibility testing was performed according to CLSI methods (2014), and disinfectant susceptibility testing was performed by microdilution. Phylogenetic analysis was carried out by average nucleotide identity (ANI), single nucleotide polymorphism analysis (snpTree), and RNA polymerase beta subunit sequence (*rpoB*) analysis. A matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) dendrogram was generated (Bruker Daltonics).

The genome includes a chromosome of 2,951,447 bp, with a 40.9% G+C content. The functional annotation of the circular chromosome showed a total of 2,795 predicted coding sequences (CDSs) and suggests that 1,281 (46%) of these CDSs could be assigned a biological function included in 380 subsystems (RAST software). There were a total of 97 RNAs genes identified. We studied the rhizome of this genome (6). In the analysis of the resistome, we found the presence of TerC family proteins from the *ter* operon (*terZABCDEF*); we also found *klaA* and *klaB* genes from the *kil* operon, which is in association with the previous one, as said by O'Gara et al. (7). One arsenic operon, arsC1-arsR-arsC2-ACR3-arsH was studied. This operon organization has only been described in the *Pseudomona stutzeri* TS44 (8). Moreover, this strain showed an MIC to arsenic of >2,048 mg/liter. Finally, an ISAba*3*-like transposase was present upstream of bla_{OXA-58}, which determines its silence state (MICs to imipenem and meropenem, 0.06 and 0.03 mg/liter, respectively) (9). For the persistome, we found four different toxin-antitoxin systems: RelEB (4 gene cassettes), ParDE, HipBA, and HigBA, with all of them related to persistence, among other things (10). For the mobilome, the percentage of insertion sequences (ISs) in this strain was about 5%, which is higher than that of clinical *Acinetobacter* species. Finally, posterior phylogenetic analysis suggested that *Acinetobacter* sp. 5-2Ac02 is a new species of *Acinetobacter*, with *A. towneri* eing the most closely related one (94.39 by ANI score, 90% by *rpoB* score gene, and 2.127 MALDI-TOF MS score).

Accession number(s). This whole-genome shotgun project has been deposited at DDBJ/ENA/GenBank under the accession no. MKQS00000000. The version described in this paper is version MKQS01000000 (BioProject PRJNA345289).

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<u>Chapter II.</u> Quorum and Light Signals Modulate Acetoin_Butanediol Catabolism in *Acinetobacter* spp.

As mentioned aboved, several tolerance and persistence mechanisms that occur under stress conditions and that favour bacterial survival have been described: the general stress response (RpoS mediated), reactive oxygen species (ROS), energy metabolism, drug efflux pumps, the SOS response, the quorum network (QS/QQ) and TA systems (*22*).

In addition, it has been found that, under stress pressure, some airborne bacteria can produce and disperse multiple small molecules (such as acetoin) called bacterial volatile compounds (BVCs). These compounds are used, among other things, in cross-kingdom interactions (plants, animals and fungi) modulating antibiotic resistance, virulence and even biofilm production (*176*).

Furthermore, QS is related to several virulence mechanisms such as the T6SS system and acetoin/butanediol metabolism (*122*). Acetoin is used for energy storage in fermentative bacteria, protecting the bacteria against cytoplasm and environmental acidification (*124*).

In the research reported in chapter II, we analysed gene expression in the airborne *Acinetobacter sp*. 5-2Ac02 (genome reported in the previous chapter) in response to environmental stress such as the SOS response (induced by mitomycin C) and quorum network (AHL). Analysis of the airborne *Acinetobacter sp*. 5-2Ac02 enabled functional characterization of the negative regulator of the acetoin/butanediol cluster (AcoN protein) in *A. baumannii* and its relation to the quorum network and photoregulation.

Array analysis in the presence of mitomycin C demonstrated the expression of genes involved in the SOS response, 6 types of TA systems, the ROS response and heavy metal resistance genes, the colicin V protein and several mobile elements. Time killing curves constructed for bacteria in the presence of mitomycin C plus ciprofloxacin indicated tolerance due to the slow growth in response to stress (the sensitivity was indicated by the MIC of this antibiotic).

Study of gene expression in the presence of quorum network signals (AHLs mix and 3-Oxo-C12-HSL) revealed overexpression of the aromatic compounds,

biodegradation cluster and, unexpectedly, the acetoin/butanediol cluster genes. This acetoin/butanediol operon was detected in *A. baumannii* ATCC 17978 as well as in 18 strains of *A. baumannii* isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010 (Bioproject PRJNA345289)". The cluster has been described as a metabolic pathway for preventing medium acidification (*122*) and improving resistance to bacterial pollution (*123*). *A. baumannii* is one of the major causes of nosocomial infections worldwide and is mainly isolated from patients in Intensive Care Units (ICUs) (*177, 178*), in whom it causes pneumonia and in some cases bacteraemia (*179, 180*). This pathogen is extremely successful due to its high genetic plasticity (*22, 181*), its resistance to several antimicrobial agents and environmental stress (*22, 182, 183*) and its high level of virulence (*184, 185*).

Interestingly, we observed that in the acetoin/butanediol cluster, the airborne strain *Acinetobacter* sp. 5-2Ac02 lacks one of the genes possessed by *A. baumannii*, AcoN. We therefore analysed the role of this gene as a negative regulator. We used an isogenic mutant of *A. baumannii* for functional characterization of the gene (*acoN*) and constructed growth curves for the strain in the presence of 5mM acetoin. The curve showed that the isogenic mutant grows in a significantly higher ratio than its wild type *A. baumannii* ATCC 17978, thus confirming the function of *acoN* as a negative regulator of the cluster. Moreover, the 2,3-butanediol dehydrogenase protein in the cluster was overexpressed in the presence of 3-Oxo-C12-HSL in the *A. baumannii* ATCC 17978 Δ*acoN* mutant.

We also showed that acetoin catabolism is induced by light, and that the BlsA photoreceptor interacts with and antagonizes the functioning of AcoN, while also integrating a temperature signal. In fact, growth on acetoin was much better supported under blue light than in darkness through BlsA and AcoN proteins. Acetoin catabolic genes were also induced under this condition in a BlsA and AcoN-dependent manner. The opposite behaviour was observed for *blsA* and *acoN* mutants, with BlsA being necessary for the observed induction and AcoN for repression, indicating that BlsA antagonizes AcoN. Finally, Y2H assays indicated that BlsA interacts with AcoN only under blue light but not in darkness. The data support a model in which BlsA interacts with and probably sequesters the acetoin repressor under these conditions, relieving acetoin catabolic genes from repression and leading to temperature-dependent increased growth under blue light. BlsA is

thus a dual regulator, antagonizing the functioning of different transcriptional regulators in darkness but also under blue light, which is a novel finding.

In this part of the research, we undertook the functional characterization of the negative regulator AcoN protein from the acetoin/butanediol cluster in an isogenic *A. baumannii* model and demonstrated that the quorum network modulators as well as light regulate the acetoin catabolic cluster, thus providing a better understanding of the mechanisms used by environmental as well as clinical bacteria.

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Quorum and Light Signals Modulate Acetoin/Butanediol Catabolism in *Acinetobacter* spp.

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Acinetobacter spp. are found in all environments on Earth due to their extraordinary capacity to survive in the presence of physical and chemical stressors. In this study, we analyzed global gene expression in airborne *Acinetobacter* sp. strain 5-2Ac02 isolated from hospital environment in response to quorum network modulators and found that they induced the expression of genes of the acetoin/butanediol catabolism, volatile compounds shown to mediate interkingdom interactions. Interestingly, the *acoN* gene, annotated as a putative transcriptional regulator, was truncated in the downstream regulatory region of the induced acetoin/butanediol cluster in *Acinetobacter* sp. strain 5-2Ac02, and its functioning as a negative regulator of this cluster integrating quorum signals was confirmed in *Acinetobacter baumannii* ATCC 17978. Moreover, we show that the acetoin catabolism is also induced by light and provide insights into the light transduction mechanism by showing that the photoreceptor BlsA interacts with and antagonizes the functioning of AcoN in *A. baumannii*, integrating also a temperature signal. The data support a model in which BlsA interacts with and likely sequesters AcoN at this condition, relieving acetoin catabolic genes from repression, and leading to better growth under blue light. This photoregulation depends on temperature, occurring at 23°C but not at 30°C. BlsA is thus a dual regulator, modulating different transcriptional regulators in the dark but also under blue light, representing thus a novel concept. The overall data show that quorum modulators as well as light regulate the acetoin catabolic cluster, providing a better understanding of environmental as well as clinical bacteria.

Keywords: acetoin, BLSA, AcoN, light, *Acinetobacter*

INTRODUCTION

Acinetobacter baumannii has recently been recognized by the World Health Organization (WHO) as one of the most threatening pathogens deserving urgent action (Tacconelli et al., 2018). With the aid of new taxonomic tools and technological advancements, other members of the *Acinetobacter* genus have also been identified as causative agents of hospital acquired infections and are gaining

clinical relevance (Turton et al., 2010; Karah et al., 2011). Key factors determining their success as pathogens include their extraordinary ability to develop resistance to antimicrobials as well as to persist in the hospital environment despite adverse conditions such as desiccation, lack of nutrients, etc. (McConnell et al., 2013; Spellberg and Bonomo, 2014; Yakupogullari et al., 2016). It is known that some members of the genus can be transmitted by air. In fact, some genotypes of *A. baumannii* have been shown to survive for up to 4 weeks in the air in intensive care units (ICUs) (Yakupogullari et al., 2016). It is becoming increasingly clear, despite not very much studied, the importance of this kind of transmission since it leads to recontamination of already decontaminated surfaces, transmission between patients, airborne contamination of healthcare providers as well as of medical instruments (Spellberg and Bonomo, 2013). We have recently reported the genome sequence of *Acinetobacter* sp. strain 5-2Ac02 (closely related to *Acinetobacter towneri*), which has been recovered from the air in an ICU of a hospital in Rio de Janeiro, Brazil (Barbosa et al., 2016). This strain was shown to harbor a much reduced genome and higher content of insertion sequences than other *Acinetobacter* sp. Moreover, four different toxin–antitoxin (TA) systems as well as heavy metal resistance operons were found encoded in its genome (Barbosa et al., 2016). Interestingly, some bacteria have been shown to produce and release a large diversity of small molecules, including organic and inorganic volatile compounds such as acetoin and 2,3-butanediol (BD), referred as bacterial volatile compounds (BVCs), which can mediate airborne bacterial interactions (Audrain et al., 2015). BVCs can mediate cross-kingdom interactions with fungi, plants, and animals, and can even modulate antibiotic resistance, biofilm formation, and virulence (Audrain et al., 2015).

Several molecular mechanisms have been associated with the development of bacterial tolerance or persistence under stress conditions (environmental or drug-related) (Trastoy et al., 2018). Among these are included the general stress response (RpoS-mediated), tolerance to reactive oxygen species (ROS), energy metabolism, drug efflux pumps, the SOS response, and TA systems, with the quorum network (quorum sensing/quorum quenching) regulating many of them (Trastoy et al., 2018). The finding that many bacterial pathogens are able to sense and respond to light modulating diverse aspects related to bacterial virulence and persistence in the environment is particularly pertinent in this context. Indeed, light has been shown to modulate biofilm formation, motility, and virulence against *C. albicans*, a microorganism sharing habitat with *A. baumannii*, at environmental temperatures in this pathogen. Moreover, light modulates metabolic pathways including trehalose biosynthesis and the phenylacetic acid degradation pathway, antioxidant enzyme levels such as catalase, and susceptibility or tolerance to some antibiotics (Ramirez et al., 2015; Muller et al., 2017). In addition, light induced the expression of whole gene clusters and pathways, including those involved in modification of lipids, the complete type VI secretion system (T6SS), acetoin catabolism, and efflux pumps (Muller et al., 2017). Many of these processes are controlled by BlsA, the only canonical photoreceptor codified in the genome of *A. baumannii*, which is a short blue light using flavin (BLUF) protein. BlsA has been shown to function at moderate temperatures such as 23°C but not at 37°C by a mechanism that includes control of transcription as well as photoactivity by temperature (Mussi et al., 2010; Abatedaga et al., 2017; Tuttobene et al., 2018). Knowledge of these mechanisms will potentially enable the implementation of several clinical or industrial applications.

In this study, we characterized the airborne *Acinetobacter* sp. strain 5-2Ac02, analyzing gene expression adjustments in response to environmental stressors such as mitomycin C and acyl-homoserine-lactones, which modulate the quorum network. The results showed that genes involved in the SOS response, TA systems, and heavy metal resistance were induced in response to mitomycin, while genes involved in acetoin and aromatic amino acid catabolism were modulated as a response to quorum sensing signals. The fact that acetoin catabolic genes were also found to be induced by light in *A. baumannii* (Muller et al., 2017) prompted us to deepen the study on this metabolism. In bacteria, the butanediol fermentation is characterized by the production of BD and acetoin from pyruvate. The production of butanediol is favored under slightly acidic conditions and is a way for the bacteria to limit the decrease in external pH caused by the synthesis of organic acids from pyruvate. The catabolic α-acetolactate-forming enzyme (ALS) condenses two molecules of pyruvate to form one α-acetolactate, which is unstable and can be converted to acetoin by α-acetolactate decarboxylase (ALDC) or diacetyl as a minor by-product by non-enzymatic oxidative decarboxylation. Diacetyl can be irreversibly transformed into its reductive state acetoin, and acetoin can be reversibly transformed into its reductive state BD, both catalyzed by 2,3-butanediol dehydrogenase (BDH). The acetoin breakdown in many bacteria is catalyzed by the acetoin dehydrogenase enzyme system (AoDH ES), which consists of acetoin:2,6-dichlorophenolindophenol oxidoreductase, encoded by *acoA* and *acoB*; dihydrolipoamide acetyltransferase, encoded by *acoC*; and dihydrolipoamide dehydrogenase, encoded by *acoL* (Xiao and Xu, 2007). Our results show that the *acoN* gene codes for a negative regulator of the acetoin/butanediol catabolic cluster and is involved in photoregulation of acetoin catabolism in *A. baumannii* through the BlsA photoreceptor. Most importantly, we provide strong evidence on the mechanism of light signal transduction, which is far from being understood for BlsA or other short BLUF photoreceptors, taking into account in addition that BlsA is a global regulator in *A. baumannii*. In this sense, we have recently shown that this photoreceptor binds to and antagonizes the functioning of the Fur repressor only in the dark at 23°C, presumably by reducing its ability to bind to acinetobactin promoters, thus relieving repression at the transcriptional level as well as growth under iron limitation at this condition (Tuttobene et al., 2018). Here, we further show that BlsA directly interacts with the acetoin catabolism negative regulator AcoN at 23°C but, in this case, in the presence of blue light rather than in the dark. In fact, growth on acetoin was much better supported under blue light than in the dark through BlsA and AcoN. Moreover, acetoin catabolic genes were induced at this condition in a BlsA- and AcoN-dependent manner. Opposite behavior was observed for Δ*blsA* and Δ*acoN* mutants, being BlsA necessary

for the observed induction while AcoN for repression, thus indicating that BlsA antagonizes AcoN. Finally, yeast two-hybrid (Y2H) assays indicate that BlsA interacts with AcoN only under blue light but not in the dark. The results strongly suggest that BlsA interacts with and likely sequesters the acetoin repressor under blue light but not in the dark. Thus, in the presence of light, acetoin catabolic genes are relieved from repression resulting in much better bacterial growth in this condition. Here again, the phenomena depends on temperature, occurring at low–moderate temperatures such as 23°C but not at 30°C, consistent with previous findings of our group for BlsA functioning (Mussi et al., 2010; Abatedaga et al., 2017; Tuttobene et al., 2018).

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

Bacterial strains and plasmids used in this work are listed in **Table 1**. Luria-Bertani (LB) broth (Difco) and agar (Difco) were used to grow and maintain bacterial strains. Broth cultures were incubated at the indicated temperatures either statically or with shaking at 200 rpm.

TABLE 1 | Minimal inhibitory concentrations (MICs) of several antibiotics and heavy metals for *Acinetobacter* sp. strain 5-2Ac02.

Antimicrobial	MIC (μg/mL)	Category[a]
Sulbactam	0.5	Susceptible
Piperacillin	0.06	Susceptible
Ceftazidime	4	Susceptible
Imipenem	0.06	Susceptible
Meropenem	0.03	Susceptible
Doripenem	0.015	Susceptible
Ciprofloxacin	1	Susceptible
Amikacin	1	Susceptible
Gentamicin	0.25	Susceptible
Tobramycin	0.5	Susceptible
Netilmicin	0.25	Susceptible
Tetracycline	0.5	Susceptible
Minocycline	<0.002	Susceptible
Doxycycline	0.03	Susceptible
Tigecycline	0.25	Susceptible
Colistin	0.125	Susceptible
Clavulanic acid	4	Susceptible
Azithromycin	16	Susceptible
Heavy metal	**MIC (μg/mL)**	**Category[b]**
Arsenic	>2048	**Resistant**
Cadmium	64	Susceptible
Cobalt	16	Susceptible
Cooper	266	**Resistant**
Chromium	128	Susceptible
Tellurite	2	Susceptible
Zinc	256	Susceptible

[a]MICs were determined by the microdilution method, in accordance with general procedures recommended by CLSI. For specific details, please refer to the section "Materials and Methods." [b]Determined according to Taylor et al. (2002) and Akinbowale et al. (2007). In bold are shown the resistance category.

Plasmid Construction

Y2H Plasmid Construction

PCR amplification of *blsA* and *acoN* coding sequences was performed from *A. baumannii* ATCC 17978 genomic DNA using primers blsAdh and acoNdh (**Supplementary Table S2**). The amplification products were subsequently cloned into the BamHI and XhoI sites of Gateway entry vector pENTR3C (Invitrogen) (**Supplementary Table S1**). The cloned fragments were then transferred to pGBKT7-Gw and pGADT7-Gw Y2H vectors (Clontech) by using LR Clonase (Cribb and Serra, 2009; Tuttobene et al., 2018). In the yeast host, these plasmids express the cloned coding sequences as fusion proteins to the GAL4 DNA-binding domain (DB) or activation domain (AD), respectively, under the control of the constitutive ADH1 promoter. Automated DNA sequencing confirmed correct construction of each plasmid.

pWHAcoN Plasmid Construction

acoN coding sequence and its promoter were amplified by PCR using *A. baumannii* ATCC 17978 genomic DNA as template and primers PAcoNF and PAcoNR (**Supplementary Table S2**), which contained BamHI restriction site tails. The amplification product was cloned into pWH1266 through the BamHI sites. Automated DNA sequencing confirmed the proper construction of pWHAcoN plasmid.

Susceptibility to Antimicrobials and Heavy Metals (MICs)

The antibiotic and heavy metal susceptibility profile by microdilution was determined according to CLSI recommendations (**Table 1**). Heavy metal susceptibility was determined by broth microdilution following CLSI instructions for cobalt, chromium, copper, arsenic, and zinc (Akinbowale et al., 2007). The susceptibility to tellurite was determined by serial plate dilution, with concentrations ranging from 1 to 1024 μg/mL *Escherichia coli* K12 were used as reference strain (Akinbowale et al., 2007). The breakpoints adopted for resistance phenotype were as follows: ≥100 μg/mL for cadmium; ≥200 μg/mL for copper, arsenic, and zinc; ≥400 μg/mL for cobalt; ≥800 μg/mL for chromium; and >128 μg/mL for tellurite.

Growth curves in the presence of heavy metals were performed as follow: one colony of *Acinetobacter* sp. strain 5-2Ac02 was grown overnight, diluted 1:100 in 20 mL of low nutrient LB broth, and incubated at 37°C with shaking (180 rpm) (Lopez et al., 2018). The cultures were grown for 4 h to the exponential phase; and then, the heavy metals were added. For each isolate, the proportion of survivors was determined: (i) in the control without heavy metals, (ii) in the presence of arsenic (0.50× MIC), (iii) in the presence of cupper (0.5× MIC). Bacterial concentrations ($\log_{10}$ CFU/mL) were determined at 0, 2, 4, 24, and 48 h by serial dilution and plating on LB agar. All experiments were performed in duplicate.

Gene Expression by Microarrays Under Stress Conditions: Mitomycin and AHLs

Acinetobacter sp. strain 5-2Ac02 cells were grown in LB medium to an exponential phase about OD_{600} = 0.5 before addition of 10 μg/mL of mitomycin C (SOS response) or a mixture of 1 μM each acyl-homoserine lactones composed by *N*-(butyl, heptanoyl, hexanoyl, β-ketocaproyl, octanoyl, and tetradecanoyl)-DL-homoserine lactones or 10 μM 3-oxo-dodecanoyl-HSL (3-oxo-C12-HSL) (Quorum Network). After incubation of the mixtures for 2 h, 1 mL of each culture was used for RNA extraction. RNA was purified using the High Pure RNA Isolation Kit (Roche, Germany). The microarrays were specifically designed for this strain using eArray (Agilent). The microarray assays were performed with 12,664 probes to study 2,795 genes. Labeling was carried out by two-color microarray-based prokaryote analysis and Fair Play III labeling, version 1.3 (Agilent). Three independent RNAs per condition (biological replicates) were used in each experiment. Statistical analysis was carried out using Bioconductor, implemented in the RankProd software package for the R computing environment. A gene was considered induced when the ratio of the treated to the untreated preparation was 1.5 and the *p*-value was <0.05 (Lopez et al., 2017b).

Bacterial Killing Curves

The MICs of ampicillin, ciprofloxacin, and mitomycin C were determined for *Acinetobacter* sp. strain 5-2Ac02 (0.5, 1, and 0.5 μg/mL) versus *A. baumannii* strain ATCC 17978 (8, $\leq$0.12, and 2 μg/mL). Briefly, an initial inoculum of 5×10^5 CFU/mL was incubated at 37°C with shaking (250 rpm) in 20 mL of low nutrient LB broth (LN-LB; 2 g/L tryptone, 1 g/L yeast extract, and 5 g/L NaCl) (Lopez et al., 2017a,b). The cultures were grown for 4 h to the exponential phase; and then, the antibiotics were added. For each isolate, the proportion of survivors was determined: (i) in the control without antibiotic, (ii) in the presence of mitomycin C (0.25× MIC), (iii) in the presence of ampicillin (10× MIC), (iv) in the presence of ciprofloxacin (10× MIC), (v) in the presence of mitomycin C and ampicillin (0.25× MIC and 10× MIC), and (vi) in the presence of mitomycin C and ciprofloxacin (0.25× MIC and 10× MIC). Bacterial concentrations ($\log_{10}$ CFU/mL) were determined at 0, 1, 2, 3, 4, 20, 24, 28, and 48 h by serial dilution and plating on Mueller-Hinton agar. All experiments were performed in triplicate. This protocol was performed following previously described indications (Hofsteenge et al., 2013). Finally, the persister sub-population was determined from the percentage of survivors.

Gene Deletion in *A. baumannii* ATCC 17978

The negative regulator of the acetoin operon was deleted following the double recombination method, using the pMO-TelR plasmid and *E. coli* DH5α strain to multiply the plasmid with the construct (Hamad et al., 2009; Aranda et al., 2010). All primer sequences used were designed in this study and are listed in **Supplementary Table S2**.

Isolation of RNA and Analyses of Genes Expression by qRT-PCR

Acinetobacter baumannii cells were grown stagnantly in LN-LB at 37°C with the addition of 10 μM of 3-oxo-C12-HSL or 10 μM of 3-hydroxy-dodecanoyl-HSL (3-OH-C12-HSL) when appropriate, or in M9 liquid medium supplemented with 15 mM acetoin as carbon source at 23 or 30°C until an OD_{600} of 0.4–0.6 was reached, as indicated. RNA extraction and qRT-PCR were performed following procedures described in Lopez et al. (2018) and Tuttobene et al. (2018). Results are informed as normalized relative quantities (NRQs) calculated using qBASE (Hellemans et al., 2007), with *recA* and *rpoB* genes as normalizers (Muller et al., 2017). The UPL Taqman Probes (Universal Probe Library-Roche, Germany) and primers used are listed in **Supplementary Table S3**.

Growth in the Presence of Acetoin

Wild-type and derivative strains *A. baumannii* ATCC 17978 were grown on acetoin as the sole carbon source. To test the ability of the *A. baumannii* strains used in this work to grow on acetoin as the sole carbon source, 1/100 dilutions of overnight cultures grown in LB Difco were washed and inoculated in M9 liquid medium supplemented with 5, 10, or 15 mM acetoin or in LB Difco medium and grown without shaking, under blue light or in the dark at 23 or 30°C. Aliquots were removed at the times indicated in the figures in order to measure the A660 of the culture.

Blue Light Treatments

Blue light treatments were conducted as reported before (Mussi et al., 2010; Golic et al., 2013; Abatedaga et al., 2017; Muller et al., 2017; Tuttobene et al., 2018). Briefly, cells were grown in the dark or under blue light emitted by an array composed of 3 × 3-LED module strips emitting an intensity of 6–10 μol photons/m^2/s, with emission peaks centered at 462 nm (Mussi et al., 2010).

Yeast Two-Hybrid (Y2H) Assays

Yeast two-hybrid experiments were conducted following procedures described before (Cribb and Serra, 2009; Tuttobene et al., 2018). *Saccharomyces cerevisiae* Mav 203 strain (MATa, *leu*2-3,112, *trp*1-901, *his*3-D200, *ade*2-101, *gal*4D, *gal*80D, SPAL10::URA3, GAL1::*lacZ*, HIS3UAS GAL1::HIS3, LYS2, *can*1R, and *cyh*2R) was transformed with the different expression vectors. First, BlsA and AcoN were analyzed for self-activation. For this purpose, MaV203 yeast strain containing the pGAD-T7 empty vector was transformed with the DNA DB-fusion protein expressing vectors (pGBK-X) (X = BlsA or AcoN). Conversely, MaV203 yeast strain containing the pGBK-T7 empty vector was then transformed with the AD-fusion protein expressing vectors (pGAD-Y) (Y = BlsA or AcoN). In addition, these strains were used for determination of the optimal 3-amino-1,2,4-triazole (3AT) concentration required to titrate basal *HIS3* expression. MaV203/pGBK-X strains were afterward transformed with each pGAD-Y plasmids. Transformations using one or both Y2H plasmids were performed by the lithium acetate/single-stranded carrier DNA/polyethylene glycol method described in Gietz and Woods (2002), and plated in convenient minimal selective

medium [synthetic complete (SC) medium without leucine (-leu) for pGAD-Y transformants, SC without tryptophan (-trp) for pGBK-X transformants, and SC-leu-trp transformants carrying both plasmids]. The plates were then incubated at 23°C for 72 h to allow growth of transformants. A "Master Plate" was then prepared using SC-leu-trp media, in which we patched: four to six clones of each pGBK-X/pGAD-Y containing yeasts, four to six self-activation control clones pGBK-X/pGAD and pGBK/pGAD-Y (Y DNA-binding negative control), and two isolated colonies of each of the five yeast control strains (A–E). The plates were incubated for 48–72 h at 23°C. This Master Plate was then replica plated to SC–leu–trp–his+3AT and to SC–leu–trp–ura to test for growth in the absence of histidine (*his*) and uracil (*ura*), respectively (*his*3 and *ura*3 reporter activation), under the different conditions analyzed, i.e., dark/light; 23/30°C, for at least 72 h. For development of blue color as a result of β-galactosidase (β-Gal) expression, transformed yeasts were replica plated on a nitrocellulose filter on top of a YPAD medium plate and grown at the different conditions (dark/light; 23/30°C). Then, the cells on the nitrocellulose filter were permeabilized with liquid nitrogen and soaked in X-Gal solution (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, pH 7.0) maintaining the different incubation conditions to be tested.

Accession Numbers

The genome of the *Acinetobacter* sp. 5-2Ac02 is deposited in GenBank database (GenBank accession number MKQS00000000; Bioproject PRJNA345289). The genome of *A. baumannii* ATCC17978 is deposited in GenBank (accession number CP018664.1). Finally, the gene expression microarray results are deposits in GEO database (GEO accession number GSE120392).

RESULTS

Transcriptome Adjustments in Response to Mitomycin C Show Induction of Defense and Stress Response Systems in *Acinetobacter* sp. Strain 5-2Ac02

The airborne *Acinetobacter* sp. 5-2Ac02 isolate was first characterized to learn about its antibiotic as well as heavy metal susceptibility profiles, since its genome harbored genes of the *ter* (tellurite resistance) operon (*terZABCDEF*); *klaA* and *klaB* genes from the *kil* operon, which is associated with the previous one (O'Gara et al., 1997); as well as the arsenic-resistance operon *arsC1-arsRarsC2-ACR3*-arsH (**Table 1**). The data presented in **Table 1** show that *Acinetobacter* sp. 5-2Ac02 is susceptible to all antibiotic tested but resistant to copper as well as to arsenic, as previously reported (Barbosa et al., 2016). This information was confirmed by growth curves in the presence of these heavy metals (**Supplementary Figure S1**).

Arrays performed in the presence of the stressor mitomycin C revealed induction of SOS genes such as those coding for recombinases, polymerases, as well as DNA repair proteins, all with a fold change (FC) > 3 in *Acinetobacter* sp. strain 5-2Ac02. Also, genes coding for components of six TA systems were found to be induced with a FC > 4.9 in all cases: the RelBE systems (x2), the HigBA system, the ParDE system, and two new putative systems (x2). The data also showed induction of genes involved in heavy metal resistance genes, among which can be highlighted cobalt–zinc–cadmium, copper, and arsenic resistance genes. In addition, the gene coding for colicin V protein was induced with a FC of 3.716 (**Table 2**). Finally, many mobile element genes, which are extraordinarily abundant in the genome of *Acinetobacter* sp. 5-2Ac02 strain, were also induced (not shown).

The TA systems have been shown to be involved both in tolerance and persistence (Fernandez-Garcia et al., 2018). We next analyzed the fraction of tolerant or persister cells in populations of *Acinetobacter* sp. strain 5-2Ac02 by determining the time-kill responses in the presence of ampicillin, ciprofloxacin, mitomycin C, and combinations of these (**Figure 1**), following protocols described in Hofsteenge et al. (2013). The data show a large decrease in colonies of *Acinetobacter* sp. strain 5-2Ac02 during the first 24 h in the presence of ampicillin, ciprofloxacin, as well as in the presence of the combination of ampicillin and mitomycin C. Interestingly, the presence of a combination of mitomycin C with ciprofloxacin showed a tolerant population displaying slow growth at 4, 24, and 48 h (**Figure 1**) under this stress condition, which may result from activation of defense mechanisms such as the toxins and antitoxins systems as well as SOS response.

Quorum Sensing Signals Modulate Expression of the Acetoin/Butanediol Catabolic Cluster in *Acinetobacter* spp., Being AcoN a Negative Regulator in *A. baumannii*

Array expression studies of *Acinetobacter* sp. 5-2Ac02 in the presence of a mixture of *N*-acyl-homoserine lactones (AHLs) or 3-oxo-C12-HSL, which are modulators of the quorum network in *A. baumannii* (Lopez et al., 2018), indicated induction of the acetoin/butanediol catabolic pathway genes, each with a FC > 1.5 (**Tables 3**, **4**, respectively). We show the genomic arrangement of this cluster in the genomes of *Acinetobacter* sp. 5-2Ac02 and *A. baumannii* ATCC 17978 strain (**Figures 2A,B**). The same genomic configuration in *A. baumannii* strain ATCC 17978 was observed in 18 clinical *A. baumannii* strains isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000–2010" which included 45 Spanish hospitals with 246 patients (GenBank Umbrella Bioproject PRJNA422585) (**Supplementary Table S4**).

Ten genes were identified in the ATCC 17978 cluster, likely coding for a putative transcriptional regulator (gene 1) followed by a putative lipoyl synthase (gene 2), two oxidoreductases homologous to *acoA* and *acoB* (genes 3 and 4), a deaminase homologous to *acoC* (gene 5), a dehydrogenase homologous to *acoD* (gene 6), a BDH reductase (gene 7), and a BDH (gene 8), all of which are followed by a hypothetical protein (gene 9) and a putative transcriptional regulator (gene 10) (**Figure 2A**). Gene 2 is homologous to *acoK* (**Figure 2A**) and gene 1 is

TABLE 2 | Gene expression adjustments in response to mitomycin C in *Acinetobacter* sp. strain 5-2Ac02.

Protein ID (RAST server)[a]	Description	Fold change	System	Mechanism
202956.5.peg.1643	*recA*	5.7671	Recombinases	SOS response
202956.5.peg.129	*recT*	25.3753		
202956.5.peg.1972	*recF*	13.1351		
202956.5.peg.2285	*umuD*	5.0572	Polymerases V	
202956.5.peg.1220	*umuD*	21.6016		
202956.5.peg.2284	*umuC*	3.7093		
202956.5.peg.1221	*umuC*	4.1229		
202956.5.peg.1236	*rmlC-like cupin*	29.8694	DNA repair protein	
202956.5.peg.2036	*rmlC-like cupin*	10.1388		
202956.5.peg.274	*relB*	5.3331	RelEB system	Toxin–antitoxin modules
202956.5.peg.2563	*relB*	7.4618		
202956.5.peg.275	*relE*	5.8337		
202956.5.peg.2564	*relE*	7.8490		
202956.5.peg.411	*higA*	7.3751	HigBA system	
202956.5.peg.412	*higB*	14.9347		
202956.5.peg.2515	Antitoxin	6.7492	New putative TA system	
202956.5.peg.2516	Toxin	9.5133		
202956.5.peg.2550	Antitoxin	8.6098		
202956.5.peg.2551	Toxin	10.5546		
202956.5.peg.797	*parD*	5.8254	ParDE system	
202956.5.peg.796	*parE*	4.9563		
202956.5.peg.984	*aphC*	2.3370	Reductase	Oxidant tolerance (ROS response)
202956.5.peg.319	*rpoS* regulon	28.4676	Regulatory system	
202956.5.peg.1019	*arsC* (arsenate reductase)	3.7665	Reductase	Heavy metals resistance
202956.5.peg.1000	*copA* (*copD*)	2.1898	Copper resistance operon	
202956.5.peg.1009	*copB*	2.3034		
202956.5.peg.1001	*copC*	3.2015		
202956.5.peg.2458	*czcA*	4.6191	Cobalt–zinc–cadmium resistance operon	
202956.5.peg.1476	*czcD*	3.1468		
202956.5.peg.2744	*cvpA* (colicin V)	3.7163	Bacteriocin protein	Antibiotic peptides

Genes showing FC >2 are indicated. [a]*RAST server was used to identify the protein-coding genes, rRNA and tRNA genes, and to assign predictive functions to these genes.*

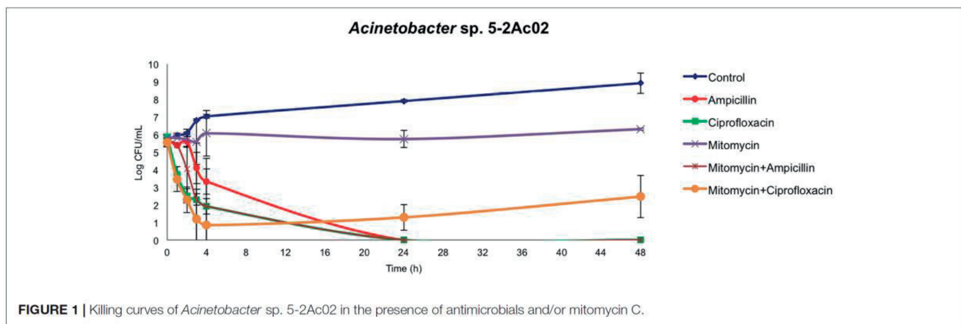

FIGURE 1 | Killing curves of *Acinetobacter* sp. 5-2Ac02 in the presence of antimicrobials and/or mitomycin C.

TABLE 3 | Expression of genes in *Acinetobacter* sp. strain 5-2Ac02 by quorum network molecules (AHLs mix).

Protein ID (RAST server)[a]	Gene/predicted protein description	Fold change	System	Mechanism
202956.5.peg.1419	*acoA*/acetoin dehydrogenase E1 alpha-subunit	3.9966	Acetoin/butanediol cluster (degradation)	QS system
202956.5.peg.1420	*acoB*/acetoin dehydrogenase E1 beta-subunit	3.7291		
202956.5.peg.1421	*acoC*/dihydrolipoamide acetyltransferase (E2) acetoin	3.6752		
202956.5.peg.1422	*acoD*/dihydrolipoamide dehydrogenase of acetoin dehydrogenase	3.3919		
202956.5.peg.1423	2,3-BDH/2,3-butanediol dehydrogenase, S-alcohol forming, (S)-acetoin-specific	2.9770		
202956.5.peg.1424	2,3-BDH/2,3-butanediol dehydrogenase, R-alcohol forming, (R)- and (S)-acetoin-specific	1.9456		
202956.5.peg.2091	1,2-Dihydroxycyclohexa-3,5-diene-1-carboxylate dehydrogenase	2.4396	Aromatic compounds biodegradation cluster	QS system
202956.5.peg.2092	Benzoate dioxygenase, ferredoxin reductase	2.8198		
202956.5.peg.2093	Benzoate 1,2-dioxygenase beta-subunit	3.2909		
202956.5.peg.2094	Benzoate 1,2-dioxygenase alpha-subunit	3.3731		
202956.5.peg.2095	Catechol 1,2-dioxygenase	3.5774		
202956.5.peg.2096	Muconolactone isomerase	3.1273		

[a] *RAST server was used to identify the protein-coding genes, rRNA and tRNA genes, and to assign functions to these genes.*

homologous to a positive transcriptional regulator (activator) homologous to *acoR* in different organisms (**Figure 2A**). The genomic configuration in *Acinetobacter* sp. strain 5-2Ac02 is similar to that of ATCC 17978 except that genes coding for the hypothetical protein and the putative transcriptional regulator (9 and 10 in ATCC 17978, respectively) are absent, while three genes coding for putative transposases were identified following gene 8 (**Figure 2B**).

Finally, in presence of the AHL mixture, the arrays also revealed increased expression (FC > 2) of genes involved in biodegradation of aromatic compounds (**Table 4**).

We suspected that the absence of the putative transcriptional regulator in *Acinetobacter* sp. strain 5-2Ac02, designated as gene 10 in the genome locus of *A. baumannii* ATCC17978 (**Figure 2A**) and renamed here from now on as *acoN*, might be responsible for the induced expression of the acetoin catabolic genes in response to quorum network signals.

We reasoned that whether this was the case, then a knockout mutant in *acoN* in *A. baumannii* ATCC 17978, which would resemble the situation in the so far genetically intractable *Acinetobacter* sp. strain 5-2Ac02, would result in induction of the acetoin catabolic genes in the presence of quorum sensing signals. As can be observed in **Figure 3**, the presence of quorum sensing signals resulted in induction of the transcript levels of BDH (*bdh*, acetoin/butanediol cluster) (RE > twofold) in the *A. baumannii* ATCC 17978 Δ*acoN* mutant with respect to the wild-type strain. This provides the first clue that AcoN functions as a negative regulator of acetoin catabolic genes.

Further studies showed that the Δ*acoN* mutant grew much better in media supplemented with acetoin (5 mM) as sole carbon source than the wild-type strain in the dark at 23°C (**Figure 4A**), which barely grew at this condition. The Δ*acoN* mutant containing the pWHAcoN plasmid, which expresses *acoN* directed from its own promoter, behaved as the wild type showing a reduced ability to grow on acetoin as sole carbon source at 23°C in the dark, restoring therefore the wild-type phenotype (**Figure 4B**). Similar results were obtained at 30°C and are discussed later in the manuscript. These results provide further evidence of the role of AcoN gene as a negative regulator of the acetoin catabolic cluster.

Finally, expression of acetoin catabolic genes such as *acoA*, *acoB*, and *acoC* was induced approximately 150-folds in the Δ*acoN* mutant with respect to the wild type at 23°C in the dark (**Figure 5**). These results confirm the functioning of AcoN as a negative regulator of the acetoin catabolic pathway in *A. baumannii*.

Light Modulates Acetoin Catabolism Through BlsA and AcoN at Moderate Temperatures in *A. baumannii*

Acetoin catabolic genes such as *acoA*, *acoB*, *acoC*, and *acoD* have been previously shown to be induced by light at moderate temperatures in *A. baumannii* ATCC 19606 by RNA-seq studies (Muller et al., 2017). We thus studied whether light modulated acetoin catabolism in ATCC 17978 at 23°C and found a differential ability of this strain to grow in the presence of acetoin as sole carbon source between light and dark conditions (**Figure 4** and **Supplementary Figure S2**).

Figure 4A shows that *A. baumannii* ATCC 17978 grows much poorer in 5 mM acetoin in the dark rather than under blue light at 23°C. The Δ*blsA* mutant, which lacks the only traditional photoreceptor encoded in the *A. baumannii* genome, behaved as the wild type in the dark both under blue light or in the dark (**Figure 4A**), as also did the mutant containing the empty vector pWH1266 (**Figure 4B**). In contrast, the Δ*blsA* mutant containing pWHBlsA, which expresses *blsA* directed from its own promoter, grew better on acetoin under blue light than in the dark, restoring thus the wild-type phenotype (**Figure 4B**). The Δ*acoN* mutant,

TABLE 4 | Expression of genes in *Acinetobacter* sp. strain 5-2Ac02 by quorum network molecules (3-oxo-C12-HSL).

Protein ID (RAST server)[a]	Gene/predicted protein description	Fold change	System	Mechanism
202956.5.peg.1419	*acoA*/acetoin dehydrogenase E1 alpha-subunit	2.3803	Acetoin/butanediol cluster	QS system
202956.5.peg.1420	*acoB*/acetoin dehydrogenase E1 beta-subunit	2.5212		
202956.5.peg.1421	*acoC*/dihydrolipoamide acetyltransferase (E2) of acetoin dehydrogenase complex	2.7127	Acetoin/butanediol cluster	QS system
202956.5.peg.1422	*acoD*/dihydrolipoamide dehydrogenase of acetoin dehydrogenase	2.2546		
202956.5.peg.1423	2,3-BDH/2,3-butanediol dehydrogenase, S-alcohol forming, (S)-acetoin-specific	2.0217		
202956.5.peg.2196	Aminoacid transporter	3.6649		Others
202956.5.peg.2630	Short-chain dehydrogenase	2.8179		
202956.5.peg.2505	Amide	2.6736		
202956.5.peg.2388	Transporter (DMT) superfamily	2.6240		
202956.5.peg.2137	Alcohol dehydrogenase	2.5237		
202956.5.peg.517	Ribonucleotide reductase	2.3017		
202956.5.peg.2100	dcaP	2.2854		
202956.5.peg.1418	Lipoate synthase	2.2626		
202956.5.peg.691	Monooxygenase	2.1241		
202956.5.peg.2775	Cyclic AMP receptor	2.0731		
202956.5.peg.2753	*cspA*	2.0635		
202956.5.peg.451	NAD(P)	2.0352		
202956.5.peg.434	Protein-export membrane protein SecD	2.0152		
202956.5.peg.1121	Putrescine importer	2.0026		

[a]*RAST server was used to identify the protein-coding genes, rRNA and tRNA genes, and to assign functions to these genes.*

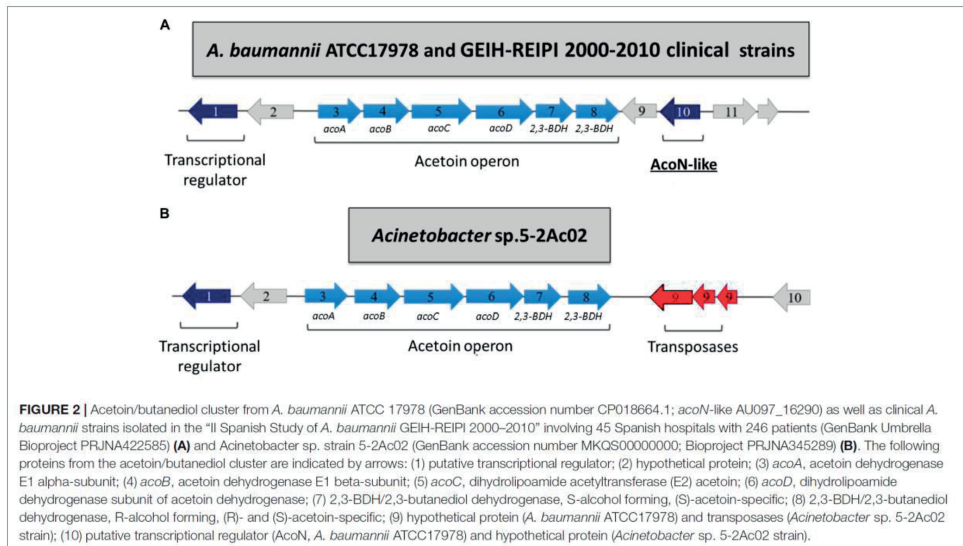

FIGURE 2 | Acetoin/butanediol cluster from *A. baumannii* ATCC 17978 (GenBank accession number CP018664.1; *acoN*-like AU097_16290) as well as clinical *A. baumannii* strains isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000–2010" involving 45 Spanish hospitals with 246 patients (GenBank Umbrella Bioproject PRJNA422585) **(A)** and Acinetobacter sp. strain 5-2Ac02 (GenBank accession number MKQS00000000; Bioproject PRJNA345289) **(B)**. The following proteins from the acetoin/butanediol cluster are indicated by arrows: (1) putative transcriptional regulator; (2) hypothetical protein; (3) *acoA*, acetoin dehydrogenase E1 alpha-subunit; (4) *acoB*, acetoin dehydrogenase E1 beta-subunit; (5) *acoC*, dihydrolipoamide acetyltransferase (E2) acetoin; (6) *acoD*, dihydrolipoamide dehydrogenase subunit of acetoin dehydrogenase; (7) 2,3-BDH/2,3-butanediol dehydrogenase, S-alcohol forming, (S)-acetoin-specific; (8) 2,3-BDH/2,3-butanediol dehydrogenase, R-alcohol forming, (R)- and (S)-acetoin-specific; (9) hypothetical protein (*A. baumannii* ATCC17978) and transposases (*Acinetobacter* sp. 5-2Ac02 strain); (10) putative transcriptional regulator (AcoN, *A. baumannii* ATCC17978) and hypothetical protein (*Acinetobacter* sp. 5-2Ac02 strain).

both under blue light and in the dark, behaved as the wild type under blue light, i.e., showed enhanced growth with respect to the wild type in the dark, congruent with the absence of the negative regulator (**Figure 4A**); as also did the Δ*acoN* mutant containing pWH1266 (**Figure 4B**). The Δ*acoN* mutant containing pWHAcoN, which expresses *acoN* directed from its

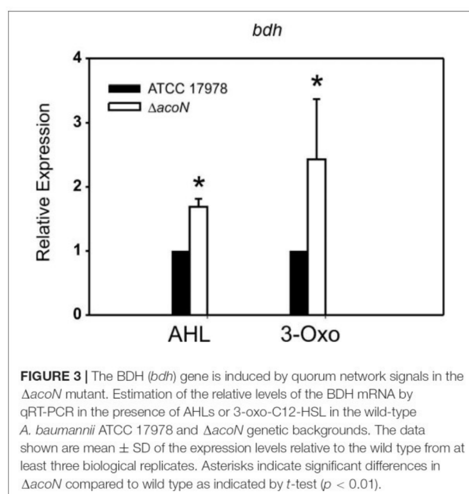

FIGURE 3 | The BDH (*bdh*) gene is induced by quorum network signals in the Δ*acoN* mutant. Estimation of the relative levels of the BDH mRNA by qRT-PCR in the presence of AHLs or 3-oxo-C12-HSL in the wild-type *A. baumannii* ATCC 17978 and Δ*acoN* genetic backgrounds. The data shown are mean ± SD of the expression levels relative to the wild type from at least three biological replicates. Asterisks indicate significant differences in Δ*acoN* compared to wild type as indicated by *t*-test ($p < 0.01$).

own promoter, grew better on acetoin under blue light than in the dark, therefore restoring the wild-type phenotype (**Figure 4B**). Similar results were obtained when acetoin 10 and 15 mM was used as sole carbon source (**Supplementary Figure S2**).

These results show that light modulation of acetoin catabolism depends on the BlsA photoreceptor and the AcoN negative regulator in *A. baumannii* ATCC 17978. Opposite behavior is observed for Δ*blsA* and Δ*acoN* mutants regarding modulation of growth on acetoin by light, indicating that BlsA is necessary for the observed induction, while AcoN for repression. The overall evidence prompts us to postulate a model in which BlsA interacts with AcoN under blue light at 23°C antagonizing this repressor, with the concomitant induction of acetoin catabolic genes' expression as well as growth on acetoin in this condition. It is important to mention that the viability of cells was not affected by light, as similar growth curves were obtained for the different strains in the complex media LB under blue light and in the dark (**Figures 4C,D**).

Light Regulates Expression of the Acetoin Catabolic Pathway Through BlsA and AcoN at Moderate Temperatures in *A. baumannii*

We then monitored AcoN functioning in response to light by measuring the expression of AcoN-regulated genes under different illumination conditions and genetic backgrounds. To this end, the expression of the acetoin catabolic genes *acoA*, *acoB*, and *acoC* (**Figures 5A–C** respectively) was analyzed by qRT-PCR at different light conditions at moderate temperatures in *A. baumannii* strain ATCC 17978. Our results show that the expression levels of these genes were basal in the dark at 23°C in M9 minimal medium with acetoin as sole carbon source. However, their expression was significantly induced in the presence of blue light (**Figure 5**). In Δ*blsA* mutants, expression of *acoA–C* genes was basal and comparable between blue light and dark, and similar to that observed for the wild type in the dark at 23°C (**Figure 5**). Thus, light modulates the expression of the acetoin catabolic genes, *acoA–C* through BlsA. On its side, the Δ*acoN* mutant also lost photoregulation, i.e., expression levels of *acoA–C* genes were similar between the illuminated or dark conditions. However, for this mutant, expression levels were much higher even than those registered in the wild-type under blue light, i.e., in the induced condition (**Figure 5**). Indeed, *acoA* expression levels in the Δ*acoN* mutant were approximately twofold higher than in the wild type under blue light, while *acoB* and *acoC* expression levels were about threefold higher, and >100-folds higher than the wild type in the dark. Opposite behavior is observed for Δ*blsA* and Δ*acoN* mutants regarding modulation of *acoA–C* genes' expression, suggesting that BlsA is necessary for the observed induction while AcoN for repression. Altogether, BlsA antagonizes the functioning of AcoN under blue light at 23°C, with the concomitant induction of the expression of AcoN-regulated genes at this condition. By analogy with a mechanism described previously for BlsA and Fur (Tuttobene et al., 2018), we hypothesized that BlsA might interact with the AcoN negative regulator, antagonizing its functioning.

BlsA Interacts With the Acetoin Catabolic Negative Regulator AcoN Under Illumination at Moderate Temperatures in *A. baumannii*

Yeast two-hybrid assay experiments were conducted to study if BlsA interacts with AcoN, using an adapted system from ProQuest™ Two-Hybrid System, as previously described (Tuttobene et al., 2018). The system includes strain Mav 203, which harbors three reporter genes with different promoters to avoid false positives: *lacZ* and two auxotrophic markers HIS3 and URA3. If the two proteins studied do interact, the appearance of blue color as well as growth in the absence of histidine or uracil would be observed. Gateway-system vectors pGAD-T7Gw and pGBK-T7Gw adapted to Y2H express each of the studied genes, *blsA* and *acoN*, as fusions to GAL4 DNA DB or AD. In each plate were also included self-activation controls (pGAD-T7Gw and pGBK-T7Gw empty vectors) as well as different strength interaction controls (A–E), to give an indication of the reporter genes' expression levels. In our previous report (Tuttobene et al., 2018), we observed that BlsA protein interactions depend on illumination and temperature conditions, so we decided to test its interaction with AcoN, the acetoin catabolism negative regulator, under different conditions. **Figure 6** shows results of Y2H assay experiments at the different conditions analyzed. At 23°C under blue light (**Figure 6**), the interaction between BlsA and AcoN was demonstrated by the appearance of blue color and growth in SC defined media without the supplementation of histidine or uracil, i.e., results were consistent for the three reporters analyzed. The interactions occurred independently of the vector used, as both pGAD*blsA*/pGBK*acoN* and pGAD*acoN*/pGBK*blsA* combinations produced signals (**Table 5**). Growth on SC–Ura

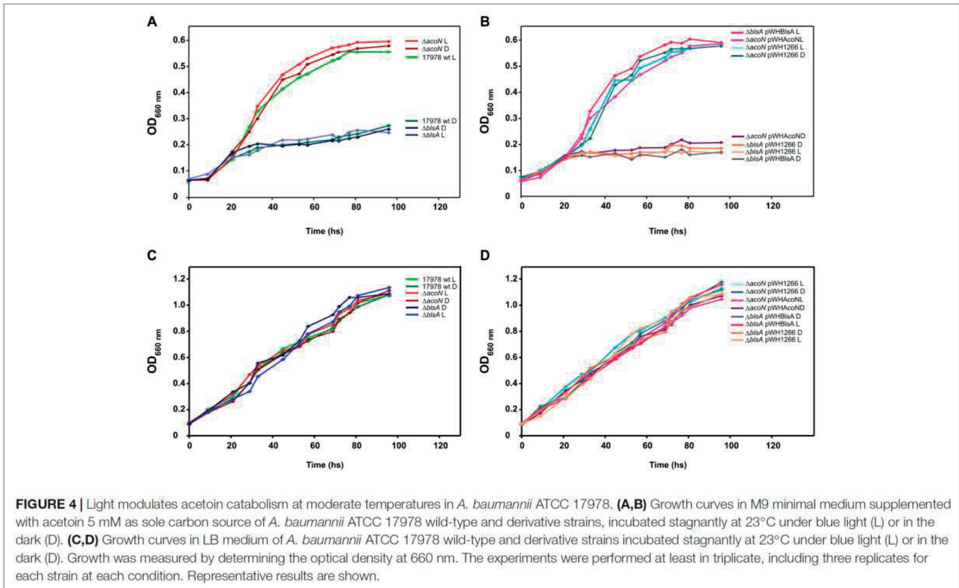

FIGURE 4 | Light modulates acetoin catabolism at moderate temperatures in *A. baumannii* ATCC 17978. **(A,B)** Growth curves in M9 minimal medium supplemented with acetoin 5 mM as sole carbon source of *A. baumannii* ATCC 17978 wild-type and derivative strains, incubated stagnantly at 23°C under blue light (L) or in the dark (D). **(C,D)** Growth curves in LB medium of *A. baumannii* ATCC 17978 wild-type and derivative strains incubated stagnantly at 23°C under blue light (L) or in the dark (D). Growth was measured by determining the optical density at 660 nm. The experiments were performed at least in triplicate, including three replicates for each strain at each condition. Representative results are shown.

plates indicates a strong interaction between BlsA and AcoN in the conditions analyzed, since the *URA3* reporter is the least sensitive[1]. Moreover, controls indicated absence of self-activation of each protein fused to DB or AD: (pGAD-T7/pGBK*blsA* or pGBK*acoN*) or (pGBK-T7/pGAD*blsA* or pGAD*acoN*) (**Table 5**). The overall data provide convincing evidence indicating that BlsA interacts with AcoN at 23°C under blue light. However, no positive signal was detected for AcoN–BlsA interaction by Y2H assays for any of the reporters tested at 23°C in the dark, while interaction controls behaved as expected (**Figure 6** and **Table 5**). Altogether, the data account for BlsA interacting with AcoN in a light-dependent manner at moderate temperatures. **Table 5** summarizes the results obtained for Y2H.

AcoN Does Not Modulate A1S_1697 Expression in Response to Light

We next analyzed the possibility that AcoN would be directly controlling the expression of the other putative transcriptional regulator identified in this cluster (gene 1, A1S_1697) in *A. baumannii* (**Figure 2**), which by analogy with *acoR* from *B. subtilis* might be an activator of the acetoin cluster. Whether this hypothesis is correct, AcoN would modulate *acoA–C* in response to light indirectly by modulation of the functioning of the putative activator. For this purpose, we studied A1S_1697 expression at different illumination conditions and genetic backgrounds. If AcoN functions as a negative regulator of A1S_1697 expression in a light-dependent manner, then A1S_1697 transcripts levels would vary between light and dark conditions. This variation would level in the Δ*acoN* mutant between light and dark, and reach higher expression levels than the wild type, had it been the negative regulator. However, and as seen in **Figure 7**, A1S_1697 transcripts levels were similar between light and dark for all the genetic backgrounds analyzed, namely, the wild-type strain, and the Δ*blsA* and Δ*acoN* mutants. These results indicate that AcoN does not regulate A1S_1697 expression in response to light.

BlsA–AcoN Interaction Is Significantly Reduced at Higher Temperatures

Since BlsA and AcoN interact at 23°C under blue light, we wondered whether this interaction is conserved at higher temperatures. Thus, BlsA–AcoN interactions were studied by Y2H at a temperature that supports yeast growth such as 30°C. A control at 23°C under blue light was always included for each repetition. **Figure 6** shows representative Y2H results indicating null or negligible BlsA–AcoN interactions at 30°C, neither in the dark nor under blue light.

Light Does Not Modulate Acetoin Catabolism at Higher Temperatures

We next studied whether acetoin catabolic gene expression and growth was modulated by light at 30°C, since no interaction between BlsA and AcoN was detected at this temperature. As

[1] http://www.invitrogen.com/content/sfs/manuals/10835031.pdf

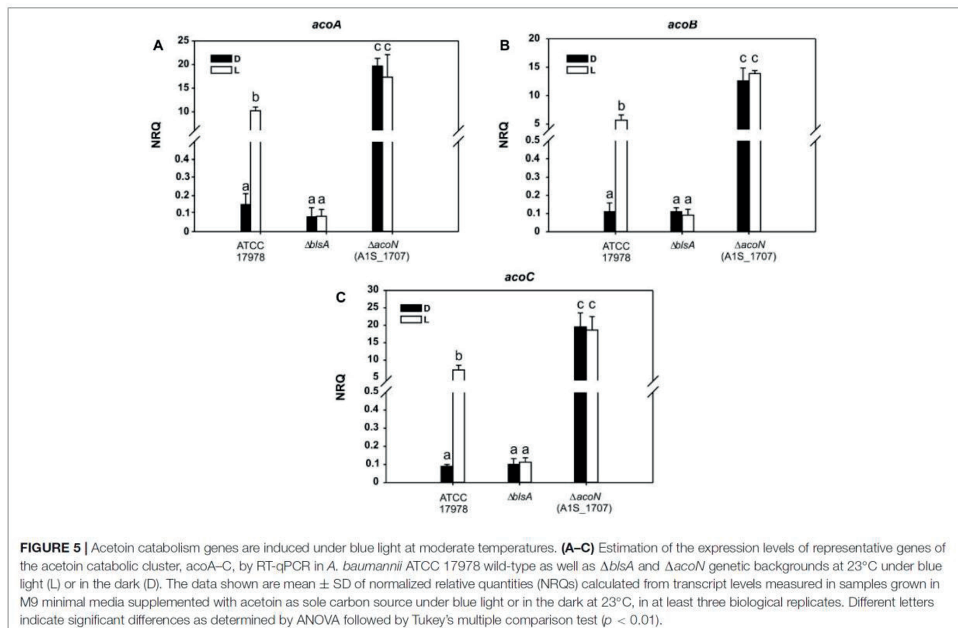

FIGURE 5 | Acetoin catabolism genes are induced under blue light at moderate temperatures. **(A–C)** Estimation of the expression levels of representative genes of the acetoin catabolic cluster, acoA–C, by RT-qPCR in *A. baumannii* ATCC 17978 wild-type as well as Δ*blsA* and Δ*acoN* genetic backgrounds at 23°C under blue light (L) or in the dark (D). The data shown are mean ± SD of normalized relative quantities (NRQs) calculated from transcript levels measured in samples grown in M9 minimal media supplemented with acetoin as sole carbon source under blue light or in the dark at 23°C, in at least three biological replicates. Different letters indicate significant differences as determined by ANOVA followed by Tukey's multiple comparison test ($p < 0.01$).

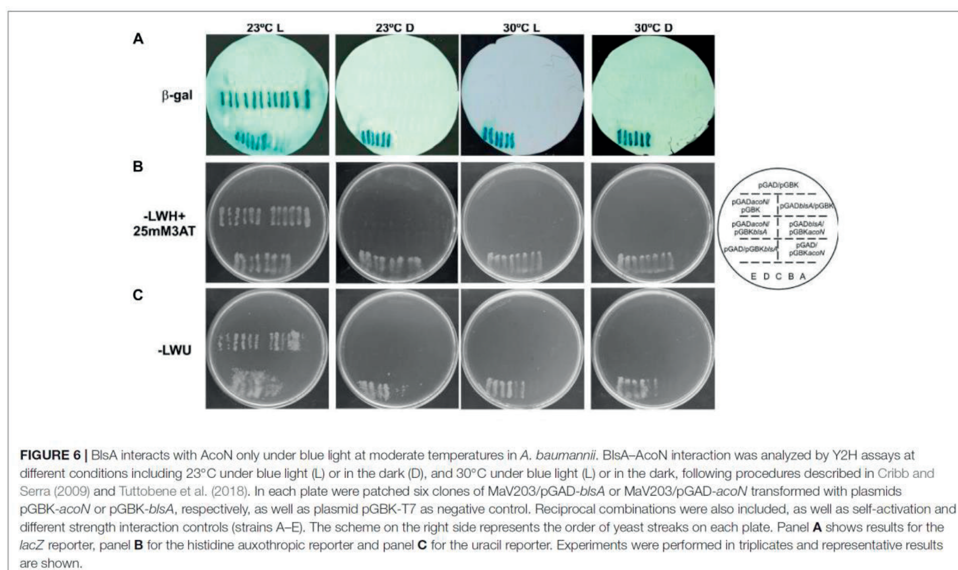

FIGURE 6 | BlsA interacts with AcoN only under blue light at moderate temperatures in *A. baumannii*. BlsA–AcoN interaction was analyzed by Y2H assays at different conditions including 23°C under blue light (L) or in the dark (D), and 30°C under blue light (L) or in the dark, following procedures described in Cribb and Serra (2009) and Tuttobene et al. (2018). In each plate were patched six clones of MaV203/pGAD-*blsA* or MaV203/pGAD-*acoN* transformed with plasmids pGBK-*acoN* or pGBK-*blsA*, respectively, as well as plasmid pGBK-T7 as negative control. Reciprocal combinations were also included, as well as self-activation and different strength interaction controls (strains A–E). The scheme on the right side represents the order of yeast streaks on each plate. Panel **A** shows results for the *lacZ* reporter, panel **B** for the histidine auxothropic reporter and panel **C** for the uracil reporter. Experiments were performed in triplicates and representative results are shown.

TABLE 5 | The interaction between AcoN and BlsA was determined by the yeast two hybrid assay, using GAL4 activation domain (AD) and DNA-binding domain (BD) fusion proteins.

β-Gal	Empty vector pGAD-T7	BlsA_AD	AcoN_AD
Empty vector pGBK-T7	–	–	–
AcoN BD	–	+	ND
BlsA_BD	–	ND	+
HIS 3			
Empty vector pGBK-T7	–	–	–
AcoN BD	–	+	ND
BlsA_BD	–	ND	+
URA3			
Empty vector pGBK-T7	–	–	–
AcoN BD	–	+	ND
BlsA_BD	–	ND	+

Both combinations of fusion proteins (AcoN_BD vs. BlsA_AD and BlsA _BD vs. AcoN_AD) were assayed giving the same results with all three reporter genes (β-Gal, HIS 3, and URA 3). +, means reporter gene expression induced by a positive interaction; –, means no interaction, confirming that no "self-activation" of the fusion proteins may result in the reporters expression (interactions using pGAD-T7 and pGBK-T7 empty vectors in combination with the fusion proteins); ND, means that self-interactions of AcoN and BlsA were not determined.

expected, *acoA*, *acoB*, and *acoC* gene expression showed no differential modulation by light neither in *A. baumannii* ATCC 17978 wild type, nor in the Δ*blsA* or Δ*acoN* mutants at this condition (**Figure 8A**). At 30°C, *acoA–C* expression levels in the Δ*blsA* mutant were similar to the wild-type strain both under blue light and in the dark, i.e., were repressed; while they were induced in the Δ*acoN* mutant both under blue light and in the dark. This behavior was congruent with growth curves performed in M9 minimal media supplemented with acetoin as sole carbon source, which showed no significant difference between light and dark for any of the studied strains (**Figures 8B,C**). Here again, the Δ*acoN* mutant showed enhanced growth consistent with the absence of the negative regulator, as also did the Δ*acoN* mutant containing pWH1266 (**Figures 8B,C**). The overall data indicate that light does not influence acetoin catabolism at 30°C or above, and are in agreement with available knowledge regarding BlsA functioning (Mussi et al., 2010; Golic et al., 2013; Abatedaga et al., 2017).

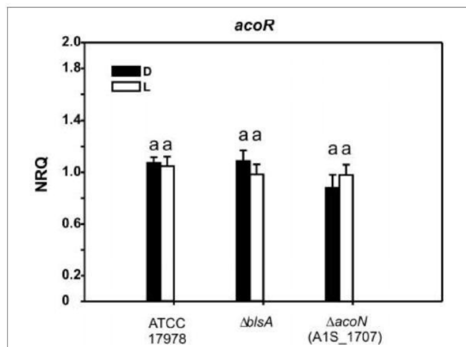

FIGURE 7 | A1S_1697 expression does not depend on light nor on AcoN. Estimation of the expression levels of A1S_1697 by RT-qPCR in *A. baumannii* ATCC 17978 wild-type as well as Δ*blsA* and Δ*acoN* genetic backgrounds at 23°C under blue light (L) or in the dark (D). The data shown are mean ± SD of NRQs calculated from transcript levels measured in samples grown in M9 minimal media supplemented with acetoin as sole carbon source under blue light or in the dark at 23°C, in at least three biological replicates. Different letters indicate significant differences as determined by ANOVA followed by Tukey's multiple comparison test ($p < 0.01$).

DISCUSSION

Acinetobacter sp. are extremely well adapted to different hostile environments thanks to several molecular mechanisms that enable survival under stress conditions. Here, we characterized the *Acinetobacter* sp. 5-2Ac02 strain isolated from the air in a hospital from Brazil. *Acinetobacter* sp. 5-2Ac02 showed an antibiotic susceptible profile. It includes a bla_{oxa-58} gene as well as *tet* genes, which have been related to resistance to tetracycline, coded in its genome. This susceptible strain carrying these cryptic genes hence represents a clinical threat as it may act as a reservoir of resistance genes. The high arsenic MIC for *Acinetobacter* sp. strain 5-2Ac02 may be attributed to the arsenic operon, arsC1–arsR–arsC2–ACR–arsH, which has only been described in the *Pseudomonas stutzeri* TS44 (Barbosa et al., 2016).

We further analyzed the global gene expression adjustments in this strain in response to environmental stressors such as mitomycin C and found induction of genes coding for components of the SOS response, genes involved in numerous TA systems (RelBE, HigBA, parDE, and other two new TA systems) (Barbosa et al., 2016), and resistance to heavy metals and antioxidant enzymes. The TA systems have been shown to be involved both in tolerance and persistence, which presuppose the ability of the bacteria to grow slowly or enter into a dormant state, respectively, to cope with the presence of a stressor (Fernandez-Garcia et al., 2018). It is thus not surprising that in the presence of mitomycin C and ciprofloxacin a tolerance phenotype was observed in killing curves (**Figure 1**). Furthermore, the ability of *A. baumannii* to survive for long periods of desiccation has been related to the achievement of dormant states, via mechanisms affecting control of cell cycling, DNA coiling, transcriptional and translational regulation, protein stabilization, antimicrobial resistance, and toxin synthesis (Gayoso et al., 2014). The fact that this airborne strain, in which desiccation is a common feature in its lifestyle, harbors and modulates numerous determinants leading to persistence in adverse environmental conditions is thus aligned with this notion.

Under pressure from the quorum network, both AHLs and 3-oxo-C12-HSL compounds induced the expression of a cluster involved in acetoin/butanediol metabolism in *Acinetobacter* sp. 5-2Ac02, which was also shown to be induced by light

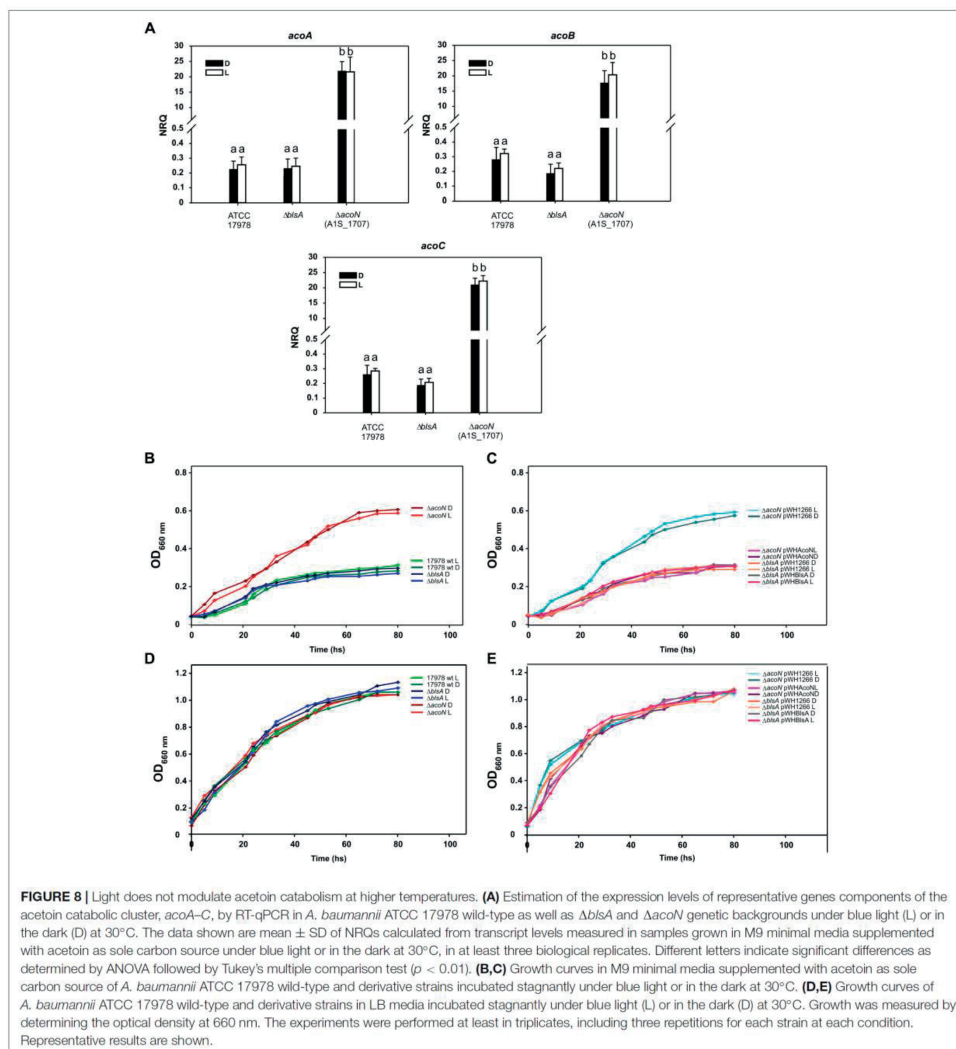

FIGURE 8 | Light does not modulate acetoin catabolism at higher temperatures. **(A)** Estimation of the expression levels of representative genes components of the acetoin catabolic cluster, *acoA–C*, by RT-qPCR in *A. baumannii* ATCC 17978 wild-type as well as Δ*blsA* and Δ*acoN* genetic backgrounds under blue light (L) or in the dark (D) at 30°C. The data shown are mean ± SD of NRQs calculated from transcript levels measured in samples grown in M9 minimal media supplemented with acetoin as sole carbon source under blue light or in the dark at 30°C, in at least three biological replicates. Different letters indicate significant differences as determined by ANOVA followed by Tukey's multiple comparison test ($p < 0.01$). **(B,C)** Growth curves in M9 minimal media supplemented with acetoin as sole carbon source of *A. baumannii* ATCC 17978 wild-type and derivative strains incubated stagnantly under blue light or in the dark at 30°C. **(D,E)** Growth curves of *A. baumannii* ATCC 17978 wild-type and derivative strains in LB media incubated stagnantly under blue light (L) or in the dark (D) at 30°C. Growth was measured by determining the optical density at 660 nm. The experiments were performed at least in triplicates, including three repetitions for each strain at each condition. Representative results are shown.

in *A. baumannii* (Muller et al., 2017). Acetoin (3-hydroxy-2-butanone) is a four carbon neutral molecule used as substrate by various microorganisms, with multiple usages in flavor, cosmetic, and chemical synthesis (Xiao et al., 2007). In *B. subtilis*, acetoin is a significant product generated from glucose metabolism in aerobiosis. Given its neutral nature, acetoin allows the consumption of important quantities of glucose without acidification of the medium. It can also serve as a carbon reserve which can be expelled to the exterior and later re-internalized (Ali et al., 2001). Acetoin and BD are also BVCs, which can influence bacterial pathogenesis (Audrain et al., 2015) by altering the production of virulence factors (Venkataraman et al., 2014) or by affecting host cell functions (Kurita-Ochiai et al., 1995). In addition to the

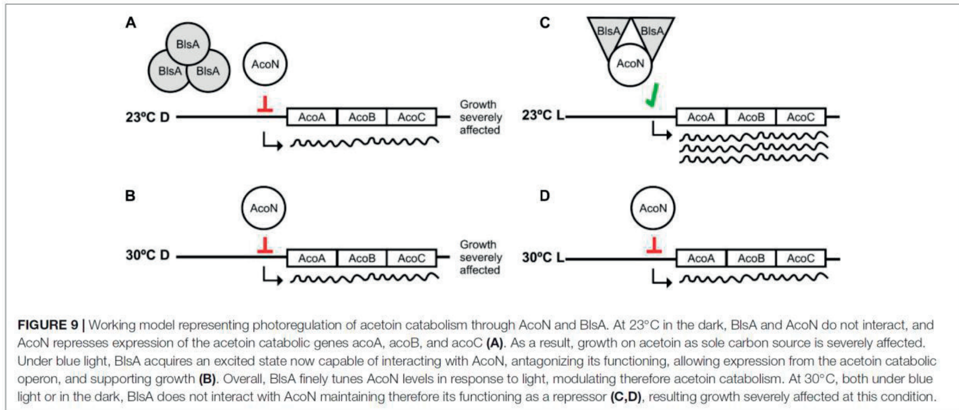

FIGURE 9 | Working model representing photoregulation of acetoin catabolism through AcoN and BlsA. At 23°C in the dark, BlsA and AcoN do not interact, and AcoN represses expression of the acetoin catabolic genes acoA, acoB, and acoC **(A)**. As a result, growth on acetoin as sole carbon source is severely affected. Under blue light, BlsA acquires an excited state now capable of interacting with AcoN, antagonizing its functioning, allowing expression from the acetoin catabolic operon, and supporting growth **(B)**. Overall, BlsA finely tunes AcoN levels in response to light, modulating therefore acetoin catabolism. At 30°C, both under blue light or in the dark, BlsA does not interact with AcoN maintaining therefore its functioning as a repressor **(C,D)**, resulting growth severely affected at this condition.

fundamental ecological interest, a better understanding of environmental bacteria and of the roles of BVCs (including BD), metabolic pathways, and mechanisms involved could provide new information about the bacterial response to the environment, thus potentially leading to clinical or industrial applications.

Comparisons of the genetic organization of this cluster from *Acinetobacter* sp. 5-2Ac02 with that of *A. baumannii* ATCC 17978 guided us to further study a gene annotated as a putative transcriptional regulator, then designated AcoN by us. We show here that it behaves as a negative regulator of the acetoin/butanediol cluster in an *A. baumannii* and is involved in photoregulation of acetoin catabolism in *A. baumannii* through the photoreceptor BlsA. In this context, we have recently shown that BlsA binds to and antagonizes the functioning of the transcriptional repressor Fur only in the dark at 23°C, likely by reducing its ability to bind to acinetobactin promoters with the concomitant enhanced gene expression and growth under iron deprivation at this condition (Tuttobene et al., 2018). In this work, we have broadened our understanding of BlsA functioning by showing that this photoreceptor can antagonize the functioning of other transcriptional regulators also under blue light such as AcoN. Our results support a model in which the system is at a basal level or repressed state in most conditions, for example in the dark at 23°C as well as at 30°C both in the dark or under blue light, i.e., AcoN is repressing acetoin catabolic genes' transcription resulting in basal gene expression levels as well as severely affected growth on acetoin (**Figure 9**). However, under blue light at 23°C the system gets derepressed: BlsA binds to the acetoin repressor AcoN antagonizing its functioning, likely by reducing its ability to bind to acetoin catabolic genes' promoters, allowing thus their expression at this condition (**Figure 9**). Overall, the global regulator BlsA functions both under blue light and in the dark at low–moderate temperatures modulating different transcriptional regulators, such as Fur and AcoN, as well as the corresponding sets of regulated genes and the corresponding cellular processes. In this sense, BlsA probes to be unique among described photoreceptors regarding its dual activity under illumination and in the dark. Indeed, many photoreceptors have been shown to antagonize transcriptional repressors (Tuttobene et al., 2018), such as AppA from *Rhodobacter sphaeroides* (Pandey et al., 2017), PixD from *Synechocystis* sp. PCC6803 (Fujisawa and Masuda, 2017), and YcgF from *E. coli* (Tschowri et al., 2012). However, their functioning has been reported to occur in the dark for the first two or under blue light for the last one. This constitutes therefore the first report showing that a single photoreceptor can act both under blue light and in the dark for differential modulation by light of diverse cellular processes.

The fact that BlsA-Fur modulates photoregulation of iron uptake, while BlsA–AcoN modulates photoregulation of acetoin catabolism in *A. baumannii* at low–moderate temperatures such as 23°C but not 30°C, is consistent with previous findings of our group. In fact, we have previously showed that BlsA integrates a temperature signal in addition to light by mechanisms affecting different points of regulation. On the one side, *blsA* expression levels are very much reduced at 30 or 37°C with respect to 23°C, which correlates with negligible photoreceptor levels in the cells at 37°C (Abatedaga et al., 2017; Tuttobene et al., 2018), while the other point of control by temperature affects BlsA photoactivity (Abatedaga et al., 2017).

The mechanism by which BlsA perceives light and differentially binds to transcriptional regulators is not clear and could result from differential properties displayed by the photoreceptor at each condition, for example regarding the oligomerization state. In this sense, our results show that BlsA forms oligomers both under blue light or in the dark at 23°C (Tuttobene et al., 2018). Yet, variations in the composition or order level of these oligomers at each condition could account

for differential functioning, as is the case of *Synechocystis* sp. PCC6803 PixD (Fujisawa and Masuda, 2017).

Many questions arise from our findings such as why photoregulation of acetoin catabolism at moderate temperatures has evolved in this pathogen. Likely, the answer lies in the lifestyle carried out by the microorganism at this condition. In this context, and as mentioned before, it has been shown that utilization of BD, a common fermentation product of *P. aeruginosa* co-habitant bacteria, significantly increases virulence and infection of the microorganism (Venkataraman et al., 2014; Nguyen et al., 2016; Liu et al., 2018). The activation of the pathway of BD utilization through acetoin by light observed could plausibly go in this same sense too in *A. baumannii*. Indeed, we have already seen that light induces factors related to virulence and/or persistence in the environment such as the type VI secretion system T6SS, the phenylacetic acid catabolic pathway, trehalose biosynthesis, tolerance to antibiotics, production of antioxidant enzymes, etc. (Muller et al., 2017), which could ultimately contribute to persistence and competition with other microorganisms in the habitat.

Future experiments will be devoted to provide a detailed characterization of the mechanism of photoregulation directed by BlsA, AcoN, and their targets. First, we will conduct gel mobility assays (EMSA) to prove that AcoN is a DNA-binding transcriptional regulator, as is strongly suggested by BLAST sequence homology analyses, which show 97–100% identity with proteins annotated as sigma-54-dependent Fis family DNA-binding transcriptional regulators in *A. baumannii*. If BlsA interacts with AcoN under blue light avoiding or reducing its ability to bind to target promoter regions, as proposed by the evidence accumulated in this work, then the addition of BlsA to these EMSA assays should reduce the delay observed for the AcoN-DNA probe. DNase protection assays will further characterize the AcoN-DNA binding region. Furthermore, by solving the 3D structures and conducting ultrafast structural dynamic studies of BlsA alone as well as bound to AcoN under blue light, we expect to gain detailed knowledge on structural as well as photochemical aspects of the light signal transduction mechanism.

Finally, we show in this work that quorum network modulators as well as light both regulate the acetoin catabolic cluster. Whether these are independent signals or share totally or partially the signal transduction cascade components is actually under study in our laboratories.

DATA AVAILABILITY

The datasets generated for this study can be found in GEO, GSE120392.

AUTHOR CONTRIBUTIONS

MRT and GLM performed the experiments. PC performed the experiments and collaborated in writing the manuscript. LF-G, LB, and AA performed the experiments and mutant strain. RER and RL-R analyzed the experiments. FF-C and IB analyzed the array studies. BB, RT, ML, and GB developed the RT-PCR experiments. MT designed the experiments. MAM and MT designed the experiments, wrote the manuscript, and provided funding.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01376/full#supplementary-material

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<u>Chapter III.</u> Relationship between the quorum network (sensing/quenching) and clinical features of pneumonia and bacteraemia caused by *Acinetobacter baumannii*

In the research reported in chapter III, we continued to study this important mechanism of tolerance and bacterial persistence, i.e. the quorum network in the *A. baumannii*. We examined the relationship between the quorum network (QS/QQ) (*abaR*/*aidA* genes), and the development of pneumonia and secondary bacteraemia in 30 clinical strains of *A. baumannii* isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010 (Bioproject PRJNA345289)". In addition, we explored the relationship between the quorum network and mortality in an *in vivo* model of *Galleria mellonella,* using a mutant *A. baumannii* lacking the *abaI* gene and the wild-type *A. baumannii* ATCC 17978.

Analysis of the expression of these genes, *abaR* (QS gene) and *aidA* (QQ gene) by RTq-PCR did not reveal any significant differences between the 17 isolates from colonized patients and the 13 isolates from pneumonia patients. However, there were significant differences between patients with pneumonia and bacteraemia (Pn-B) and patients with pneumonia but not bacteraemia (Pn-NB). The *abaR* gene was overexpressed in Pn-B patients relative to Pn-NB patients, and the *aidA* gene was overexpressed in Pn-NB patients relative to the Pn-B patients. Furthermore, analysis of these quorum network genes under different conditions such as 3-Oxo-C12-HSL and H_2O_2 in the strains from Pn-NB patients showed regulation of *aidA* gene in the presence of 3-Oxo-C12-HSL (overexpression) and H_2O_2 (underexpression). In adition, there was a significant decrease in expression of the *abaR* gene due to the effect of 3-Oxo-C12-HSL. Analysis of these genes in the strains from Pn-B patients revealed that the *aidA* gene was not regulated by either of the compounds; nevertheless, the *abaR* gene was overexpressed under H_2O_2 pressure. These findings indicate that the isolates from Pn-B patients did not possess a functional *aidA* gene, in contrast to the strains that did not cause bacteraemia secondary to pneumonia.

On the other hand, clinical analysis of the risk factors associated with the development of pneumonia vs colonization demonstrated that diabetes mellitus is the only significant variable. Nevertheless, the only important factor associated with

the development of bacteraemia following pneumonia caused by *A. baumannii* is the lack of the functional *aidA* gene. These results are consistent with those reported by Lopez *et al*., who showed that 67% of clinical *A. baumannii* were non-mobile, isolated from the respiratory tract and expressed the AidA protein. However, the only non-motile isolate from blood lacks the *aidA* gene (*33, 102*).

Finally, *in vivo* analysis revealed 100% mortality of *Galleria mellonella* in 24h when the moth was infected with *A. baumannii* ATCC 17978, whereas infection with *A. baumannii* ATCC 17978 Δ*abaI* only produced 70% mortality in 24h.

The research findings showed that the QN (QS/QQ) is involved in the development of bacteraemia in patients with pneumonia caused by *A. baumannii*. This is the first study to analyse the relationship between the absence of the functional AidA QQ enzyme and the development of secundary bacteraemia following pneumonia.

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Relationship Between the Quorum Network (Sensing/Quenching) and Clinical Features of Pneumonia and Bacteraemia Caused by *A. baumannii*

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Acinetobacter baumannii (Ab) is one of the most important pathogens associated with nosocomial infections, especially pneumonia. Interest in the Quorum network, i.e., Quorum Sensing (QS)/Quorum Quenching (QQ), in this pathogen has grown in recent years. The Quorum network plays an important role in regulating diverse virulence factors such as surface motility and bacterial competition through the type VI secretion system (T6SS), which is associated with bacterial invasiveness. In the present study, we investigated 30 clinical strains of *A. baumannii* isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010" (*Genbank Umbrella Bioproject* PRJNA422585), a multicentre study describing the relationship between the Quorum network in *A. baumannii* and the development of pneumonia and associated bacteraemia. Expression of the *aidA* gene (encoding the AidA protein, QQ enzyme) was lower ($P < 0.001$) in strains of *A. baumannii* isolated from patients with bacteraemic pneumonia than in strains isolated from patients with non-bacteraemic pneumonia. Moreover, *aidA* expression in the first type of strain was not regulated in the presence of environmental stress factors such as the 3-oxo-C12-HSL molecule (substrate of AidA protein, QQ activation) or H_2O_2 (inhibitor of AidA protein, QS activation). However, in the *A. baumannii* strains isolated from patients with non-bacteraemic pneumonia, *aidA* gene expression was regulated by stressors such as 3-oxo-C12-HSL and H_2O_2. In an *in vivo* *Galleria mellonella* model of *A. baumannii* infection, the *A. baumannii* ATCC 17978 strain was associated with higher mortality (100% at 24 h) than the mutant, *abaI*-deficient, strain

(carrying a synthetase enzyme of Acyl homoserine lactone molecules) (70% at 24 h). These data suggest that the QS (*abaR* and *abaI* genes)/QQ (*aidA* gene) network affects the development of secondary bacteraemia in pneumonia patients and also the virulence of *A. baumannii*.

Keywords: quorum, sensing/quenching, pneumonia, bacteraemia, *Acinetobacter*

INTRODUCTION

Acinetobacter baumannii is a major cause of hospital-acquired infections associated with high mortality rates (Fuchs, 2016),s usually affecting patients in Intensive Care Units (ICU) (del Mar Tomas et al., 2005; Lee et al., 2017). In these patients, *A. baumannii* causes infections such as pneumonia or, to a lesser extent, serious infections of the bloodstream (around 10% of clinical isolates of *A. baumannii* cause bacteraemia) (Cisneros and Rodríguez-Baño, 2002; El Kettani et al., 2017).

The success of this bacterium as a nosocomial pathogen, has been attributed to the following factors, amongst others: (i) high genetic versatility, facilitating rapid adaptation to stressful or unfavorable situations (Gayoso et al., 2014; Trastoy et al., 2018); (ii) ability to acquire new genes horizontally by the acquisition of plasmids and phages (López et al., 2018); (iii) ability to persist for a long time on animate and inanimate surfaces (resistance to desiccation) (Gayoso et al., 2014), which is generally attributed to biofilm formation; (iv) resistance to antimicrobial agents, including broad-spectrum antibiotics such as carbapenems, colistin, and tigecycline (Fernández-Cuenca et al., 2015), as well as to disinfectants and biocides (Fernández-García et al., 2018); and (v) high virulence (colonization, invasiveness, and cytotoxicity) (Rumbo et al., 2014; Wong et al., 2017). These characteristics contribute to the fact that nosocomial outbreaks caused by *A. baumannii* are difficult to control and that therapeutic options to treat infections are scarce or non-existent (Fernández-Cuenca et al., 2013). In February, 2017, the World Health Organization (WHO) published a list of "priority pathogens." The list includes antibiotic resistant bacteria, considered a serious threat to human health and for which new antibiotics are urgently needed, and is headed by carbapenem-resistant *A. baumannii* (Tacconelli et al., 2018).

The Quorum Sensing (QS) network is generally used by Gram-negative bacterial pathogens to regulate biological processes such as virulence, conjugation, resistance, biofilm formation (which also depends on other factors such as the lytic enzymes responsible for peptidoglycan recycling: Vijayakumar et al., 2016), motility and bacterial competition, via secretion systems (T6SS), which are associated with greater invasiveness (LaSarre and Federle, 2013; López et al., 2017a,b). Two proteins (AbaI /AbaR) identified in *A. baumannii* have been described as homologs of the LuxI/LuxR system found in *Vibrio fischeri*. This system comprises a signal or autoinducer molecule (acyl-homoserine lactone, AHL), an enzyme that synthesizes signaling molecules (AbaI) and a receptor protein activator of the QS (AbaR), which forms a complex with N-(3-hydroxydodecanoil)-L-homoserine lactone (3-OH-C12-HSL) to regulate virulence factors, biofilm formation, surface motility, and bacterial competence (T6SS) (Stacy et al., 2012). When a threshold concentration is reached, the AHL molecules present inside the cell are transported to its receptor (AbaR), putatively joining the lux-box, which is located 67 bp upstream of the ATG of AbaI, resulting in the synthesis of more AHL molecules (López et al., 2017b). The QS mechanism, on the other hand, acts naturally under environmental stress conditions such as the presence of bile salts in the gastrointestinal tract and H_2O_2 (ROS response) in the respiratory tract (López et al., 2017b).

A new enzyme (AidA) has recently been cloned in *E. coli* BL21 (DE3) and functionally characterized in clinical strains of *A. baumannii* capable of inhibiting their own QS (by Quorum Quenching) (López et al., 2017b). This enzyme acts by degrading signaling molecules such as N-(3-Oxo-dodecanoyl), L-homoserine lactone (3-Oxo-C12-HSL), and N-dodecanoyl-L-homoserine lactone (C12-HSL), as confirmed by observation of inhibition of motility, biofilm formation and other virulence factors associated with activation of the Quorum Sensing system (López et al., 2017b; Mayer et al., 2018). Other QQ enzymes have also recently been described in *A. baumannii* ATCC17978 (A1S_0383, A1S_2662, A1S_1876) (Mayer et al., 2018). Multiple QQ enzymes have been analyzed in diverse pathogens such as *Pseudomonas aeruginosa* (Zhang et al., 2011), *Deinococcus radiodurans*, *Hyphomonas neptunium*, *Photorhabdus luminicencens,* and *Rhizobium* spp. (Kalia et al., 2011; Krysciak et al., 2011).

Based on these findings, in the present study, we examined the relationship between the global Quorum regulatory network (QS/QQ) mediated by the *abaR* (QS) and *aidA* (QQ) genes and the development of pneumonia and bacteraemia in clinical strains of *A. baumannii* isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010," a multicentre study involving 45 Spanish hospitals and 246 patients. In addition, we used an *in vivo* infection model consisting of larvae of the wax moth *Galleria mellonella* to examine the relationship between the global QS/QQ and the development of mortality by a mutant *abaI* (QS)-deficient strain of *A. baumannii* (*A. baumannii* ATCC17978Δ*abaI*) relative to that of the wild-type *A. baumannii* ATCC17978 strain.

MATERIALS AND METHODS

Bacteria and Samples

To carry out this study, we analyzed 30 clinical strains of *A. baumannii* from the 465 strains isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010" multicentre

study (Genbank Umbrella Bioproject PRJNA422585). The multicentre study included 45 hospitals in Spain, in which new cases of colonization or infection by *A. baumannii* were analyzed between February and March 2010 (Villar et al., 2014). The 30 *A. baumannii* strains were all isolated from respiratory samples from patients with nosocomial pneumonia ($n = 13$: 6 with and 7 without bacteraemia) or *A. baumannii* colonization of the lower respiratory tract ($n = 17$) (Sánchez-Encinales et al., 2017). Molecular typing was performed by Multilocus Sequence Typing (MLST) (Mosqueda et al., 2014). In addition, we used a killing assay with the *Galleria mellonella* infection model and an *A. baumannii* ATCC17978Δ*abaI* mutant strain (identified by Castañeda-Tamez et al., 2018).

The main clinical study variables included demographics, underlying diseases, mechanical ventilation, tracheostomy, colonization of lower respiratory airways, bacteraemic pneumonia (Pn-B), non-bacteraemic pneumonia (Pn-NB) (Horan et al., 2008) and any cause of death during hospitalization.

To design the primers and probes of the QS genes and QQ enzymes, we analyzed the presence of QS genes (*abaR* and *abaI*) and the QQ enzyme (*aidA*) in *A. baumannii* ATCC 17978 (Genbank genome accession numbers CP000521.1 [CP018664.1]) and in 1000 *A. baumannii* genomes by consulting the "Integrated Microbial Genomes and Microbiomes" web page (https://img.jgi.doe.gov) and using nucleotide BLAST. The gene sequences used in the search were selected from the *Acinetobacter baumannii* ATCC 17978 genome. A threshold of 1e-50 was used as the limit for analysis of the nucleotide sequence, where the e-value was defined as the probability of random alignments with the same score. We also calculated the percentage presence of these genes in the genomes (**Figure S1**).

RNA Extraction

RNA Extraction to Analyze the Quorum Regulatory Network (QS/QQ)

All clinical strains of *A. baumannii* were cultured on solid Luria-Bertani (LB) plates and incubated at 37° C for 24 h. One colony was removed and inoculated in liquid LB medium and incubated overnight at 37° C under stirring at 180 rpm. The inoculum was diluted (1:100) and allowed to grow until an optical density (OD_{600} nm) of 0.4–0.6 (corresponding to the logarithmic growth phase) was reached. The RNA was then extracted using the High Pure RNA Isolation kit (Roche, Germany) and the extract was treated with Dnase (Roche, Germany). The extracted RNA was subsequently quantified in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), and the concentration was adjusted to 50 ng/µl in order to yield efficiencies of 90-110% (Rumbo et al., 2013). All extractions were carried out in duplicate.

RNA Extraction to Analyze the Quorum Regulatory Network (QS/QQ) Under Stress Conditions (3-Oxo-C12-HSL and H_2O_2)

The 13 strains of *A. baumannii*, isolated from patients with pneumonia, were cultured on solid Luria-Bertani (LB) plates and incubated at 37°C for 24 h. One colony was then removed, inoculated in liquid LB medium and incubated overnight at 37°C under stirring at 180 rpm. The preinoculum was diluted (1:100) and allowed to grow until an optical density (OD600 nm) of 0.3 was reached. Aliquots of 10 µM of 3-Oxo-C12-HSL (QS-inactivating molecule by expression of the AidA protein) (Stacy et al., 2012; López et al., 2017b) and (10 µl) H_2O_2 were then added for 5 min (QS-activator by ROS response) (López et al., 2018). All controls were prepared by adding the same volumes of DMSO (dimethyl sulfoxide), 3-Oxo-C12-HSL and of sample, but with no H_2O_2. After incubation of the samples for 4 and 5 h in the presence of 3-Oxo-C12-HSL, to study the regulatory QS/QQ genes (*abaR* and *aidA*), as well as 5 min under H_2O_2 in static at 37°C, RNA was extracted using the High Pure RNA Isolation kit (Roche, Germany) and treated with Dnase. The extracted RNA was subsequently quantified as described above (Rumbo et al., 2013).

RT-qPCR

The studies were carried out with a Lightcycler 480 RNA MasterHydrolysis Probe (Roche, Germany), under the following conditions: reverse transcription at 63°C for 3 min, denaturation at 95°C for 30 s, followed by 45 cycles of 15 s at 95°C and 45 s at 60°C and, finally, cooling at 40°C for 30 s. The UPL primers and probes from conserved DNA regions identified by PCR (Universal Probe Library-Roche, Germany) used in the analysis are shown in **Table 1**.

All of the experiments were carried out in a final volume of 20 µl per well (18 µl of master mix and 2 µl of RNA). Each experiment was carried out in duplicate with two RNA extracts (50 ng/µl). For each strain, the expression of all genes, primers, and probes was normalized relative to the reference or housekeeping gene, *rpoB*, for RT-qPCR studies of Quorum sensing Primer sequences (5′-3′) with Taqman probes (Rumbo et al., 2013; López et al., 2017b). Analysis of the controls without reverse transcriptase confirmed the absence of DNA contamination.

Galleria mellonella Infection Model

The *Galleria mellonella* model was an adapted version of that developed by Peleg et al. (2009), Yang et al. (2015). The procedure was as follows: twelve *G. mellonella* larvae, acquired from TruLarvTM (Biosystems Technology, Exeter, Devon, UK), were each injected with 10 µl of a suspension of *A. baumannii* ATCC17978, or its isogenic deficient mutant *A. baumannii* ATCC17978Δ*abaI*, diluted in sterile phosphate buffer saline (PBS) and containing 8×10^4 CFU ($\pm$ 0. 5 log). The injection was performed with a Hamilton syringe (volume 100 µl) (Hamilton, Shanghai, China). In addition, a control group of twelve larvae were injected with 10 µl of sterile PBS. After being injected, the groups of larvae were placed in Petri dishes and incubated in darkness at 37°C. The number of dead larvae was recorded twice a day (morning and afternoon) for 6 days. The larvae were considered dead when they showed no movement in response to touch (Peleg et al., 2009).

TABLE 1 | Primers and Probes used in this study.

		Sequence (5′-3′)	Probe	Reference
QUORUM SENSING				
abaR	Forw	TGGCAAGAAGATTTATTATCAGCA	119/TTGGTGGT	This study
	Rev	TGCGGTAGATTTAACGATCTCA		
	Forw	AGAGGCGTTACGTTGGACTG	155/GAAGGCAA	This study
	Rev	CCAAGAATCTGAGCTATTGC		
QUORUM QUENCHING				
aidA	Forw	GGGAACTTCTTTCGGTGGAG	145/CAGCGACC	López et al., 2017b
	Rev	AACAGCAGCAAGTCGATTATCA		
	Forw	CCTAACCTTGCATTAGGGCTATTA	53/TGGCAGAG	López et al., 2017b
	Rev	CGGTAAACCACAGGTCGGTA		
HOUSEKEEPING				
rpoB	Forw	CGTGTATCTGCGCTTGG	131/CTGGTGGT	Rumbo et al., 2014
	Rev	CGTACTTCGAAGCCTGCAC		

Statistical Analysis

The gene expression studies were carried out in duplicate, and the data obtained were analyzed by Student's *t*-test, implemented with GraphPad Prism v.6 software (GraphPad Software Inc. San Diego, CA). The graphs were constructed using the GraphPad program, and the results were represented as means and their respective standard deviations.

The mortality curves corresponding to the *in vivo Galleria mellonella* infection model were constructed using GraphPad Prism v.6 and the data were analyzed using the Log-rank test (Mantel-Cox). In both cases, *p*-values < 0.05 were considered statistically significant, and the data were expressed as mean values.

The statistical analyses were applied to the following categorical variables: age, sex, immunosuppressive treatment, surgery, ICU stay, mechanical ventilation, tracheostomy, severe sepsis, septic shock, and expression of the Quorum genes in *A. baumannii* clinical strains (Bone et al., 1992). In addition, the severity of co-morbidities was assessed using the Charlson score (Charlson et al., 1987) and the McCabe score (McCabe and Jackson, 1962). Chi-square and Fisher tests were used in the univariate analysis of categorical variables. Continuous variables were analyzed using two-sample *t*-test or Mann Whitney, as appropriate. A logistic regression analysis was performed to identify factors independently associated with pneumonia and bacteraemia. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using SPSS v.16.0 (SPSS Inc., Chicago, IL).

RESULTS

Study of the Gene Expression of the *abaR* and *aidA* Genes of the Quorum Network (QS/QQ)

The Relative Expression (RE) of the *abaR* and *aidA* genes of the Quorum network (QS/QQ) was quantified by RT-qPCR analysis of the 17 isolates of *A. baumannii* from colonized patients and of the 13 isolates of *A. baumannii* from patients with pneumonia (**Figure 1A**). The mean values (of two biological replicates) are presented in **Tables 2**, **3**. These values were first used to determine any significant differences between the two types of strains in terms of gene expression in the Quorum network.

The results did not reveal any significant differences in the RE of the Quorum network genes (*abaR*, *aidA*) between clinical strains of *A. baumannii* isolated from colonized patients and strains of *A. baumannii* isolated from patients with pneumonia (0.086/0.094 vs. 0.071/0.095, $p > 0.05$).

We then proceeded to study the RE of the *abaR* and *aidA* genes in strains of *A. baumannii* from patients with pneumonia, differentiating the strains isolated from patients with bacteraemia (Pn-B) from those isolated from patients without bacteraemia (Pn-NB). The resulting graphs are shown below (**Figure 1B**). The findings reveal significant differences in the expression of the *abaR* and *aidA* genes between clinical strains of *A. baumannii* from patients with bacteraemic pneumonia (Pn-B) and those with non-bacteraemic pneumonia (Pn-NB). We observed that *abaR* gene was overexpressed in *A. baumannii* isolates from Pn-B patients relative to Pn-NB patients (0.047 vs. 0.097, $p < 0.05$). By contrast, the *aidA* gene was overexpressed in *A. baumannii* clinical strains in Pn-NB patients relative to Pn-B patients (0.173 vs. 0.0045, $p < 0.001$) (**Figure 1B**). Only one strain, Ab 148_GEIH-2010 (ST-2), isolated from Pn-B patients, showed an *aidA* gene profile different from the other isolates of this group, although the RE of this gene was lower (0.022) than that of isolates from Pn-NB patients.

Study of *abaR*/*aidA* Genes (QS/QQ) Under Stress Conditions (3-Oxo-C12-HSL and H_2O_2)

The values of the RE of the *abaR* and *aidA* genes (Quorum network) in the presence of 3-Oxo-C12-HSL (Inhibition of the QS) and H_2O_2 (Activation of the QS), obtained by RT-qPCR of the 13 isolates of *A. baumannii* from patients with pneumonia (differentiated from Pn-NB) are shown in **Table 3**, expressed as

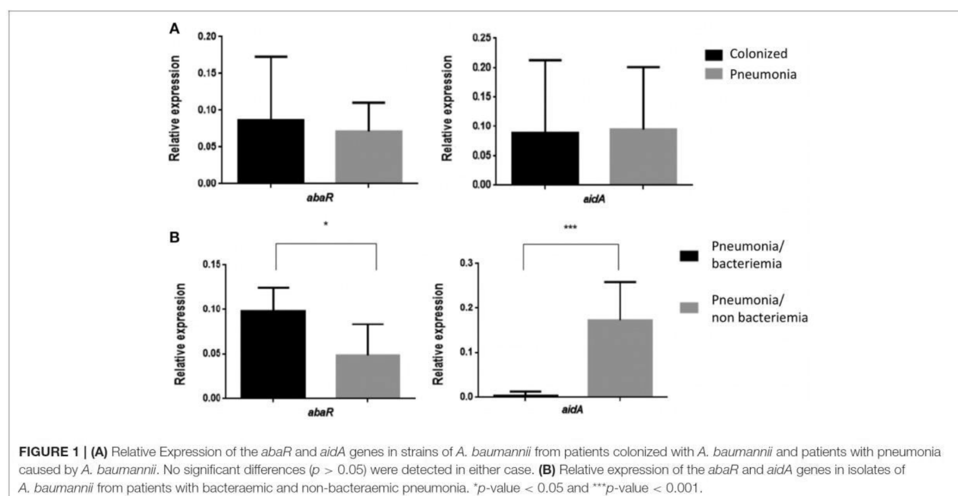

FIGURE 1 | (A) Relative Expression of the *abaR* and *aidA* genes in strains of *A. baumannii* from patients colonized with *A. baumannii* and patients with pneumonia caused by *A. baumannii*. No significant differences ($p > 0.05$) were detected in either case. **(B)** Relative expression of the *abaR* and *aidA* genes in isolates of *A. baumannii* from patients with bacteraemic and non-bacteraemic pneumonia. *p-value < 0.05 and ***p-value < 0.001.

TABLE 2 | Results of RT-qPCR analysis of the Relative Expression (RE) of the *abaR* and *aidA* genes (Quorum network genes) in the *A. baumannii* isolates from colonized patients.

Strain (MLST[a])	*abaR* (RE)	*aidA* (RE)
STRAINS OF *A. baumannii* ISOLATED FROM COLONIZED PATIENTS		
Ab 22_GEIH-2010 (ST-52)	0.043	0.042
Ab 38_GEIH-2010 (ST-2)	0.078	0.146
Ab 59_GEIH-2010 (ST-269)	0.081	0.003
Ab 64_GEIH-2010 (ST-2)	0.076	0.213
Ab 77_GEIH-2010 (ST-261)	0.018	0.112
Ab 112_GEIH-2010 (ST-263)	0.112	0.038
Ab 141_GEIH-2010 (ST-264)	**0.001**	0.010
Ab 177_GEIH-2010 (ST-2)	**0.001**	0.067
Ab 205_GEIH-2010 (ST-2)	0.131	0.126
Ab 288_GEIH-2010 (ST-263)	0.067	0.199
Ab 290_GEIH-2010 (ST-264)	0.141	0.006
Ab 294_GEIH-2010 (ST-2)	0.123	0.481
Ab 326_GEIH-2010 (ST-2)	0.123	0.015
Ab 354_GEIH-2010 (ST79)	0.052	0.020
Ab 364_GEIH-2010 (ST-79)	**0.001**	0.081
Ab 399_GEIH-2010 (ST-79)	0.061	0.050
Ab 456_GEIH-2010 (ST-269)	0.368	**0.001**

The results are expressed as the mean value of the two biological replicates. [a]MLST (Mutilocus Sequence Typing by Pasteur database, https://pubmlst.org/) (Villar et al., 2014). In bold, RE ≤0.001 not detected by RT-PCR.

the mean value of the two biological replicates. These values were then analyzed to determine any significant differences in the RE of the *abaR*/*aidA* (QS/QQ) genes between the different clinical isolates (**Figures 2**, **3**).

In the clinical strains of *A. baumannii* isolated from Pn-NB (**Figure 2**), we observed regulation of expression of the *aidA* gene in the presence of 3-Oxo-C12-HSL (overexpression, RE ≥ 1.5) (**Figure 2A**) and of H_2O_2 [underexpression, RE ≤ 0.5 (**Figure 2B**)]. Expression of the *abaR* gene decreased significantly in the presence of the 3-Oxo-C12-HSL molecule (RE ≤ 0.5, **Figure 2A**).

In the clinical strains *A. baumannii* isolated from Pn-B (**Figure 3**), expression of the *aidA* gene was not regulated in the presence of 3-Oxo-C12-HSL or H_2O_2. However, the *abaR* gene was overexpressed in the presence of H_2O_2 (RE ≥ 1.5, **Figure 3B**).

These results indicate that the isolates of *A. baumannii* from Pn-NB may harbor a functional AidA protein (QQ enzyme), in contrast to the isolates of *A. baumannii* from Pn-B, which did not have this functional protein. Therefore, in the *A. baumannii* strains isolated from Pn-B, overexpression of the *abaR* gene (activation of the QS) in the presence of H_2O_2 (ROS response) would enable the development of the virulence factors favoring invasiveness, such as type VI secretion system (T6SS) and motility.

Quorum Network (QS/QQ) Genes and Clinical Variables

Analysis of the risk factors associated with the development of pneumonia vs. colonization by clinical strains of *A. baumannii* revealed only one statistically significant variable, i.e., diabetes mellitus (**Table 4**).

However, analysis of the risk factors associated with the development of bacteraemia in pneumonia caused by *A. baumannii* revealed underexpression of the *aidA* gene as the only statistically significant variable ($p < 0.05$) (**Table 5**).

TABLE 3 | Results of RT-qPCR analysis of the Relative Expression (RE) of the *abaR* and *aidA* genes (Quorum network genes) in the *A. baumannii* isolates from patients with bacteraemic pneumonia (Pn-B) or non-bacteraemic pneumonia (Pn-NB).

***A. baumannii* strains from Pn-NB patients**			***A. baumannii* strains from Pn-B patients**		
Strain (MLST[a])	***abaR* (RE)**	***aidA* (RE)**	**Strain (MLST[a])**	***abaR* (RE)**	***aidA* (RE)**
Ab 8_GEIH-2010 (ST-2)	0.035	0.108	Ab 148_GEIH-2010 (ST-2)	0.094	0.022
	0.592*	1.545*		1.032*	1.204*
	0.940**	0.873**		1.407**	0.598**
Ab 73_GEIH-2010 (ST-2)	0.036	0.361	Ab 215_GEIH-2010 (ST-2)	0.085	**0.001**
	0.564*	1.695*		0.883*	**0.001***
	0.763**	0.450**		1.376**	**0.001****
Ab 125_GEIH-2010 (ST-257)	0.047	0.156	Ab 232_GEIH-2010 (ST-2)	0.139	**0.001**
	0.431*	1.582*		0.692*	**0.001***
	1.366**	1.076**		0.845**	**0.001****
Ab 157_GEIH-2010 (ST-2)	**0.001**	0.172	Ab 275_GEIH-2010 (ST-181)	0.110	**0.001**
	0.329*	2.308*		0.638*	**0.001***
	1.272**	0.685**		1.430**	**0.001****
Ab 240_GEIH-2010 (ST-2)	0.078	0.150	Ab 371_GEIH-2010 (ST-79)	0.059	**0.001**
	0.561*	0.858*		0.503*	**0.001***
	0.553**	0.703**		1.233**	**0.001****
Ab 268_GEIH-2010 (ST-181)	0.034	0.139	Ab 461_GEIH-2010 (ST-2)	0.099	**0.001**
	0.683*	1.530*		1.221*	**0.001***
	0.845**	0.717**		2.713**	**0.001****
Ab 276_GEIH-2010 (ST-181)	0.108	0.126			
	0.553*	1.007*			
	0.586**	0.722**			

*The results are expressed as the mean values of the two biological replicates. [a]MLST (Mutilocus Sequence Typing by Pasteur database, https://pubmlst.org/) (Villar et al., 2014). In bold, RE ≤0.001, not detected by RT-PCR. * Results of RT-qPCR analysis of the Relative Expression (RE) of the abaR/aidA genes (Quorum network genes) in the presence of 3-Oxo-C12-HSL in strains of A. baumannii from patients with bacteraemic pneumonia (Pn-B) or non-bacteraemic pneumonia (Pn-NB). ** Results of RT-qPCR analysis of the Relative Expression (RE) of the abaR/aidA genes (Quorum network genes) in the presence of H_2O_2 in strains of A. baumannii from patients with bacteraemic pneumonia (Pn-B) or non-bacteraemic pneumonia (Pn-NB).*

Mortality in the *in vivo Galleria mellonella* Model

Injection of *G. mellonella* larvae with *A. baumannii* ATCC17978 at a concentration of 8 x 10^4 CFU/larva ($\pm$ 0.5 log) caused 100% mortality after 24 h, whereas injection of the larvae with the same concentration of *A. baumannii* ATCC17978Δ*abaI* resulted in 70% mortality after 24 h (**Figure 4**; $p < 0.05$, Mantel-Cox analysis).

DISCUSSION

In this study, we analyzed the expression of Quorum network (QS/QQ) genes that differed between genomes of clinical isolates of *A. baumannii*, *abaR* and *abaI* (QS system) and *aidA* (QQ mechanism) in relation to clinical features of pneumonia and bacteraemia. Although other QQ enzymes have been described in *A. baumannii* ATCC 17978 (Mayer et al., 2018), these were not analyzed in the present study due to the lack of any differences between *A. baumannii* genomes.

In clinical strains of *A. baumannii* isolated from patients with bacteraemic pneumonia (Pn-B), the *abaR* gene was overexpressed ($p < 0.05$). The AbaR protein was the receptor activator of the Quorum Sensing system (QS), and the *aidA* gene was not expressed. Moreover, we observed regulation of *aidA* gene expression in clinical strains of pneumonia-causing *A. baumannii* (non-bacteraemic pneumonia, Pn-NB) by the 3-Oxo-C12-HSL molecule (which is an AidA enzyme substrate in QQ activity) and H_2O_2 (an activator of the QS system). However, there was no difference in the expression of Quorum network genes between colonized and pneumonia patients, as previously described (Stones and Krachler, 2016).

On the other hand, clinical analysis of the risk factors associated with pneumonia caused by *A. baumannii* revealed diabetes mellitus as only statistically significant risk factor (Kim et al., 2014). In relation to bacteraemia in *A. baumannii* pneumonia ($P < 0.05$), underexpression of the *aidA* gene was also the only statistically significant variable ($P < 0.05$).

In several pathogens, such as *Yersinia pseudotuberculosis*, *Proteus mirabilis,* and *Vibrio cholerae*, the QS system is the main regulatory mechanism of bacterial competence via T6SS, which is involved in the invasiveness and motility that favor the development of bacteraemia (Zhang et al., 2011; Debnath et al., 2018; Jaskólska et al., 2018; Trastoy et al., 2018). Moreover, in 86% of ICU patients, gastrointestinal tract colonization by a clinical strain of *A. baumannii* led to development of bacteraemia caused by genetically similar strains (Thom et al., 2010). This implies that clinical isolates of *A. baumannii* most capable of

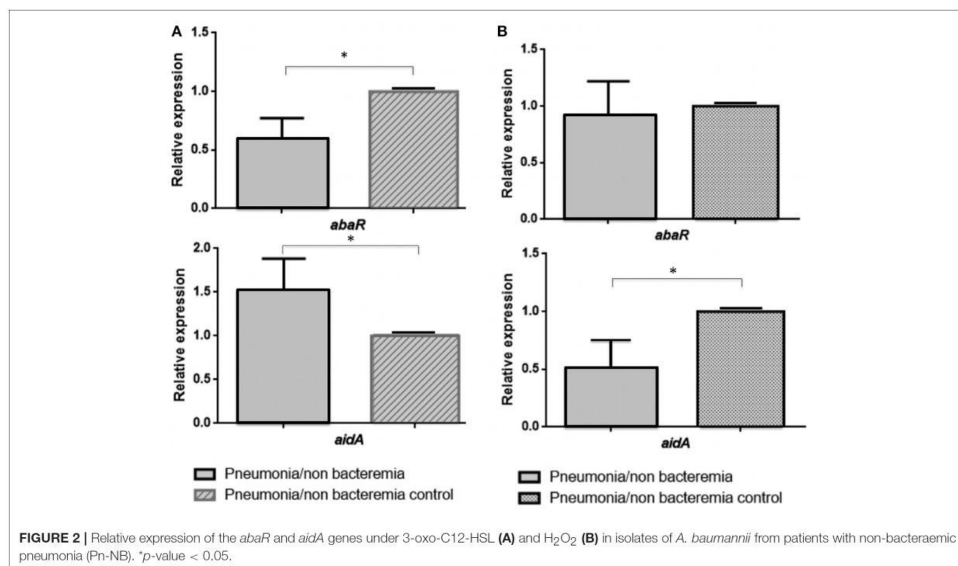

FIGURE 2 | Relative expression of the *abaR* and *aidA* genes under 3-oxo-C12-HSL **(A)** and H_2O_2 **(B)** in isolates of *A. baumannii* from patients with non-bacteraemic pneumonia (Pn-NB). *p-value < 0.05.

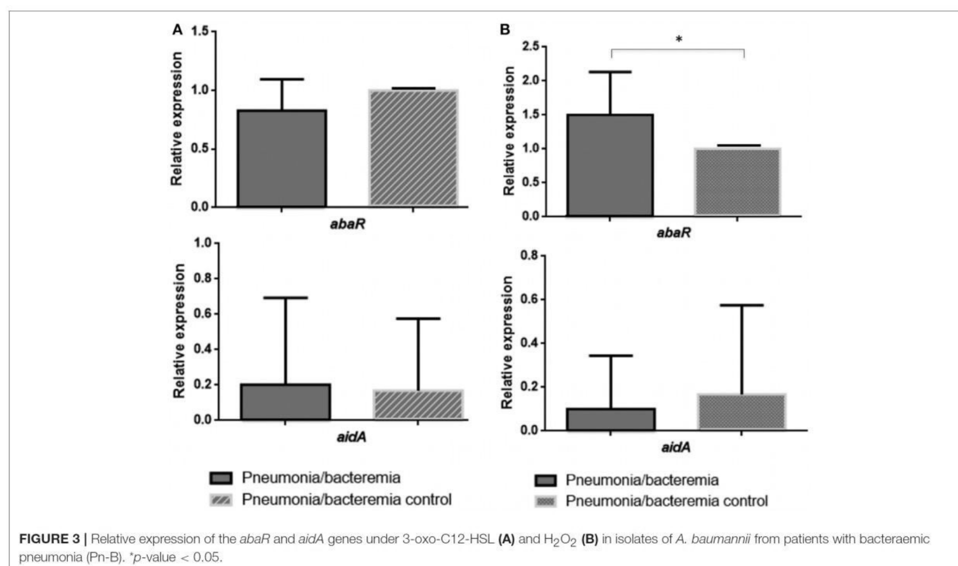

FIGURE 3 | Relative expression of the *abaR* and *aidA* genes under 3-oxo-C12-HSL **(A)** and H_2O_2 **(B)** in isolates of *A. baumannii* from patients with bacteraemic pneumonia (Pn-B). *p-value < 0.05.

TABLE 4 | Univariate analysis of risk factors associated with development of pneumonia relative to colonization by clinical strains of *A. baumannii*.

Variable	Colonized patients (*n* = 17)	Patients with pneumonia (*n* = 13)	*P*-value
Age, Med ± SEM	55.06 ± 5.12	59.31 ± 5.90	0.590
Female sex	5 (29.41)	6 (46.15)	0.287
Charlson score, Med ± SEM	2.12 ± 0.67	2.46 ± 0.69	0.729
Comorbity condition, no. (%)			
McCabe score, ultimately or rapidly	8 (47.06)	5 (38.46)	0.840
Cancer	1 (5.88)	2 (15.38)	0.397
Diabetes	**3 (17.65)**	**7 (53.85)**	**0.045**
Cirrhosis	0 (0)	0 (0)	NA
AIDS	0 (0)	1 (7.69)	0.433
Chronic lung disease	2 (11.67)	4 (30.78)	0.204
CRF	0 (0)	1 (7.69)	0.433
Immunosuppression	1 (5.88)	2 (15.38)	0.397
Surgery, No. (%)	5 (29.41)	4 (30.78)	0.623
ICU stay, No. (%)	15 (88.23)	11 (84.61)	0.591
Tracheostomy	4 (23.53)	2 (15.38)	0.469
Mechanical ventilation	10 (58.82)	7 (53.85)	0.538
Death	3 (17.65)	3 (23.08)	0.531
abaR	0.09 ± 0.02	0.07 ± 0.04	0.547
aidA	0.09 ± 0.03	0.09 ± 0.03	0.919

In bold and highlighted the variables that showed a p < 0.05.

TABLE 5 | Univariate analysis of risk factors associated with the development of bacteraemia in pneumonia caused by *A. baumannii* relative to the non-bacteraemic pneumonia control.

Variable	Pn-NB patients (*N* = 7)	Pn-B patients (*N* = 6)	*P*-value
Age, Med ± SEM	58.43 ± 10.13	60.33 ± 6.08	0.880
Female sex	4 (57.14)	2 (33.33)	0.383
Charlson score, Med ± SEM	2.00 ± 0.79	3.20 ± 1.24	0.497
Comorbity condition, no. (%)			
McCabe score, ultimately or rapidly	3 (42.86)	2 (33.33)	0.380
Cancer	1 (14.28)	1 (20)	0.731
Diabetes	2 (28.57)	5 (83.33)	0.078
Cirrhosis	0 (0)	0 (0)	NA
AIDS	0 (0)	1 (20)	0.462
Chronic lung disease	3 (42.86)	1 (20)	0.343
CRF	1 (14.28)	0 (0)	0.538
Immunosuppression	2 (28.57)	0 (0)	0.269
Surgery, No. (%)	3 (42.86)	1 (20)	0.343
ICU stay, No. (%)	5 (71.43)	6 (100)	0.269
Tracheostomy	0 (0)	2 (33.33)	0.192
Mechanical ventilation	3 (42.86)	4 (66.67)	0.383
Severe sepsis and septic shock, No. (%)	2 (28.57)	3 (50)	0.565
Death	3 (42.86)	0 (0)	0.122
abaR	0.78 ± 0.015	0.06 ± 0.02	0.522
aidA	**0.15 ± 0.04**	**0.03 ± 0.03**	**0.045**

In bold and highlighted the variables that showed a p < 0.05.

surviving under stress conditions (such as the presence of bile salts in the gastrointestinal tract or H_2O_2 in the respiratory tract) (Zheng et al., 2018) may have a higher invasive capacity due to virulence factors, such as the type VI secretion system (T6SS), previously activated under stressful conditions. Motility is also a crucial virulence factor, allowing penetration of the bacteria into the host's body and subsequent colonization (Gellatly and Hancock, 2013). Previous studies have demonstrated the existence of a relationship between motility and the origin of the isolates. Indeed, blood isolates of *A. baumannii* have been found to be more mobile than sputum isolates (Vijayakumar et al., 2016). Interestingly, 67% of the clinical isolates of *A. baumannii* were non-mobile and all of them had the AidA protein and were of respiratory origin (López et al., 2017a,b). In addition, the *aidA* gene was not located in the genome of the only mobile strain (clone ST79/PFGE-HUI-1) isolated from blood and which was the origin of a bacteraemic outbreak (López et al., 2017a,b).

Finally, multiple studies carried out with the *abaI* mutant of the M2 strain of *Acinetobacter nosocomialis* have analyzed the role of the *abaI* gene (responsible for the synthesis of quorum sensing synthesizing molecules) in various virulence factors such as biofilm formation and motility. In both cases, *abaI* deficiency led to a decrease in biofilm production and motility (Niu et al., 2008; Bhargava et al., 2012). The mutant lacking *abaI* is believed to be less virulent than the wild strain. This result was confirmed in our study in which injection of *G. mellonella* larvae with the reference *A. baumannii* ATCC17978 strain caused higher mortality than injection with the mutant *A. baumannii* ATCC17978Δ*abaI*. Regarding the mortality of the reference strain (*A. baumannii* ATCC17978), similar effects have been observed in other studies, in which injection of *G. mellonella* larvae with the reference strain *A. baumannii* ATCC17978 resulted in rapid death. Mortality was significantly dependent on the number of cells injected. More than 75% of the larvae died in the first 48 h of injection with at least 3.7×10^5 CFU / larva, while very few of the larvae died after being injected with a concentration equal to or lower than 3.7×10^4 CFU/larva ($p < 0.01$) (Clemmer et al., 2011). The results regarding the mutant *A. baumannii* ATCC17978Δ*abaI* are consistent with those obtained in a study of *Pseudomonas aeruginosa* (Steindler et al., 2009) in which a mutant Δ*rhLI* Δ*lasI* (QS systems homologous to *abaI*) was obtained, demonstrating that inactivation of both QS systems leads to a significant reduction in pathogenicity ($p < 0.01$) when virulence factors are not activated, such as the type VI secretion system (T6SS) and motility (Jaskólska et al., 2018).

In conclusion, our findings suggest that the QS (*abaR* and *abaI* genes)/QQ (*aidA* gene) network plays a role in the development of bacteraemia in patients with pneumonia caused by *A. baumannii.* This is the first study reporting a relationship between reduced expression of this bacterial QQ enzyme gene (AidA protein) and bacteraemia. Further studies

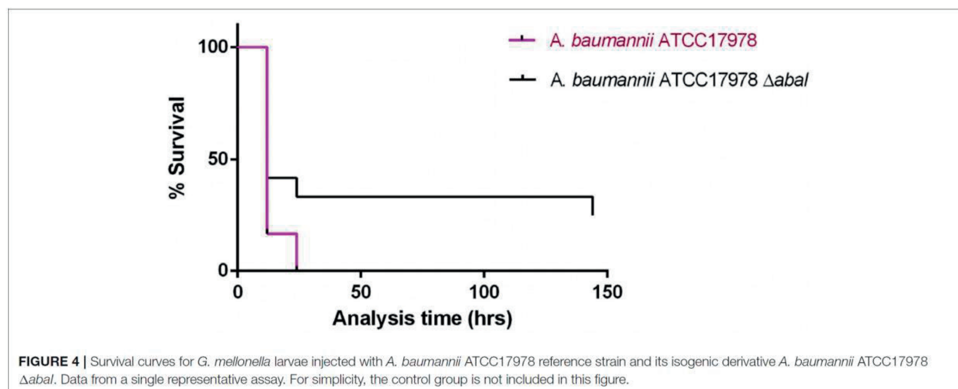

FIGURE 4 | Survival curves for *G. mellonella* larvae injected with *A. baumannii* ATCC17978 reference strain and its isogenic derivative *A. baumannii* ATCC17978 Δ*abaI*. Data from a single representative assay. For simplicity, the control group is not included in this figure.

of this relationship in the same and other bacterial QQ enzymes would be of great interest.

AUTHOR CONTRIBUTIONS

LF-G, AA, LB, IB, ML and RA-M developed the experiments. FF-C, LM-M, JV, JR-B, JG-M, JMC, AP, JP, GB, and YS wrote the manuscript and provided the strains. MT led the experiments and manuscript redaction.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03105/full#supplementary-material

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<u>Chapter IV.</u> Relationship between tolerance and persistence mechanisms in *Acinetobacter baumannii* strains with AbkB/AbkA toxin-antitoxin system

In hospitals, bacteria must survive stressors such as disinfectants and biocides in addition to the important antimicrobial stress (*17*). Among the biocides most frequently used in the hospital environment, we highlight chlorhexidine (*186*).

In the research reported in chapter IV, we used transcriptomic and phenotypic assays to analyse the tolerance and persistence mechanisms in response to chlorhexidine in *A. baumannii* isolates previously characterized in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010 (Bioproject PRJNA345289)".

Transcriptomic analysis of strain Ab-2_clon_2010-CHLX (tolerant to chlorhexidine) in the presence of chlorhexidine, showed overexpression of the following: i) Efflux pumps genes encoding AdeABC, arsenite and AceI chlorhexidine; ii) Plasmid PMMCU3p genes, such as OXA 24/40 β-lactamase, OriV and DNA replication protein; iii) CsuA/BABCDE operon, taurine and CydAB operons; and iv) Quorum sensing genes. However, the analysis also revealed repression of some genes, the metabolic pathways of which were analysed by reference to the Kyoto Encyclopedia of Genes and Genomes (KEGG): i) the ppGpp network, which is mediated by a variety of RelA/SpoT homologe (RSH) proteins (*187*), the accumulation of ppGpp affects resource-consuming cell processes and produces cell arrest under nutrient starvation (*188*); and ii) oxidative phosphorylation, in which α/β subunits of the ATP synthase as well as rho factor (a transcription termination factor) were downregulated.

Additionally, time-kill curves showed that, unlike Ab-2_clon_2010, Ab-2_clon_2010-CHLX suffered a massive depletion in the presence of imipenem; confirmed by the lower expression levels of OXA 24/40 β-lactamase, *abkA* antitoxin gene and the overexpression of *abkB* toxin gene. In addition, we observed regrowth of persister cells in the Ab-2_clon_2010-CHLX isolate in the presence of imipenem plus chlorhexidine. Moreover, we used two *A. baumannii* ATCC strains as controls (both susceptible to carbapenems) and surprisingly found that growth of *A. baumannii* ATCC 17978 (which contains the AbkB/AbkA toxin-antitoxin system) was reactivated in the presence of imipenem and chlorhexidine, in contrast to *A. baumannii* ATCC

19606 (which lacks the TA system). These data were confirmed by enzymatic analysis with the cell proliferation reagent WST-1.

These data confirm the importance of tolerance and persistence mechanisms under chlorhexidine and imipenem pressure in clinical strains of *A. baumannii* carrying the $bla_{OXA\ 24/40}$ β-lactamase gene and the AbkB/AbkA toxin-antitoxin system in a plasmid. They thus demonstrate the importance of the TA systems in persister cell formation under stress conditions.

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MECHANISMS OF ACTION: PHYSIOLOGICAL EFFECTS

Relationship between Tolerance and Persistence Mechanisms in *Acinetobacter baumannii* Strains with AbkAB Toxin-Antitoxin System

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ABSTRACT The molecular mechanisms of tolerance and persistence associated with several compounds in *Acinetobacter baumannii* clinical isolates are unknown. Using transcriptomic and phenotypic studies, we found a link between mechanisms of bacterial tolerance to chlorhexidine and the development of persistence in the presence of imipenem in an *A. baumannii* strain belonging to clinical clone ST-2 (OXA-24 β-lactamase and AbkAB toxin-antitoxin [TA] system carried in a plasmid). Interestingly, the strain *A. baumannii* ATCC 17978 (AbkAB TA system from plasmid) showed persistence in the presence of imipenem and chlorhexidine.

KEYWORDS tolerance, persistence, chlorhexidine, imipenem, *Acinetobacter*, toxin-antitoxin

The importance of preventing the development of tolerance and/or persistence has recently been highlighted as a new strategy for delaying the emergence of resistance (1–4). In this context, it is essential to distinguish between bacterial resistance, tolerance, and persistence (5). Resistance refers to the ability of bacterial populations to grow at the same rate in the presence of antibiotic-induced or environmental stress. Tolerance is the ability of a bacterial population to grow slowly in response to stress. Finally, persistence is the latent state of a bacterial subpopulation, which is activated under certain conditions (5).

Several bacterial tolerance mechanisms develop during stress and antibiotic exposure (6). These mechanisms include (p)ppGpp signaling accumulation, reactive oxygen species (ROS) and SOS responses, bacterial communication (quorum sensing), efflux pumps, and energy metabolism (6).

Carbapenem-resistant *Acinetobacter baumannii* (CRAb) is currently a major source of nosocomial infections and is considered a highly successful human pathogen (7). Among the different mechanisms associated with carbapenem resistance in *A. baumannii*, the production of acquired carbapenem-hydrolyzing class D β-lactamases (CHDLs) and class B metallo-β-lactamases (MBLs) has been widely studied (8). On the other hand, the main mechanisms of development of persister cells in the presence of antibiotics (such as imipenem [IMP]) involve toxin-antitoxin (TA) modules (6, 9).

Studies about molecular mechanisms of tolerance and persistence from *A. baumannii* strains in response to several compounds are scarce. In this study, we used transcriptomic and phenotypic assays to analyze the tolerance and persistence mech-

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anisms of *A. baumannii* isolates in response to chlorhexidine and imipenem (resistance and susceptibility to carbapenems).

In a previous work of the REIPI-GEIH Ab-2010 project (10), we worked with the *A. baumannii* clinical strains Ab-2_clon_2010 (belonging to clone ST-2) and Ab-2_clon_2010-CHLX, which showed the absence of an increase of MICs to antibiotics after exposure to subinhibitory concentrations of chlorhexidine digluconate (CHLX) (0.25× MIC) during 4 weeks (see Table S1 in the supplemental material). The genome of this Ab-2_clon_2010 strain, together with 17 other clinical strains from this ST-2 clone, were sequenced by Lopez et al. (11) in the Umbrella GenBank BioProject number PRJNA422585. All strains from this ST-2 clone belonged to the REIPI-GEIH Ab-2010 project and had a plasmid with the *bla*$OXA_{24/40}$ β-lactamase gene (conferring resistance to carbapenems), as well as the *abkAB* genes from a toxin-antitoxin system (12). RNA assays by transcriptomics had a number of reads assigned to the different genes and were analyzed using the EdgeR and DESeq2 packages and reverse transcription PCR (RT-PCR) techniques using UPLs Probe (see Table S3 in the supplemental material; Roche, Germany) of both clinical isolates (DNase-treated RNA of Ab-2_clon_2010 and Ab-2_clon_2010-CHLX) (GenBank BioProject number PRJNA433173 and GEO series number GSE110207), the results of which are shown in Table S2 and Fig. S1 and S2 in the supplemental material.

The results showed the activation of tolerance molecular mechanisms (known as "tolerome") in response to chlorhexidine in strain Ab-2_clon_2010-CHLX (Table 1). In relation with the tolerome, in the strain Ab-2_clon_2010-CHLX, we observed overexpression (1.5- to 6-fold change [FC]) of genes encoding the AdeABC, arsenite, and AceI chlorhexidine efflux pumps (10, 13–16). Some of these additional protective mechanisms, such as the production of efflux pumps, may also reduce the effective concentration of the antibiotic, which increases the MIC and results in a mixed phenotype of resistance and tolerance (5). We also observed an increase in the expression of genes involved in tetracycline and aminoglycoside resistance (FC, 3.4 to 6). The genes with the highest level of overexpression in this study were those carried by the AbATCC329 plasmid (PMMCU3p), such as OXA24/40 β-lactamase, DNA replication protein, and OriV (FC, 5.2 to 12) (12) (Table 1). Interestingly, the gene expression FCs of *abkA* (antitoxin gene) and *abkB* (toxin gene) from this plasmid were 0. 63 and 1.25, respectively. In addition, we observed the overexpression of genes associated with molecular mechanisms of bacterial tolerance (FC, 3.5 to 10), namely, the CsuA/BABCDE operon (17, 18), the CydAB operon (cytochrome *d* ubiquinol oxidase complex) (19, 20, 21), the taurine operon complex (taurine metabolism/electron carrier activity) (22, 23), and finally, regulatory genes involved in the quorum-sensing (QS) system, i.e., *abaR* and *abaI* (Table 1) (22–25).

We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) tool to analyze those genes that were downregulated (FC, ≤0.5-fold) in Ab-2_clon_2010-CHLX. We studied two metabolic pathways. The first one was the ppGpp network (KEGG accession numbers ec00230 2.7.7.6 and ec00230 2.7.7.7) involving RNA polymerases, DNA polymerases, and finally, 50S ribosomal protein. The ppGpp network is mediated by a variety of RelA/SpoT homologue (RSH) proteins with a nucleotidyl transferase domain, with some displaying only synthetic or hydrolytic activities, and others displaying both (Rel) (26, 27). Accumulation of (p)ppGpp affects resource-consuming cell processes, such as replication, transcription, and translation. Furthermore, (p)ppGpp is thought to bind RNA polymerase and alter the transcriptional profile, decreasing the synthesis of translational machinery (such as rRNA and tRNA) and increasing transcription of the biosynthetic gene (28). Additionally, initiation of new rounds of replication is inhibited, and the cell cycle arrests until nutrient conditions improve (29). Translational GTPases involved in protein biosynthesis are also affected by ppGpp, with initiation factor 2 (IF2) being the main target (30). Although these proteins are scarcely known in *A. baumannii*, in this study we describe RelA-SpoT-homologous (RSH) proteins associated with these functions that show repression in Ab-2_clon_2010-CHLX isolate. The second metabolic pathway studied was that of oxidative phosphorylation (KEGG accession

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TABLE 1 Mechanisms of bacterial tolerance to chlorhexidine in strain Ab-2_clon_2010-CHLX, revealed by transcriptomic studies[a]

GenBank[b] protein accession no.	Gene expression fold change determined by: DESeq2	EdgeR	Functional description	Defense mechanism (reference no.)	Tolerome type (reference no.)
ODA53993.1	6.933753475	6.982635042	AdeA protein	AdeABC system (RND-type) (10)	Transporter/efflux pump (5)
ODA53994.1	6.149907892	6.175526694	AdeB protein		
ODA53995.1	4.257153566	4.270842036	AdeC protein		
ODA55718.1	6.119321647	6.133494454	Tetracycline resistance protein	MFS system	
ODA54617.1	5.377292457	7.227172031	Arsenite efflux pump	ACR3 system (13)	
ODA56577.1	3.498098186	3.528728206	Aminoglycoside phosphotransferase	APT family	
ODA54814.1	3.605781331	3.649808635	Chlorexidine efflux pump	AceI system (16)	
ODA56167.1	5.265550668	7.054151044	MFS transporter	MFS system	
ODA53764.1	12.16763575	14.92175121	OXA 24/40 β-lactamase	AbATCC329p/pMMCU3	Plasmid (5)
ODA53763.1	8.975633873	11.30715263	DNA replication protein A		
ODA53762.1	5.273985066	5.329333593	RepB family plasmid replication initiator		
ODA54084.1	3.511019975	3.547062538	CsuA protein	CsuABCDE (17, 18)	Biofilm (14)
ODA54083.1	3.199749378	3.259685195	CsuB protein		
ODA54082.1	2.575094974	2.584527435	CsuC protein		
ODA54081.1	2.810613341	2.819199271	CsuD protein		
ODA54080.1	2.782552791	2.791313686	CsuE protein		
ODA53940.1	2.037734523	2.053934504	Cytochrome *b*	Cytochrome operon (19–21)	Stress oxidative (ROS) (21)
ODA57053.1	2.173049691	2.184371809	Cytochrome bd biosynthesis protein		
ODA56663.1	2.405101873	2.428897655	Sodium/proline symporter		
ODA56171.1	10.75708444	13.32903693	Cytochrome bd biosynthesis protein		
ODA56172.1	10.35093438	12.86380541	Cytochrome *d* ubiquinol oxidase subunit		
ODA54604.1	10.07652823	12.56398102	Taurine ABC transporter substrate-binding	Taurine transporter (22, 23)	Electron transport
ODA54605.1	9.758316312	12.21616998	Taurine transporter-binding subunit (TauB)		
ODA54606.1	8.966908008	11.30350134	Taurine ABC transporter permease (TauC)		
ODA54607.1	10.85324686	13.44475271	Taurine dioxygenase (TauD)		
ODA55153.1	−6.486154998	−6.530626555	Hypothetical protein	Replication	ppGpp network (28)[c]
ODA54592.1	0.932475218	0.929842277	DNA polymerase I		
ODA54625.1	0.931688577	0.929428817	DNA polymerase III subunit alpha		
ODA54730.1	−1.77207536	−1.816317901	Response regulator		
ODA55878.1	0.500078184	0.506140675	50S ribosomal protein L17		
ODA55763.1	0.438241011	0.436148678	RNA polymerase subunit omega		
ODA55654.1	0.582523178	0.580921263	50S ribosomal protein L7/L12		
ODA55933.1	−0.523115918	−0.531876133	ATP synthase subunit beta	ATP metabolism	Energy production (31, 32)[c]
ODA55935.1	−0.570647356	−0.579254835	ATP synthase subunit alpha		
ODA54585.1	0.422390483	0.418615357	Transcription termination factor rho		

[a]The relative expression (expressed as fold change [FC]) of *abaI* (3.05) and *abaR* (2.88) genes, determined by RT-PCR, indicated activation of the quorum-sensing system.
[b]Sanger sequencing of these genes from the Ab-2_clon_2010-CHLX strain, as well as of the regulatory genes *adeR* and *adeS*, did not show mutations with respect to the sequence of strain Ab-2_clon_2010.
[c]Genes that belonged to the ppGpp network and energy production categories showed downregulation (FC, <1).

no. ec00190 3.6.3.14, ATP phosphohydrolases), in which the alpha/beta ATP synthase subunit and transcription termination factor rho were downregulated. Finally, energy production by ATP metabolism has been associated with the development of tolerant cells in *Escherichia coli* (31). Moreover, Wang et al., described how genes mapped in this pathway have an important role in the survival of clinical strains of *Staphylococcus aureus* (32).

The time-kill curves for strains Ab-2_clon_2010 and Ab-2_clon_2010-CHLX were performed following the indications of Hofsteenge and colleagues (33) in low-nutrient Luria-Bertani broth (LN-LB; 2 g/liter tryptone, 1 g/liter yeast extract, and 5 g/liter NaCl) (13, 16). The cultures were incubated for 4 h to ensure logarithmic growth, and CHLX (0.25× MIC) and IMP (10× MIC) were then added alone or in combination to the cultures. We observed a lower growth rate of the Ab-2_clon_2010 strain in the presence of CHLX than in its absence (Fig. 1), as well as faster growth rate in the presence of IMP. Interestingly, the time-kill curves for isolate Ab-2_clon_2010-CHLX showed a massive killing in the presence of IMP (Fig. 1). The results of RT-PCR analysis confirmed a lower expression of $OXA_{24/40}$ β-lactamase and *abkA* antitoxin genes (FC, 0.06 and 0.04, respectively) in Ab-2_clon_2010-CHLX, as well as overexpression of the *abkB* toxin gene (FC, 2.77) relative to that in Ab-2_clon_2010 (known as the persistome) (34–36). This

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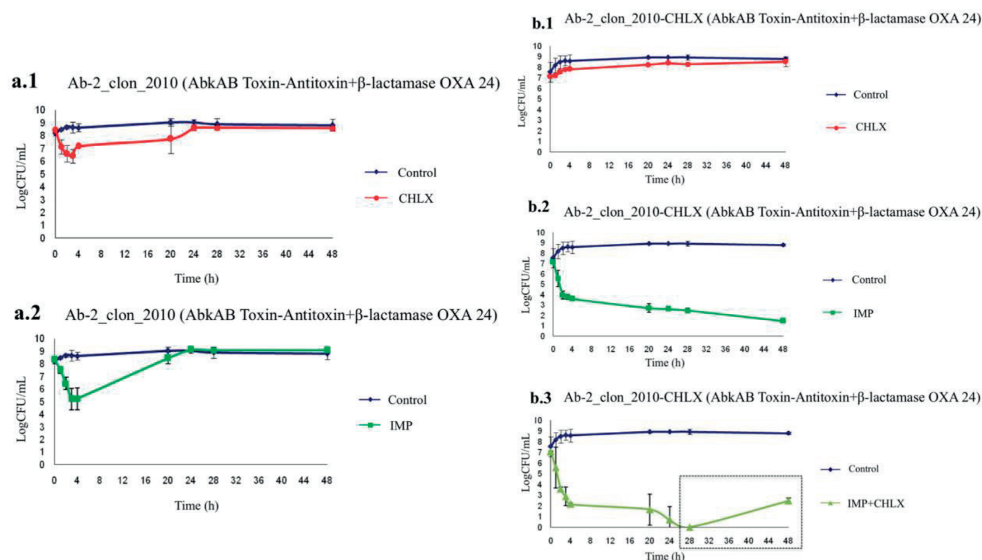

FIG 1 Time-kill curves in the presence of biocides (CHLX) and antibiotics (IMP) in Ab-2_clon_2010 (carbapenem-resistant) and Ab-2_clon_2010-CHLX isolates. Box in panel b.3, regrowth is due to putative reactivation of persister cells.

toxin protein belongs to the AbkAB toxin-antitoxin module in the AbATCC329p/pMMCU3 plasmid (12). Mosqueda et al. located the AbkB/AbkA TA system (the so-called SplTA) in the most prevalent plasmids (GenBank KJ534568 and KJ534569) found in clinical isolates of *A. baumannii* (12, 37). Finally, we observed regrowth of persister cells in the Ab-2_clon_2010-CHLX isolate grown in the presence of IMP+CHLX for 28 h (Fig. 1). Moreover, we used two *A. baumannii* ATCC isolates as controls (both susceptible to carbapenems) whose complete genomes have been sequenced, *A. baumannii* strain ATCC 17978 (which harbors the AbkAB toxin-antitoxin system encoded by plasmid pAB2, GenBank number CP000523.1) and *A. baumannii* strain ATCC 19606 (which does not have this AbkAB toxin-antitoxin system). In Fig. 2, we observed that in the *A. baumannii* strain ATCC 17978, there was a reactivation of growth in the presence of IMP+CHLX for 28 h, in contrast to the lack of growth of the *A. baumannii* ATCC 19606 under the same conditions. These results of regrowth in the *A. baumannii* strain ATCC 17978 and in Ab-2_clon_2010-CHLX with IMP (10× MIC) and CHLX (0.25× MIC) at 48 h were confirmed by enzymatic analysis using the cell proliferation reagent WST-1 protocol (Roche, Germany) and calculating the serial dilutions of each culture (CFU/ml; Fig. 3).

In conclusion, this is the first study describing the important link between mechanisms of bacterial tolerance and persistence under chlorhexidine and imipenem pressure in a clinical isolate of *A. baumannii* ST-2 harboring the $bla_{\text{OXA 24/40}}$ β-lactamase gene and *abKA/abkB* genes (toxin-antitoxin system) in a plasmid. The study of these mechanisms (bacterial tolerance and persistence) is key to the development of new anti-infective treatments which will allow for the eradication of multidrug resistant pathogens.

Accession number(s). The whole-genome sequence (WGS) studies of GEIH-2010 isolate Ab-2_clon_2010 comprise part of the II Spanish Multicenter Study. GEIH-REIPI *A. baumannii* 2000 to 2010 project (umbrella GenBank BioProject number PRJNA422585), as well as the transcriptomic results shown in GenBank BioProject

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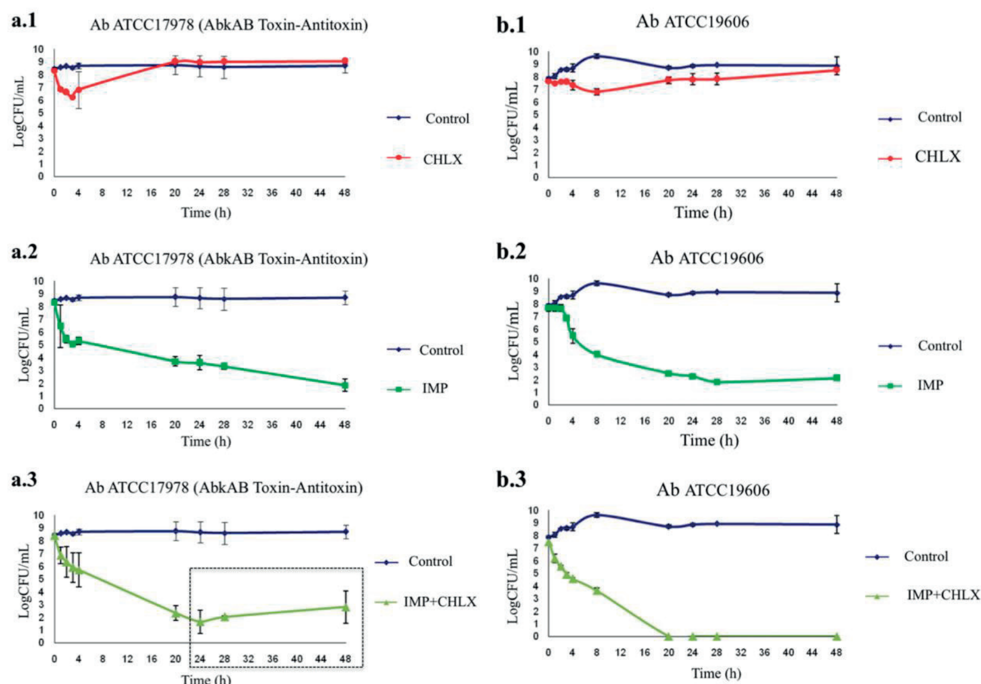

FIG 2 Time-kill curves in the presence of antibiotics (IMP) and biocides (CHLX) in susceptible *A. baumannii* ATCC strains. (a) *A. baumannii* strain ATCC 17978, which harbors the plasmid with the AbKA/AbkB toxin-antitoxin system (positive control); (b) *A. baumannii* ATCC 19606 strain without this AbKA/AbkB toxin-antitoxin system (negative control). Box in panel a.3, regrowth is due to putative reactivation of persister cells.

number PRJNA433173 (GEO series number GSE110207). The WGSs of the *A. baumannii* strain ATCC 17978 complete genome and *A. baumannii* strain ATCC 19606 complete genome are deposited under GenBank accession numbers CP018664.1 and GG704575.1, respectively.

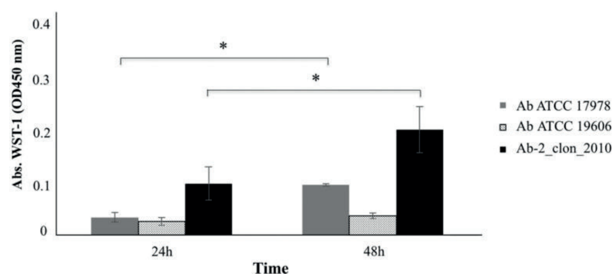

FIG 3 Enzymatic activity by colorimetric assay (WST-1-based) of the isolates *A. baumannii* ATCC 17978, *A. baumannii* ATCC 19606, and *A. baumannii* Ab-2_clon_2010-CHLX in the presence of IMP and CHLX. The *x* axis represents absorbance (optical density at 450 nm [OD_{450}]), and the *y* axis represents time (h). *, $P < 0.05$ (Student's *t* test).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00250-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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We declare that we have no competing interests.

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<u>Chapter V.</u> Toxin inactivation in toxin/antitoxin systems

In the previous chapter we demonstrated the relationship between TA systems and persistence/tolerance mechanisms. TA systems are widely distributed in bacteria, but they are also found in Archaea and even fungi (*131*); the broad distribution and myriad types of TA systems implies an evolutionary advantage for their use. Nevertheless, TA systems require tight regulation, to prevent undesired effects on metabolism.

In this fifth chapter, we provide insights into how bacteria respond to TA toxin activity, by identifying the mutations that occur in bacteria to inactivate toxins, when antitoxins do not function or are not present. We produced each toxin (an RNase, membrane-damaging peptide, DNase, and transcriptional regulator) from a common plasmid backbone and promoter.

We constructed growth curves for *E. coli* K12 BW25113 strain with a plasmid pCA24N, which carried a toxin gene (*mqsR, ghoT, ralR,* and *hha*) that was overexpressed in the presence of IPTG. The growth curves showed inhibition of growth during the first hours; however, after 12 h the bacteria had regrown. Surprisingly, a second curve with these strains showed that they were no longer affected by overexpression of the toxin, confirming the presence of a stable mutation.

Moreover, in order to determine the type of modification that had occurred, we sequenced the plasmids and found a deletion in the promoter of some of them. The genomes of those strains in which the plasmid was not mutated were sequenced, revealing several SNPs in different genes, highlighting mutations in *lacI, iraM* and *mhpR*. Furthermore, we determined the rate of conservation of these toxin genes in 1000 genomes of *E. coli,* noting a high rate of preservation in almost all of them, except RalR, in which the sequence was more variable.

The research findings demonstrate that bacteria inactivate toxins by mutating the promoter of the toxin or by inactivating chromosomal copies of *lacI*, *iraM* and *mhpR*, thus preventing modification of the toxin sequence.

Attached is the corresponding paper in review at Journal of Applied Microbiology:

Toxin Inactivation in Toxin/Antitoxin Systems

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ABSTRACT

Aims: Given the extreme toxicity of some of the toxins of toxin-antitoxin (TA) systems, we were curious how the cell silences toxins, if the antitoxin is inactivated or when toxins are obtained without antitoxins via horizontal gene transfer.

Methods and Results: Growth curves using *E. coli* K12 BW25113 harboring plasmid pCA24N, carrying RalR, MqsR, GhoT or Hha toxins, showed cessation of growth inhibition after 3 hours. Plasmids from these cultures were purified and sequenced, finding that most of them had a deletion in the plasmid promoter. In those strains that lack of mutation in the plasmid a SNPs analysis were performed showing chromosomal mutations at *iraM* and *mhpR* genes. Besides, a bioinfromatic analysis using IMG/M were performed to determine the conservation of these toxins in the population.

Conclusion: We find that the RalR (type I), MqsR (type II), GhoT (type V), and Hha (type VII) toxins are inactivated primarily by a mutation that inactivates the toxin promoter or via the chromosomal mutations *iraM* and *mhpR*.

Significance and Impact of the Study: This study was the first to mention the inhibition of toxins from TA systems by mutations that affect the plasmid promoter or the chromosome instead of the toxin gene.

Keywords: toxin, antitoxin, mutation, toxicity inhibition, TA systems

INTRODUCTION

Toxin-antitoxin (TA) systems have been related to gene regulation during the stress response (Wang et al., 2011), persister cell generation (Kim and Wood, 2010, Shah et al., 2006), bacteriophage protection (Pecota and Wood, 1996), and other functions (Schuster and Bertram, 2013). TA systems were originally found on plasmids (Ogura and Hiraga, 1983), but they have also been found in bacterial chromosomes and bacteriophages. Almost all bacteria have TA systems in their genomes, reaching 88 TA loci in case of *Mycobacterium tuberculosis* (Page and Peti, 2016). Moreover, TA systems have been related to pathogenicity in a wide range of nosocomial pathogens (Trastoy et al., 2018). Furthermore, TA systems have also been described in 86 Archaea and even in some fungi, in which there have been identified fourteen Doc toxin homologs (Yamaguchi et al., 2011).

TA systems are primarily two component systems, composed of a toxin, which disrupts important cellular mechanisms, and an antitoxin, which blocks the toxin action (Schuster and Bertram, 2013). Depending on how the antitoxin interacts with the toxin, TA systems are classified into seven types. In type I systems (e.g., Hok/Sok), the antitoxin is an antisense RNA of the toxin. In type II (e.g., CcdB/CcdA) and III (e.g., ToxN/ToxI) systems, the antitoxin protein or RNA, respectively, inhibits the toxin by direct binding (Schuster and Bertram, 2013). In type IV systems (e.g., CbtA/CbeA), the antitoxin competes with the toxin for the target (Masuda et al., 2012), and in type V systems (e.g., GhoT/GhoS), the antitoxin is an enzyme that cleaves specifically the toxin mRNA (Wang et al., 2012a). In type VI systems (e.g., SocB/SocA), the antitoxin protein facilitates toxin degradation as an adaptor protein (Page and Peti, 2016). Recently, the type VII system has been described (Hha/TomB) in which the antitoxin is an enzyme that inactivates the toxin by oxidizing a cysteine residue (Marimon et al., 2016, Song and Wood, 2018).

The broad distribution and myriad types of TA systems implies there is an evolutionary advantage for utilizing them. Also, many of the TA systems require tight regulation, to prevent undesired reductions in metabolism. For example, deletion of the gene encoding antitoxin MqsA is lethal (Brown et al., 2009). For

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this tight regulation, TA systems are often self-regulated, as (i) antitoxins of type II TAs repress expression via promoter binding, (ii) some toxins limit both antitoxin and toxin levels via post-transcriptional cleavage of mRNA (Hayes and Kędzierska, 2014, Wang et al., 2013a), and (iii) some antitoxins inhibit toxins. For example, toxin MqsR autoregulates itself by cleaving its own mRNA (Brown et al., 2012).

This study focuses on four different toxins, MqsR, GhoT, RalR, and Hha, each one from a different TA system. The MqsR/MqsA system is a type II TA system in which the toxin, MqsR, was originally characterized as a biofilm formation regulator that is quorum-sensing related (Ren et al., 2004). MqsR also regulates another TA system, GhoT/GhoS (Wang et al., 2013b). MqsA antitoxin participates in the global stress response by regulating RpoS and activating biofilm formation under oxidative stress (Wang et al., 2011). As an example of this oxidative stress response, the MqsR/MqsA system manages growth during stress due to bile acids in the gastrointestinal tract (Kwan et al., 2015). MqsR is also the first toxin that when inactivated, reduces persister cell formation (Kim and Wood, 2009). In regard to its regulation, toxin MqsR disrupts the MqsA-DNA complex to activate transcription (Brown et al., 2012).

The first type V TA system, GhoT/GhoS (Wang et al., 2012a), is named based on the ghost-cell phenotype seen when toxin GhoT is produced, which results from membrane damage and reduces ATP (Cheng et al., 2014). The RalR/RalA system is a type I TA system found in the cryptic prophage rac (Guo et al., 2014). RalR is the only known, non-specific DNase TA system toxin (many toxins are RNases); it activates the SOS response to DNA damage and increases resistance to the antibiotic fosfomycin. Hence, the RalR/RalA systems improve bacterial fitness under stress conditions (Guo et al., 2014).

Three years ago, the first type VII TA system was described (Marimon et al., 2016). The haemolysin expression modulation protein (Hha) is the toxin, and TomB is the antitoxin. Hha is a global transcriptional regulator that modulates cell physiology (Hong et al., 2010) by (i) forming the Hha-H-NS complex where it represses the pathogenicity locus of enterocyte effacement (LEE), (ii) repressing the transcription of rare codon tRNAs (bacteriolytic effect) and fimbrial genes

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which reduces biofilm formation (García-Contreras et al., 2008), (iii) having a pleiotropic effect in catabolite repression (Balsalobre et al., 1999), inducing protease ClpXP, which activates some prophage lytic genes (García-Contreras et al., 2008), and inducing excision of prophages Cp4-57 and DLP-12 of *E. coli* (Wang et al., 2009). Remarkably, instead of forming a complex between the toxin and the antitoxin, toxin Hha is inactivated by oxidation mediated by the antitoxin TomB (Marimon et al., 2016).

The aim of this study was to provide insights into how bacteria cope with TA toxin activity by identifying what mutations take place in bacteria to inactivate toxins, for cases in which antitoxins do not function or are not present, such as after horizontal gene transfer. For this goal, we produced four different toxins of TA systems from *E. coli* (an RNase, membrane-damaging peptide, DNase, and transcriptional regulator) from a common plasmid backbone with an IPTG dependent promoter, and we determined that bacteria inactivate toxins primarily by mutating the promoter of the toxin or by mutating chromosomal copies of the *iraM* and *mhpR* genes.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* K12 BW25113 (Baba et al., 2006) with multicopy plasmids pCA24N (Kitagawa et al., 2005), pCA24N-mqsR (Wang et al., 2013b), pCA24N-ghoT (Wang et al., 2012b), pCA24N-ralR (Guo et al., 2014) and pCA24N-hha (Marimon et al., 2016) was used for producing toxins and BW25113 with plasmids pCA24N-mqsRA (Wang et al., 2013b), pCA24N-ghoST (Wang et al., 2012b), pCA24N-ralRA (Guo et al., 2014), and pCA24N-hha-tomB (Marimon et al., 2016) was used for producing the toxin along with its antitoxin. Moreover, we used Keio mutants for *lacI*, *iraM*, and *mhpR* genes (*27*) with the pCA24N-based plasmids to determine possible influence of these genes on the toxin activity; using these mutants allowed us to test the toxicity of the pCA24N-based plasmids in a strain with no other mutations. All cultures were grown in lysogeny broth (LB) (Sambrook et al.) supplemented with 30 µg/mL of chloramphenicol (to maintain the plasmids) at 37°C with shaking.

Toxin inactivation. The toxicity of each toxin was confirmed by growing each strain from an overnight culture on plates with and without 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For inactivation, overnight cultures were used to inoculate fresh medium and grown until a turbidity at 600 nm of 0.05 to 0.1, then 1mM IPTG was added. The turbidity was measured every 15 minutes until it reached 0.7 to 0.8. After 12 hours, single colonies were isolated from each culture, regrown, and the plasmid purified with an E.Z.N.A plasmid DNA Minikit Omega® for sequencing. To confirm that the toxins had been inactivated, the purified strains were grown in liquid cultures in the presence of 1mM of IPTG.

Bioinformatic analysis. Sequences were analyzed by Clustal Omega. The analysis of 1,000 genomes of *E. coli* were made using "Integrated microbial genomes and microbiomes" (IMG/M) web page. Using IMG/M data base it was performed a Blast search of each of the genes to study in order to determine their conservation rate in the *E. coli* population.

Mutation analysis. Plasmids were sequenced (Quintara Biosciences) using reverse primer pCA24N-R (5'-GAACAAATCCAGATGGAGTTCTGAGGTCATT-3'). Strains that lost toxin activity but did not show any mutations in the plasmid-based toxin gene or its promoter were sequenced by Illumina HiSeq platform. Raw sequence data were trimmed by Sickle (https://github.com/najoshi/sickle) and quality was checked by FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The raw data were mapped to reference the genome by BWA (http://bio-bwa.sourceforge.net/). Mapping data were sorted, merged, and deduplicated by Picard (https://broadinstitute.github.io/picard/). Realignment and unified-genotype data were performed by GATK (https://software.broadinstitute.org/gatk/). SnpEff (http://snpeff.sourceforge.net/) was used for genome annotation. REDTools (https://bedtools.readthedocs.io/en/latest/) and Samtools (http://samtools.sourceforge.net/) were used for calculating the depth of each base and for finding unique reads. All the strains were re-grown in the presence of 1mM of IPTG after the mutation period, in order to assay the stability of the mutations, by measuring the turbidity at 600 nm.

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RESULTS

Mutation analysis. To determine general insights into how toxins are inactivated, we studied four diverse toxins by choosing one from each of the type I (RalR), type II (MqsR), type V (GhoT) and type VII (Hha) systems. Initially, each toxin inhibited cell growth completely when induced with 1 mM IPTG, and each antitoxin was able to mask each toxin, restoring growth to that seen with the empty plasmid for antitoxins MqsA, RalA, and TomB whereas GhoS did not completely restore growth (**Fig. 1**).

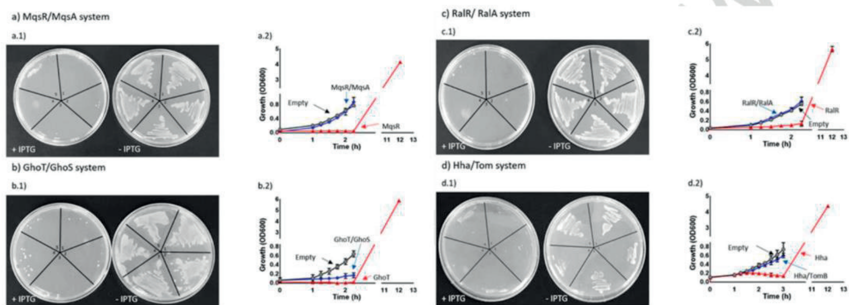

Figure 1. Inactivation of plasmid-based toxins. Plate results: BW25113 producing toxins from pCA24N-based plasmids (pCA24N-mqsR, pCA24N-ghoT, pCA24N-ralR, or pCA24N-hha) with (left) and without (right) 1mM IPTG to induce the toxin genes indicate the initial toxicity for MqsR, GhoT, RalR, and Hha. Five colonies of the original strain were streaked on each plate. Growth curves: Growth of cells harboring the empty plasmid pCA24N ("Empty", black circles and lines), the toxin and antitoxin (blue diamonds and lines), and toxin alone (triangles and red lines) with 1mM IPTG.

For all four toxins, after 3 to 12 h, growth inhibition ceased (**Fig. 1**) which indicated a mutation in the plasmid or in the chromosome that allowed the cells to resume growth. Also, purified colonies, obtained after the toxicity of each of the four toxins was inactivated, grew in the presence of IPTG (**Fig. 2**), indicating the mutations were stable.

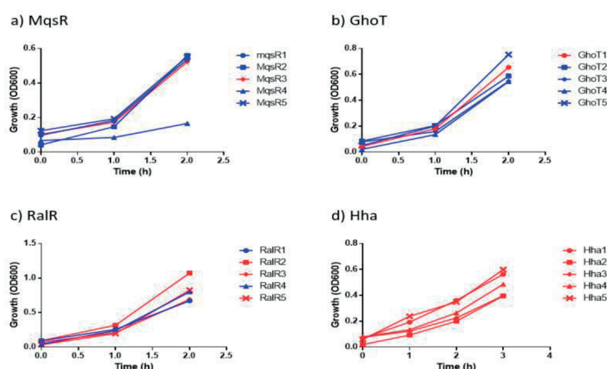

Figure 2. Stability of the toxin inactivation mutations. Growth of BW25113 containing pCA24N-based plasmids with mutated promoters or chromosomal changes that inactivate the toxin in the presence of 1 mM IPTG. Blue indicates plasmid mutations whereas red indicates chromosomal changes. Sequences of the plasmid toxin genes and promoter are shown in Fig. S1 (for mqsR), Fig. S2 (for ghoT), Fig. S3 (for ralR), and Fig. S4 (for hha).

Upon sequencing the plasmids of the strains with inactivated toxins, 10 plasmids (four encoding MqsR, four encoding GhoT, and two encoding RalR), showed a partial deletion of a core 32 nucleotides in the P*t5-lac* promotor rather than changes in the structural portion of the gene (**Fig. S1-S4**). Specifically, the core deletion included the -10 TATA box of the promotor. For 10 other strains with inactivated toxins (one MqsR, one GhoT, three RalR, and five Hha), no mutation in the promoter and toxin gene were found (**Fig. S1-S4**); hence, a stable mutation occurred in the chromosome. Note that along with the five mutants with chromosomal changes that inactivate Hha shown in **Fig. 2**, another three mutants were obtained with chromosomal changes that inactivate Hha, so in all, Hha was inactivated eight times by changes in the chromosome.

To investigate changes in the chromosome that led to growth in the presence of the four toxins in the absence of changes in the promoter and toxin genes, we selected five strains (one for MqsR, one for GhoT, two for RalR and one for Hha) and sequenced their chromosomes. We found several single nucleotide changes, including those in bacteriophage genes, RNA general metabolism genes and other metabolism genes. We focused on mutations related to regulators, specifically, those in *lacI* (lac operon repressor), *iraM* (anti-RssB factor, involved

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in RpoS stabilization during Mg starvation) and *mhpR* (transcriptional regulator) genes. Modifications in these three genes were observed in the chromosome of the five strains analyzed.

Using clean strains with single gene knockouts in either *lacI, iraM,* or *mhpR,* we electroporated plasmids with active GhoT and MqsR toxins and checked for the impact of the deletions on toxin activity. As shown in **Fig. 3**, the *lacI* and *iraM* mutations were unable to mask MqsR toxicity; however, GhoT was not toxic in both of the *iraM* and *mhpR* strains, and MqsR was inactive in the *mphR* strain. Hence, inactivating MhpR renders both GhoT and MqsR toxins inactive.

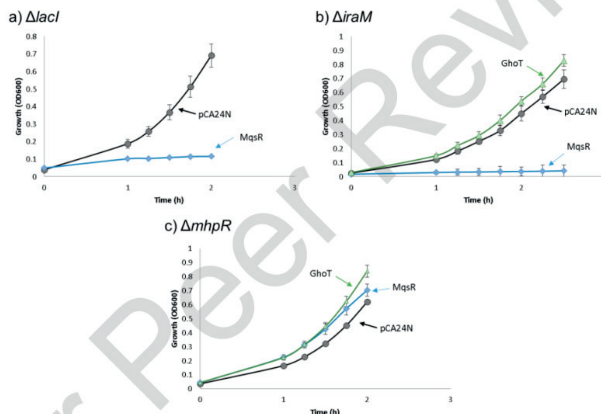

Figure 3. Relation between LacI, IraM and MhpR and toxicity of the plasmid toxin. Growth of BW25113 Keio mutants containing pCA24N-based plasmids with MqsR or GhoT toxins in the presence of 1 mM IPTG. Empty plasmid pCA24N ("Empty", black circles and lines), MqsR plasmid (blue diamonds and lines), and GhoT plasmid (triangles and green lines).

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Bioinformatic analysis. To determine the presence and conservation of these toxins in *E. coli*, we made a bioinformatic analysis of 1,000 *E. coli* genomes using the IMG/M internal database. This analysis revealed that Hha is the most conserved toxin among the four, being present in 98% of the population analyzed (**Fig. 4**). Also, GhoT was conserved since it was present in the 91% of the genomes. This analysis also shows 50% of the changes in *hha* (49% with a 99%

of homology and 1% with less) and 33% of the changes in *ghoT* (30% of the strains with a 99% of homology and 3% of strains with less than 99%), compared to 50% and 67% of the population with 100% of homology in their sequences. However, both genes were present with 100% of identity in the amino acid sequence in 99% and 98% of strains, respectively, with only 1% of the population has changes in the amino acid sequence in both cases and another 1% of the population in which the modification of the nucleotide sequence was translated with an early stop in the protein in the case of GhoT.

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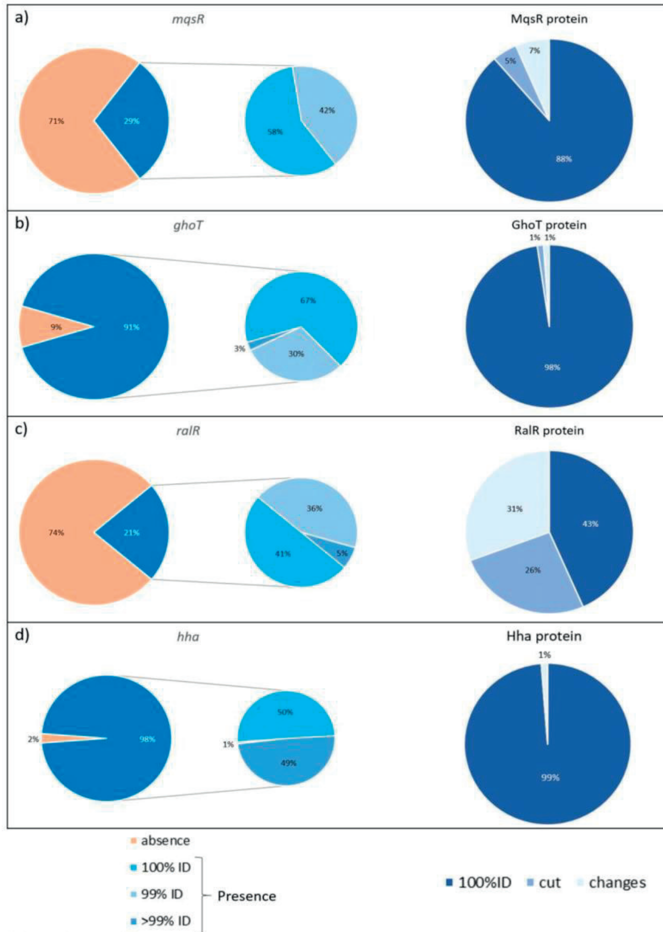

Figure 4. Conservation of the toxins in *E. coli*. Presence (%, first column), nucleotide identity (%, 2nd column) and amino acid identity (%, 3rd column) of each toxin in 1000 *E. coli* genomes.

MqsR toxin was found in 29% of the 1,000 genomes analyzed, of which 58% have 100% nucleotide sequence homology and a 99% homology in the other 42% of the population. However, these percentages of homology only implied 88% of the population has 100% of homology in its amino acid sequence, and 7% of the population has changes in the protein sequence and 5% had an early stop codon (**Fig. 4**).

RalR was the less conserved toxin since it was present in only 21% of the genomes analyzed. Only 41% of the strains had 100% homology in the nucleotide sequence compared to 36% that had 99% identity and 5% showed less than 99% homology. For the RalR sequence, only 43% of the population had 100% amino acid identity, and 31% of strains had changes in the protein sequence (some with more than one amino acid change). Furthermore, we found that 26% of the genomes had an early stop codon in their sequence.

Overall, our bioinformatic results show these four toxin genes are widely distributed and conserved in *E. coli* genomes. Hence, these data indicate clearly the importance of these TAs are to bacteria.

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DISCUSSION

In this study, we demonstrate that bacteria mutate rapidly (in the first 12 hours of toxin production) to inactivate toxins of TAs and allow the cells to resume growth, when toxin production is not regulated. Hence our data indicate the importance of tight regulation for TA systems. The mutations that inactivate the toxins were found primarily in the -10 promoter region of the plasmids that carry the toxins or in *lacI, iraM* and *mhpR*. No mutations were found in the structural part of the toxin genes. These results are novel since previous work has shown that toxins are inactivated by mutations in the toxin gene itself (Masachis et al., 2018, Silvaggi et al., 2005, Jahn et al., 2012).

The lack of changes in the toxin structural genes for all four toxins is surprising since we have shown TA systems can evolve rapidly, transforming their genes into new toxins and antitoxins via a few mutations in their genes (Soo et al., 2014). For example, a novel toxin was created from the *ghoS* antitoxin gene (with only two amino acid changes), and two novel antitoxins were created from the *mqsA* and from *toxI* antitoxin genes (Soo et al., 2014). Therefore, since small changes in the sequence of either a toxin or an antitoxin gene can radically alter the TA system, perhaps this prevents mutations in the toxin structural gene.

The whole-genome sequencing revealed the two chromosomal mutations that

we verified inactivate the toxins (**Fig. 3**). The first, *iraM* encodes an anti-adaptor protein that has been related to the stabilization of RpoS during Mg starvation (Bougdour et al., 2008, Yang et al., 2004, Hemmi et al., 1998). IraM has also been linked to the PhoP/PhoQ a two component system, which is necessary to activate IraM, and to H-NS that inhibits IraM activation (Battesti et al., 2012). The second mutation, *mhpR*, encodes a DNA-binding transcriptional activator and is located upstream of the *lacI* reperessor (Zhang et al., 2006); MhpR is the regulator of the 3-hydroxyphenyl propionate catabolic pathway-10 (Manso et al., 2011) and is activated by the cAMP-CRP complex in the absence of glucose and in the presence of 3-hydroxyphenyl propionate (Torres et al., 2003). How inactivating IraM and MhpR reduce toxin activity remains to be discerned.

Bacteria with high stress increase their mutation rate; for example, the mutation rate of *E.coli* is higher with prolonged growth arrest (Loewe et al., 2003). For antibiotic-induced resistance mutations, some studies suggest stress induces the mutations (Bjedov et al., 2003) while others suggest the mutations are due to selection (Wrande et al., 2008). Our data indicate that the appearance of toxin-inactivating mutations are due to selection rather than random mutation since the same promoter deletions were found repeatedly that inactivate the toxin and select faster-growing cells.

Overall, by using four toxins from four different type of TA systems, we found toxins are rapidly inactivated by changes in their promoters rather than changes in the structural genes. We also identified two proteins important for toxin activity, IraM and MhpR, and our results suggest selection is important for mutations.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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DISCUSSION

In the last few decades there has been a great increase in the emergence of antibiotic resistant bacteria, causing a huge problem for public health worldwide. One of the most important causes of this increase is the appearance of tolerant and persister cells, which can survive antibiotic exposure by reducing some bacterial processes (*22*). Determining the mechanisms by which bacteria become tolerant and/or persistent has become a priority target for the scientific community. It is currently known that the following mechanisms participate in tolerance and persistence: the ROS response (by the action of SOD and catalase) (*27*); the general stress response (through the RpoS response by activating bacterial TA systems) (*24*); the SOS response (by repairing DNA damage and regulating DNA recombination) (*34*); energy metabolism (by improving ROS response or adapting bacteria to different substrates) (*28, 29*); (p)ppGpp (by regulating all the DNA-RNA process) (*37*); efflux pumps (by removing toxic elements from bacteria) (*33*); the quorum network (by controlling bacteria population and regulating several metabolic routes) (*39*); and TA systems, which are closely involved in persister cell formation via inhibition of bacterial growth (*41*).

In the research reported in chapter I of the present thesis, we analysed these mechanisms in an airborne strain isolated from air samples from an ICU in a hospital in Rio de Janeiro (Brazil), identified as *Acinetobacter sp.* strain 5-2Ac02. The genome of this strain included a small chromosome (2,951,447 bp), and 5% of the genome consisted of insertion sequences (a very high percentage). Moreover, we identified several heavy metal resistant genes, six TA systems and, more surprisingly, an OXA-58 ß-lactamase that is blocked by an ISAba3-like transposase in its upstream region, explaining why this ß-lactamase OXA-58 was not expressed and therefore there was no resistance to carbapenemic antimicrobials (*189*). The presence of the β-lactamase and the heavy metal resistance genes indicates that in this strain not only has an astonishing capacity to survive in adverse environments, but also acts as an environmental reservoir of resistance genes (*190, 191*). In fact, antibiotic resistance is a natural phenomenon, as some bacteria can produce antibiotics in order to eliminate other competing bacteria from the environment (*192*). Surprisingly, β-lactam resistance genes have been found in bacteria isolated from 30,000-year-old permafrost (*193*), and other several resistance genes have been detected in 4 million-year-old sediment from a cave in New Mexico (USA) (*194*). The

coexistence of antibiotic producing and non-producing bacteria has contributed to the natural development of antibiotic resistance genes as well as the horizontal transfer of these between bacteria (*195*). In addition, the widespread use of antibiotics in clinical situations, as well as in livestock and plant cultures, in addition to the antibiotic contamination of the environment have led to an increase in the development of several new antibiotic resistance genes. However, as well as affecting environmental strains, these problems also affect clinical strains (as both types coexist), contributing to an increase in the acquisition of the resistance genes in clinical pathogens (*196*). This is well illustrated in airborne strain *Acinetobacter* sp. 5-2Ac02, in which 5% of the genome corresponds to insertion sequences (indicating its high genome plasticity), and which possesses several resistant genes such as a β-lactamase and heavy metal resistant genes. Furthermore, *A. baumannii* was a rare pathogen 25 years ago, and *Acinetobacter* sp. was considered an environmental bacteria that can persist in both in humid and dry locations and tolerate a wide range of pH, temperatures, energy and carbon resources, whereas it is now considered one of the most dangerous opportunistic pathogens worldwide (*197*).

In order to determine the mechanisms that are activated in *Acinetobacter sp*. strain 5-2Ac02, we analysed gene expression in this bacterium in response to different types of stress (chapter II). We observed that under mitomycin C (MMC) pressure, this strain expresses SOS response genes, as well as antibiotic peptides, heavy metals resistance gene operons (such as arsenate, copper, and cobalt-zinc-cadmium) and, as in strain Ab-2, the TA system (five in this case) and ROS response genes. These data show the importance of these tolerance and persistence mechanisms in different bacteria under different stress conditions. Moreover, we constructed growth curves for *Acinetobacter sp*. strain 5-2Ac02 in the presence of MMC (stress factor) as well as different antibiotics, either alone or in combination with MMC. Mitomycin C has been described as a treatment for persister cells in *A. baumannii,* and in other bacteria such as *Borrelia burgdorferi* (*198, 199*). However, we observed that the presence of this substance in combination with ciprofloxacin actually induced the production of tolerant cells. Similar results were reported by Dörr *et al.,* who observed an increase in persister cell formation in those cultures treated with MMC prior to ciprofloxacin (*200*). These findings demonstrated that MMC is not an efficient treatment against all bacterial persister cells, as

suggested by other authors (*201*). On the other hand, focusing on the QN analysis, we characterized the acetoin/butanediol cluster in the airborne *Acinetobacter sp.* strain 5-2Ac02, which lacks the *acoN* gen (negative regulator). Moreover, we studied this acetoin/butanediol operon in *A. baumannii* ATCC 17978 and in eighteen clinical *A. baumannii* strains in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010", as well as its negative regulator, AcoN, which was located in all isolates (*202*). Comparison of growth curves of the isogenic mutant *A. baumannii* ATCC 17978 Δ*acoN* in the presence of acetoin and the wild type revealed the role of the *acoN* gene as a negative regulator. We also observed a relationship between the acetoin/butanediol cluster and the quorum network. Regulation of this operon has been widely studied and is known to be associated with quorum sensing and pH or the concentration of acetoin in the environment; regulation is activated when the high cell density leads to accumulation of acetoin as a consequence of glucose metabolism (*122*). In *V. cholerae,* the QS regulator LuxO is inactivated by dephosphorylation, stabilizing hapR, which activates alsR in the cluster (*129*). However, negative regulation is more variable: in *B. subtilis* the acetoin operon is downregulated by the catabolite control protein A (CcpA) to the *cre* site of the promoter region of *acoR* (positive regulator of the cluster) (*127*); in *Serratia marcescens,* SwrR (QS receptor) downregulates two genes of the operon (*slaA* and *slaB*) that control acetoin production, although it cannot control either the 2,3-butanediol dehydrogenase (*slaC*) or the positive regulator SlaR (*203*); in *Klebsiella* and *Serratia*, BudR (positive regulator) also acts as its own negative regulator under neutral pH, in the absence of acetate and under aerobic conditions (*204, 205*). We demonstrate that the absence of the AcoN negative regulator in *Acinetobacter* sp., under certain types of environmental stress, converts the strain into a more efficient one, which can grow readily in the presence of high levels of acetoin and increase activation of the cluster in response to quorum network signals. In addition, the absence of the QS synthase has also been related to a decrease in acetoin production, demonstrating the relationship between QS and acetoin metabolism, although it has also been shown to be regulated by pH (*204*). Acetoin and 2,3 butanediol are also BVCs, which can influence bacterial pathogenesis (*120*) by altering the production of virulence factors (*115*) or by affecting host cell functions (*206*). In addition to the fundamental ecological interest, a better understanding of environmental bacteria and of the roles of BVCs (including 2,3-butanediol), metabolic

pathways and mechanisms involved could provide new information about the bacterial response to the environment, thus potentially leading to clinical or industrial applications.

For deeper analysis of the cluster and negative regulator function of the AcoN protein, we carried out photoregulation studies, in which the relationship between acetoin/butanediol metabolism and both bacterial pathogenesis and activation of virulence factors has been demonstrated (*176*). For example, in *P. aeruginosa,* in the presence of 2,3-butanediol increases elastase production and biofilm density, but also produces a reduction in swimming and swarming motility, contributing to biofilm formation. Pyocyanin levels are also stimulated by 2,3-butanediol, inducing a competitive advantage, and, a relationship between 3-Oxo-C12-HSL and 2,3-butanediol has been observed in this bacterium (*115*). Otherwise, acetoin is an important product of the physiological metabolism of bacteria produced in response to excess glucose or other fermentable carbon sources (*207*). Metabolism of acetoin controls the NAD/NADH ratio, and also prevents the accumulation of acid both in bacterial cytoplasm and in the environment (*208, 209*). The presence of this operon, particularly without its negative regulator, contributes to better environmental adaptation under stress conditions, helping the bacteria to persist for longer in an unfavourable environment (*123*). Besides, the more time bacteria could survive in an environment, the longer this bacterium will have the chance of acquiring new resistance genes (*21*). Moreover, we found that light can modulate acetoin metabolism through BlsA (a short blue light using flavin (BLUF) type protein) in a temperature dependent way, by inducing *aco ABC* genes and repressing AcoN. Certain types of light can regulate *A. baumannii* metabolism as well as biofilm formation and tolerance to antibiotics (*75, 77*). Although the short BLUF proteins are widely distributed in bacteria, a mechanism of signal transduction by short BLUF photoreceptors has only been described in few cases (*210*). Many photoreceptors, such as PixD (*210*), AppA (*211*) and YcgF (*212*), have been shown to antagonize transcriptional repressors, but BlsA is the only photoreceptor known to act both in darkness (repressor Fur inactivation) and in light (AcoN repression) (*213*). Light-induced activation of the acetoin/butanediol metabolism may occur in the same direction as in *P. aeruginosa*, in which light has been shown to increase virulence (*115*). Indeed, light induces virulence and persister factors such as the type VI

secretion system, production of antioxidant enzymes, the quorum network and tolerance to antibiotics, among others (*78*). All of these findings indicate that light may modulate bacterial persistence in the environment. Despite the absence of light in internal organs and tissues, it may be important in surface-exposed wound infections (*74*).

Given the importance of the QN as a mechanism of bacterial tolerance and persistence, we focused our research on attempting to understand the role of QN in the development of bacteraemia in patients with pneumonia caused by clinical strains of *A. baumannii* (Chapter III). We chose to study this aspect because it is precisely in the lungs of the patients where *Acinetobacter* is submitted to a high level of environmental stress by ROS, and its tolerance mechanisms, catalase and superoxide dismutase (SOD) enzymes will be activated through the QN (*103*). Furthermore, we found that the respiratory strains are less motile than the blood strains, demonstrating a correlation between the motility and origin of the strain. The authors speculate that motile strains cannot attach as firmly to alveolar cells as the non-motile strains. In addition, an oxygen-rich environment has been proposed to inhibit the factors required for motility (*214*). It has also been demonstrated that several proteins involved in the oxidative stress response are affected by QQ, such as Gor protein, a glutathione reductase, whose activity has been reduced in the absence of the QQ enzyme AiiA, a lactonase isolated from *Sinorhizobium meliloti* (*215, 216*). It has been found that strains of *A. baumannii* isolated from blood samples, are motile (in contrast to those isolated from lung), but also lack the QQ AidA protein (a dienelactone) (*102*). In the present study, analysis of 13 isolates from patients with pneumonia revealed overexpression of *abaR* in strains isolated from patients with bacteraemic-pneumonia relative to strains from patients with only pneumonia. Moreover, we observed regulation of *aidA* in the presence of H_2O_2 and 3-Oxo-C12 in pneumonia-causing strains. However, we observed overexpression of the *aidA* gene only under H_2O_2 pressure in the pneumonia-causing strains, as five of the six strains that produced bacteraemia did not express AidA. These findings are consistent with previous reports, inasmuch as AidA does not only regulates the QS of producer bacteria, but also can degrade signals from other bacteria (*88, 102*). Nevertheless, there was no difference in the expression of the analysed genes between colonized and pneumonia patients, as previously observed (*217*). In an

analysis of different variables in the patients we found that the only statistically significant risk factor for the development of pneumonia was diabetes mellitus, as previously described (*218*). This can be attributed to the effects of diabetes on the immune system, such as a reduction in chemotaxis, phagocytosis and killing by macrophages and polymorphonuclear cells (*219*). However, in relation to the development of bacteraemia secondary to pneumonia, the only significant risk factor was the underexpression of the *aidA* gene. The lack of AidA protein resulted in lack of regulation of the QS, contributing to the activation of virulence factors as T6SS and motility, which could favour the development of invasive infections such as bacteraemia (*102*).

In infection assays conducted in an *in vivo* model with larvae of wax moth *Galleria mellonella*, we observed a reduction in the mortality of the larvae infected with *abaI* gene mutants, because of the role of QS in the regulating virulence in several bacteria (*220*). In *A. baumannii,* motility requires a functional AbaI synthase, and the lack of AbaI reduced biofilm formation by 30-40% relative to the wild-type, although complementing the culture with AHL restores biofilm maturation (*221, 222*). In addition, a mutation in the synthase of *Aeromonas salmonicida* subsp. *achromogenes* reduced virulence factors such as levels of expression of a toxic metalloprotease, secretion of cytotoxic factors and generation of pigments (*223*). Nevertheless, *lasR* gene mutants of *P. aeruginosa* adapt better in certain environments, such as the lungs, where they grow better under chronic infection conditions, thus providing a selective advantage (*224*). However, cells with a defective QS are known as cheaters, as they cannot supply QS signals to the population, although they can take advantage of them (*225*). In spite of that, high levels of stress, such as oxidative stress, contribute to eliminating the cheaters from the population and selecting those cells with a functional QN (*225*).

Toxin-antitoxin systems represent another important mechanism of bacterial tolerance and persistence. Chapter IV reports a study of the molecular mechanisms involved in tolerance to chlorhexidine (a commonly used antiseptic) in a clinical isolate of *A. baumannii* (Ab-2_clon_2010-CHLX). In this strain, overexpression of efflux pumps occurred in response to chlorhexidine (in order to eliminate the antiseptic) and surprisingly to arsenite, ROS response, biofilm, ppGpp genes and an OXA-24/40 β-lactamase (which confers resistance to imipenem) together with the

module AbkB/AbkA TA system (also known as SplT/SplA) (*23*). With the object of determining whether the genomic expression modified the bacterial phenotype, we constructed growth curves for the strain in the presence of chlorhexidine, imipenem and a combination of both. Astonishingly, we found that the presence of TA systems (AbkB/AbkA proteins) in *A. baumannii* ATCC 17978 contributes to tolerance mechanisms that enable survival in the presence of antibiotics and environmental stress factors, such as chlorhexidine, but also that activation of the AbkB toxin (from module AbkB/AbkA) in the tolerant *A. baumannii* Ab2_clon_2010-CHLX strain generated the development of persister cells in the presence of imipenem and chlorhexidine and the absence of expression of the OXA-24/40 β-lactamase. In the last few decades it has been shown that certain antibiotics (such as those that prevent translation) contribute to persister cell formation (*226*), and chlorhexidine has been found to induce the production of persister cells in Archaea such as *Haloferax volcanii* (*227*). In addition, clinical isolates of *K. pneumoniae* have been found to acquire cross-resistance to colistin as a consequence of becoming resistant to chlorhexidine (*228*). In addition, a combination of imipenem and gentamicin generates the formation of persister cells at the initial stages of treatment; and revival after 12 hours, elevating the number of surviving cells, even though antibiotics are still present (*229*).

As stated above, tolerance and persister mechanisms contribute to maintaining bacteria in adverse environments, thus increasing the chance of these acquiring new resistance genes and contributing to their better adaptation to different conditions (*22, 230*). The presence of these mechanisms has been widely studied in pathogenic bacteria, and the TA systems are one of the most widely analysed. TA systems contribute to both tolerance and persister cell formation, and their contribution to one or the other depends on the balance between the toxin and the antitoxin (*231*). It is known that toxins from TA systems can act on protein synthesis, replication, DNA and RNA integrity, protein assembly, cell-wall synthesis and energy production (*232*). The persister cell formation that depends on TA systems is associated with a stringent response and the alarmone signal (p)ppGpp (*36*), as well as with the QN response (*233*); these mechanisms are activated under several types of environmental stress, in order to reprogram physiological cell functions, ranging from growth to survival functions (*234*). The (p)ppGpp inhibits the exopolyphosphatase

(ppX), which degrades polyP, thus producing an accumulation of polyP that contributes to degradation of the anitytoxins by Lon-protease (*235*). Two of the main TA systems involved in persister cell formation are MqsR/MqsA and HipB/HipA systems, which also impact motility, biofilm formation and QS (*236*). Despite the focus of research on the relation of the type II TA systems in persistence, members of the other types of systems have also been related to persister cell formation (*23*). It has been reported that MazF toxins increase the expression of stress resistant genes as well as reducing metabolic processes, producing reversible formation of persister cells, which are more resistant to antibiotics and environmental stress than the non-persisters (*237*).

All of these mechanisms can be considered possible new targets for antimicrobial treatments. In the last few decades, both natural and synthetic molecules with the capacity to reduce persister cell formation have been studied, such as inhibitors of (p)ppGpp (*238*), inhibitors of the RecA protein and the SOS response (*239*), antibiofilm activity molecules (*240*), and QS inhibitors (*22*). Some of these molecules are already used; for example, QS inhibitors are used in clinical trials and also to develop medical devices (*241*). Furthermore, TA systems have also been analysed as new antimicrobial targets, and some authors have speculated about the possibility of activating expression of the toxin or eliminating the antitoxin from the system in order to provoke bacterial death. For example, artificial activation of TA systems by PNA oligomers (which inhibit the antitoxin) has been proposed as a new antibacterial strategy (*242*). However, the proposed strategy involved artificial dysregulation of the natural balance between a toxin and its antitoxin. TA systems are tightly self-regulated, and thus as antitoxins repress expression via promoter binding, toxins limit both antitoxin and toxin levels via post-transcriptional cleavage of mRNA (*231, 243*), and antitoxins inhibit toxins. In some cases, the antitoxins repress expression via other TA systems as GhoT/GhoS, which is regulated by MqsR (*243*). The high variability of these systems has been demonstrated by the fact that only two sequence substitutions can turn the antitoxin of one system into a new toxin or even in the antitoxin of a total different toxin, thereby generating a new system (*244*).

Finally (chapter V), we analysed what occurs in bacteria when an external toxin is added and overexpressed, noting that despite initial inhibition of growth, bacteria can regrow after 12 hours even though the external factor (the IPTG that induced

overexpression of the toxin) remains in the medium. Previous research on the inactivation of external toxins has shown that these toxins are inactivated by mutations in their genes (*245-247*). However, we found that the inactivation was due to a deletion in the TATA box of the P*t5-lac* promoter of the plasmid, in most of the strains analysed. In those strains in which we did not observe deletion in the plasmid promoter, we observed chromosome modifications, specifically in the genes *lacI* (*lac* operon inhibitor)*, iraM* (anti-RssB factor involved in RpoS stabilization during Mg starvation) and *mhpR* (transcriptional activator). The *lac* system repressor (LacI) has been found to be repressed by IPTG, enabling activation of the *lac* operon (*248*). Several mutations in the *lacI* gene that modified the mechanism of action have been detected, highlighting a change in the valine 95 to alanine, so that IPTG activates LacI rather than repressing it (*249*). IraM is an anti-adaptor protein that has been related to the stabilization of RpoS during Mg starvation, and has been found to be encoded by the *ycgW* and *elbA* genes (*250-252*). This protein has been related to PhoP/PhoQ, a two component system, which is necessary for activating IraM, and H-NS (Histone-like nucleoid-structuring), which inhibit IraM activation (*253*). Finally, MhpR (a DNA-binding transcriptional activator), which is located following lac operon (*254*), regulates the 3-hydroxyphenyl propionate catabolic pathway (*255*). This operon, and more specifically MhpR, has been related to the cAMP-CRP complex, which activates the cluster when glucose is not available, but 3-hydroxyphenyl propionate (an aromatic compound) is available (*256*). We believe that the mutations in these two genes are related to the high level of stress to which the bacteria are subjected during overexpression of the external toxin.

The mutation capacity of bacteria has been shown to increase under exposure to high levels of environmental stress (*257*). The appearance of mutations in cultures under environmental stress conditions has been widely studied, especially the appearance of mutants resistant to antibiotics, such as rifampicin, in pathogens such as *E. coli* (*258, 259*). Some researchers have hypothesized that bacteria can switch between high and low mutation rates in order to adapt to environmental conditions (*258*). However, other authors consider that mutant bacteria appear as a result of selection under stress rather than as a result of random mutagenesis. Mutants accumulate under rifampicin pressure because they can grow, whereas the wild type cannot grow under these conditions (*259*). We consider that the appearance of

bacteria that inactivate the external toxin corresponds to selection rather than to random mutation, inasmuch as inhibition of the external toxin allows bacteria to resume growth, unlike those without the mutation. Moreover, the high prevalence of these toxin genes in the bacterial population, observed by bioinformatic tools, and the high plasticity of the TA systems (*244*) may explain why the toxin gene has not mutated in bacteria.

The study findings indicate that artificial modification of the balance of the TA systems is not a correct antibacterial strategy and new ways of approaching this treatment path should be sought.

CONCLUSIONS

Chapter 1

1. The genome of the airborne strain *Acinetobacter sp*. 5-2Ac02, isolated from an ICU of a hospital in Rio de Janeiro (Brazil), is described. This strain has a genome of size 2.95Mb, with a 40.9% GC content and 2,795 CDSs. The genome has a high percentage (5%) of insertion sequences, and the genes include several heavy metal resistant genes, six TA systems, and a β-lactamase OXA-58 interrupted by an ISAba3-like transposase.

2. *Acinetobacter sp*. 5-2AC02 was suggested to be a new species of *Acinetobacter*, closely related to *A. towneri*.

Chapter 2

1. Several tolerance mechanisms were activated under stress conditions such as the SOS response (mitomycin C) and quorum network (AHLs) in airborne *Acinetobacter sp*. 5-2Ac02. Among these we highlight SOS and ROS responses, heavy metal resistance, acetoin/butanediol metabolism, quorum network and toxin-antitoxin systems.

2. The AcoN protein was functionally characterized as a negative regulator of the acetoin/butanediol cluster in *Acinetobacter sp*. 5-2Ac02 and clinical strains of *A. baumannii*.

3. The relationship between the acetoin/butanediol cluster and the quorum network was demonstrated, and knowledge of acetoin metabolism as well as the ion light transduction mechanism mediated by the BLUF photoreceptor BlsA in *A. baumannii* strains is highlighted.

Chapter 3

1. The relationship between reduced expression of the QQ enzyme (AidA) from the quorum network in *A. baumannii* clinical strains and the development of bacteraemia secondary to pneumonia infections is described.

2. Quorum network deficiency in the *A. baumannii* mutant by knockout of the *abaI* gene isolate showed a reduction in mortality in an animal model.

Chapter 4

1. Efflux pumps, ROS response, CsuABCDE (biofilm genes) and ppGpp, among others, were identified as the mechanisms of tolerance to chlorhexidine in the *A. baumannii* clinical isolate ST-2 harbouring the β-lactamase OXA-24/40 gene and AbkB/AbkA TA system in a plasmid.

2. The important link between mechanisms of bacterial tolerance and persistence under chlorhexidine and imipenem pressure in a clinical isolate of *A. baumannii* ST-2 harbouring the β-lactamase OXA-24/40 gene and AbkB/AbkA TA system in a plasmid are described and attributed to the absence of activity of the OXA-24/40 ß-lactamase together with the overexpression of the abkB toxin (from AbkB/AbkA module).

Chapter 5

1. None of the toxin genes (from the TA systems) mutated, emphasizing the importance of the conservation of TA system genes in the *E. coli* genome.

2. It was demonstrated that the chromosome or the plasmid promoter sequence in *E. coli* can mutate in order to block toxins, for which antitoxins are lacking. This highlightings the importance of the regulation of TA systems.

General conclusion

The study of global mechanisms of tolerance and persistence in multiresistant bacteria may be key factors in the development of new anti-infectious treatments from new molecular targets.

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ANNEXES

Supplementary material chapter II

STRAIN/PLASMID	RELEVANT CHARACTERISTIC	SOURCE OR REFERENCE
Acinetobacter sp.strain 5-2Ac02	Airborne strain	(Barvosa *et al*. 2016)
A. baumannii		
ATCC 17978	Clinical isolate	ATCC
ATCC 17978 *ΔabaI*	Generated by mutagenesis using plasmid pMO130-telR	(Castañeda-Tamez *et al.* 2018, Hamad *et al*. 2009)
ATCC 17978 *ΔacoN*	Generated by mutagenesis using plasmid pMO130-telR	This study
18 clinical strains	Identified in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010"	Genbank Umbrella Bioproject PRJNA422585
ATCC 17978 *ΔblsA*	*blsA*::aph derivative of 17978; Km^r	(Mussi *et al*., 2010)
ATCC 17978 *ΔblsA* pWHBlsA	17978 *ΔblsA* harboring plasmid pWHBlsA; Km^r Amp^r	(Mussi *et al*., 2010)
ATCC 17978 *ΔblsA* pWH1266	17978 *ΔblsA* harboring pWH1266; Km^r Tet^r Amp^r	(Mussi *et al*., 2010)
ATCC 17978 *ΔacoN* pWHAcoN	17978 ΔacoN harboring plasmid pWHAcoN; Km^r Amp^r	This study
ATCC 17978 *ΔacoN* pWH1266	17979 ΔacoN harboring plasmid pWH1266; Km^r Tet^r Amp^r	This study
E. coli		
DH5α	Used for DNA recombinant methods	Gibco-BRL
Saccharomyces cerevisiae		
Mav 203 strain	MATa, *leu*2-3,112, *trp*1-901, *his*3-D200, *ade*2-101, *gal*4D, *gal*80D, SPAL10::URA3, GAL1::*lac*Z, HIS3UAS GAL1::HIS3, YS2, *can*1R and *cyh*2R	Thermofisher

Plasmids		
pMO130-TelR	suicide vector	(Aranda *et al.* 2010, Hamad *et al*. 2009)
pBluescript	PCR cloning vector; Ampr	Promega
pWH1266	*E. coli-A. baumannii* shuttle vector; Amp^r Tet^r	(Hunger *et al.*, 1990)
pWHBlsA	pWH1266 harboring wildtype copy of blsA from ATCC 17978 expressed under its own promoter; Amp^r	(Mussi *et al*., 2010)
pWHAcoN	pWH1266 harboring wild-type copy of acoN from ATCC 17978 expressed under its own promoter; Amp^r	This study
pENTR3C	Gateway system entry-vector	Invitrogen-Thermofisher
PGAD-T7-GW	Y2H AD-fusion vector, adapted to Gateway System	Clontech, (Cribb and Serra, 2009)
PGBK-T7-GW	Y2H DB-fusion vector, adapted to Gateway System	Clontech, (Cribb and Serra, 2009)

Table S1. Bacterial, yeast strains and plasmids used in this study.

PRIMER DELETION				
Gene		**SEQUENCE (5'-3')**	**RESTRICTION SITE**	**REFERENCE**
Up acoN	Fow	ATAAGAATGCGGCCGCTAAACTATAAAATCATGTTTCCAACTAGG	Not I	This study
	Rev	CCGGAATTCCGGTACTTGAGTTTCAAAGGAAC	Eco RI	
Down acoN	Fow	CCGGAATTCCGGTAGGTATTTAGCTCATCTAAGG	Eco RI	This study
	Rev	CGCGGATCCGCGTAGATGATGTCACTAATTTCAC	Bam HI	
Interno acoN	Fow	GCAGCGGTAATATGGTCTG	-	This study
	Rev	AAGAATCACGCCAAAGATGG		
Interno acoN	Fow	ATTTTATTTGAGCAGCTTAAAGC	-	This study
	Rev	CTTATGCTTTTGTCTGTATCG		
PAcoN	Fow	GGATCCCAAGCATATATGTATAAGTCGAACT	Bam HI	This study
	Rev	GGATCCGTTCCTTTCCAAGCAAATAAGAG	Bam HI	
blsAdh	Fow	GGATCCATGAACGTTCGCCTGTGT	-	(Tuttobene et al., 2018)
	Rev	CTCGAGTGCTAGAACGGGTTTACTC		
acoNdh	Fow	GGATCCATGGCAAAGTTAAATTTTGAGTTAG	-	This study
	Rev	CTCGAGACCTAAAGCTTATGCTTTTGTCTGTATC		

Table S2. Primers for *A. baumannii* ATCC 17978 Δ*acoN* strain mutant.

q-PCR PRIMERS				
GENE		**SEQUENCE (5'-3')**	**PROBE**	**REFERENCE**
abaR	Fow	AGAGGCGTTACGTTGGACTG	155/ GAAGGCAA	(Lopez et al., 2017a)
	Rev	CCAAGAATCTGAGCTATTTCTGC		
abaI	Fow	GGGAACTTCTTTCGGTGGAG	145/ CAGCGACC	(Lopez et al., 2017a)
	Rev	AACAGCAGCAAGTCGATTATCA		
acoB	Fow	TGCCAAATAAAAGTTTTCGTAATG	135/ ATGGCTTC	This study
	Rev	TGCCAAATAAAAGTTTTCGTAATG		
dehydorenase	Fow	TGGGTGCATCCAATTTCC	27/CAGGCAGC	This study
	Rev	CCACCGATAACGGTTCAATTA		
rpoB	Fow	CGTGTATCTGCGCTTGG	131/ CTGGTGGT	(Fernández-Cuenca et al., 2015)
	Rev	CGTACTTCGAAGCCTGCAC		
rpoB	Fow	CAGAAGTCACGCGAAGTTGAAGGT		(Muller et al., 2017)
	Rev	AACAGCACGCTCAACACGAACT		
recA	Fow	TACAGAAAGCTGGTGCATGG		(Mussi et al., 2010)
	Rev	TGCACCATTTGTGCCTGTAG		
acoA	Fow	AAGATGACGGACTATGCCGTGGAA		This study
	Rev	ACGCCGCCAGTCTTTAAGGTTT		
acoB	Fow	TCAGAAATGCGCCGTGATCCAA		This study
	Rev	TTACGCCCAATACACCACCGAAAC		
acoC	Fow	ACAATGTGCAGCCCAACCACAA		This study
	Rev	GGAAATGCGGTGCGTTACGTTT		

Table S3. *q*PCR primers and probes.

STRAIN	GENOME	ACETOIN/BUTANEDIOL CLUSTER (ACC.NUMBER GENBANK/PROTEINS ID)						
		AcoA	AcoB	AcoC	AcoD	2,3-BDH	2,3-BDH	AcoN
***Acinetobacter* sp.5-2Ac02**	MKQS00000000	OFE43540.1	OFE43541.1	OFE43542.1	OFE43543.1	OFE43544.1	OFE43545.1	ND
***A. baumannii* ATCC17978**	CP018664.1	AUO97_16255	AUO97_16260	AUO97_16265	AUO97_16270	AUO97_16275	AUO97_16280	AUO97_16290
Ab 155_GEIH-2000[a]	LJHA00000000	ODA50985.1	ODA50984.1	ODA50983.1	ODA50982.1	ODA50981.1	ODA50980.1	ODA50978.1
Ab 158_GEIH-2000[a]	MSMC00000000	OLV45015.1	OLV45014.1	OLV45013.1	OLV45012.1	OLV45011.1	OLV45010.1	OLV451680.1
Ab 161_GEIH-2000[a]	MSMB00000000	OLV49849.1	OLV49848.1	OLV49847.1	OLV49846.1	OLV49845.1	OLV49844.1	OLV49842.1
Ab 166_GEIH-2000[a]	MSMG00000000	OLV70189.1	OLV70190.1	OLV70191.1	OLV70192.1	OLV70193.1	OLV70194.1	OLV70196.1
Ab 169_GEIH-2000[a]	MSMF00000000	OLV67314.1	OLV67315.1	OLV67316.1	OLV67317.1	OLV67318.1	OLV67319.1	OLV67321.1
Ab 175_GEIH-2000[a]	MSMI00000000	OLV81247.1	OLV81246.1	OLV81245.1	OLV81244.1	OLV81243.1	OLV81242.1	OLV81240.1
Ab 177_GEIH-2000[a]	MSME00000000	OLV55538.1	OLV55539.1	OLV55540.1	OLV55541.1	OLV55542.1	OLV55543.1	OLV55545.1
Ab 183_GEIH-2000[a]	MSMJ00000000	OLV78584.1	OLV78585.1	OLV78586.1	OLV78587.1	OLV78588.1	OLV78589.1	OLV78591.1
Ab 192_GEIH-2000[a]	MSMH00000000	OLV63765.1	OLV63766.1	OLV63767.1	OLV63768.1	OLV63769.1	OLV63770.1	OLV63772.1
Ab 105_GEIH-2010[a]	LJHB00000000	ODA53436.1	ODA53435.1	ODA53434.1	ODA53433.1	ODA53432.1	ODA53431.1	ODA53429.1
Ab 33_GEIH-2010[a]	MSMK00000000	OLV78767.1	OLV78766.1	OLV78765.1	OLV78764.1	OLV78763.1	OLV78762.1	OLV78760.1
Ab 49_GEIH-2010[a]	MSMM00000000	OLV86121.1	OLV86122.1	OLV86123.1	OLV86124.1	OLV86125.1	OLV86126.1	OLV86128.1
Ab 54_GEIH-2010[a]	MSML00000000	OLV85204.1	OLV85205.1	OLV85206.1	OLV85207.1	OLV85208.1	OLV85209.1	OLV85211.1
Ab 76_GEIH-2010[a]	MSLY00000000	OLV41736.1	OLV41735.1	OLV41734.1	OLV41733.1	OLV41732.1	OLV41731.1	OLV41737.1
Ab 103_GEIH-2010[a]	MSLX00000000	OLV35374.1	OLV35375.1	OLV35376.1	OLV35377.1	OLV35378.1	OLV35379.1	OLV39499.1
Ab 104_GEIH-2010[a]	MSMA00000000	OLV45315.1	OLV45314.1	OLV45313.1	OLV45312.1	OLV45311.1	OLV45310.1	OLV45316.1
Ab 121_GEIH-2010[a]	MSLZ00000000	OLV34644.1	OLV34645.1	OLV34646.1	OLV34647.1	OLV34648.1	OLV34649.1	OLV34651.1
Ab 122_GEIH-2010[a]	MSMD00000000	OLV53193.1	OLV53194.1	OLV53195.1	OLV53196.1	OLV53197.1	OLV53198.1	OLV53200.1

Table S4. Genomes and Proteins in the acetoin/butanediol cluster. Genbank database. [a] *A. baumannii* strains isolated in the "II Spanish Study of *A. baumannii* GEIH-REIPI 2000-2010" (Genbank Umbrella Bioproject PRJNA422585). ND: Not detected in the genome.

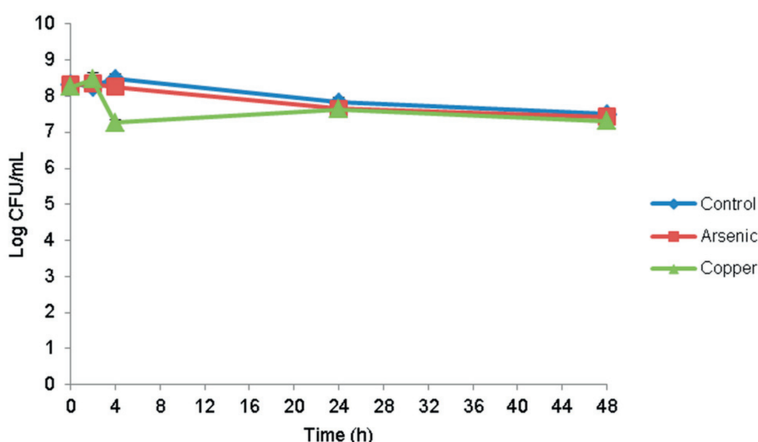

Figure S1. *Acinetobacter* sp. strain 5-2Ac02 is resistant to arsenic and copper. Growth curves of *Acinetobacter* sp. strain 5-2Ac02 in low LB browth in the absence (control) or in the presence of arsenic 1024 µg/ml and copper 133 µg/ml at 37ºC.

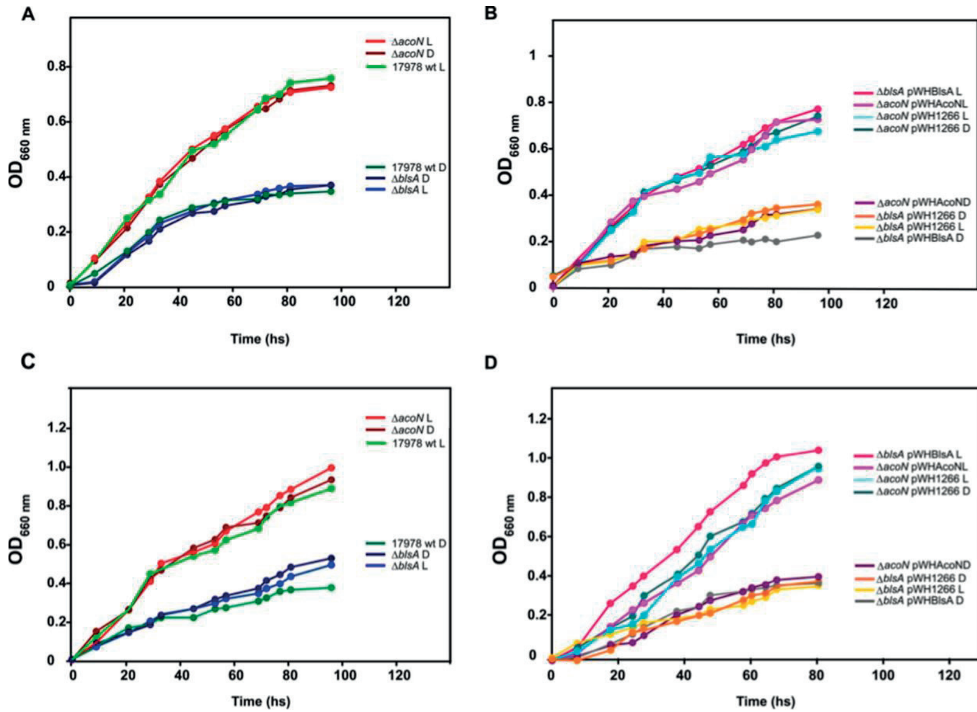

Figure S2. Light modulates acetoin catabolism at moderate temperatures in *A. baumannii* ATCC 17978. **A and B.** Growth curves in M9 minimal medium supplemented with acetoin 10 mM as sole carbon source of *A. baumannii* ATCC 17978 wild-type and derivative strains, incubated stagnantly at 23°C under blue light (L) or in the dark (D). **C and D.** Growth curves in M9 minimal medium supplemented with acetoin 15 mM as sole carbon source of *A. baumannii* ATCC 17978 wild-type and derivative strains, incubated stagnantly at 23°C under blue light (L) or in the dark (D).

Supplementary material chapter III

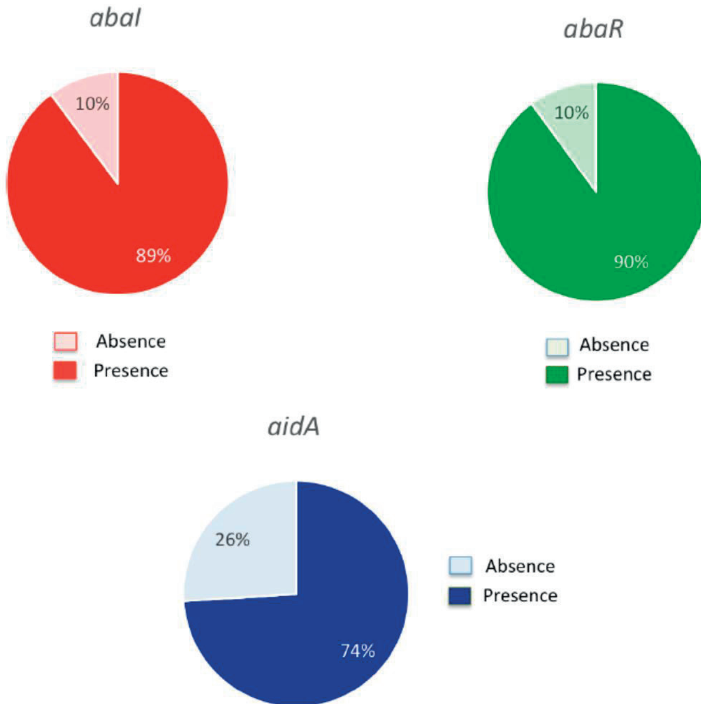

Figure 1S. Analysis of genes involved in the Quorum Sensing system (*abaR* and *abaI*) and Quorum Quenching system (*aidA*) in *A. baumannii* ATCC 17978 (Genbank genome access numbers CP000521.1 [CP018664.1]) and in 1000 *A. baumannii* genomes.

Supplementary material chapter IV

	MIC (mg/L)														
Isolate	SUL	CAZ	IMP	MEM	CIP	GEN	TOB	AMK	MIN	NET	TET	DOX	CST	RIF	TGC
Ab-2_clon_2010	64	>128	64	32	32	2	32	32	<0.5	>64	>64	16	<0.5	1	0.5
Ab-2_clon_2010-CHLX	32	>128	64	32	32	2	32	16	<0.5	64	>64	16	<0.5	1	0.5

Table S1. MICs of several antimicrobials for the bacterial isolates used in the study.

	GO. ID	Term	Annotated	Significant	Expected	ClassicFisher
1	GO:0030001	metal ion transport	27	20	11.28	0.00063
2	GO:0006813	potassium ion transport	11	10	4.6	0.00108
3	GO:0015672	monovalent inorganic cation transport	25	18	10.45	0.00206
4	GO:0006812	cation transport	46	29	19.22	0.00266
5	**GO:0019222**	**regulation of metabolic process**	**213**	**105**	**89.02**	**0.01142**
6	**GO:0032774**	**RNA biosynthetic process**	**211**	**104**	**88.18**	**0.01187**
7	**GO:0060255**	**regulation of macromolecule metabolic process**	**211**	**104**	**88.18**	**0.01187**
8	GO:0006805	xenobiotic metabolic process	5	5	2.09	0.01265
9	GO:0009410	response to xenobiotic stimulus	5	5	2.09	0.01265
10	GO:0010124	Phenylacetate catabolic process	5	5	2.09	0.01265

Table S2. Using the TopGO software tool (version 3.3) and a Fisher test, the GO term enrichment analysis was performed. This enrichment has been made from the previously made InterProtScan annotation that allowed us to relate 1852 genes with GO terms, of which 774 were included within the SDRs identified in common by the software EdgeR and DESeq2. This analysis has made it possible to identify that there is an enrichment of the differentially expressed genes that are associated with the terms GO: 0030001 ("metal ion transport"), GO: 0006813 ("potassium ion transport"), GO: 0015672 ("monovalent inorganic cation" transport ") and GO: 0006812 (" cation transport "). The reference value to consider a term as significant has been p-value <0.01. In this Table S1, it shows the enrichment values according to the GO terms.

PRIMERS and PROBES for RT-PCR STUDIES				
Genes /Function		**Primer Sequences (5´-3´)**	**Taqman Probes**	**References**
abaR/Quorum Sensing	Fow	ACCTCTTGTTTGGTCGAGTCA	96/ACAGGCAG	(1)
	Rev	CGTGCTTCCTCCCAAAAAT		
abaI/ Quorum Sensing	Fow	CCGCTACAGGGTATTTGTTGAAT	6FAM-	(1)
	Rev	GCAGGGAATAGGCATTCCATTG	TGGATTCTCTGTCTTGAGCCACGACA-BBQ	
bla_{OXA24} β-lactamase	Rev	CAAATAAAGAATATGTCCCTGC	-	This study[a]
	Fow	CTGCATTAGCTCTAGGCCAGT		
abkA antitoxin	Rev	GATAACCTTTGCCTTGTGC	-	This study[a]
	Fow	CGCTTGCTTGCTAAAGGCAC		
abkB toxin	Rev	CTCATATTTGTTTCGCTCCGC	-	This study[a]
	Fow	GATCCATTACGGATCTCAATCC		

Table S3. Primers and probes used in this study. [a]Using LightCycler FastStrand DNA Master SYBR green I (Roche, Mannheim, Germany).

REFERENCES

1. López M, Mayer C, Fernández-García L, Blasco L, Muras A, Ruiz FM, Bou G, Otero A, Tomás M; GEIH-GEMARA (SEIMC). 2017. Quorum sensing network in clinical strains of *A. baumannii*: AidA is a new quorum quenching enzyme. PLoS One. 12(3):e0174454.

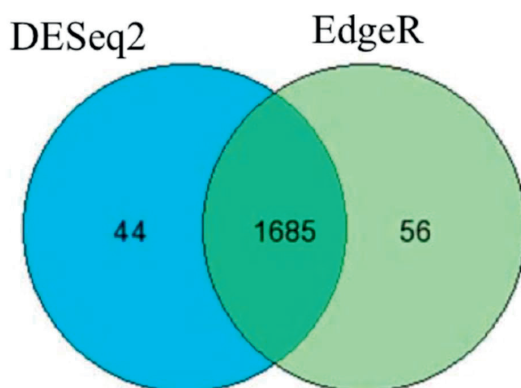

Fig 1S. Venn Diagram. Total number of genes differentially expressed between the groups (RNAs from Ab-2_clon_2010 *versus* Ab-2_clon_2010-CHLX) identified in common between both methods. With respect to the previous report, we observed a greater number of differentially expressed genes identified by both packages (1685 vs 1204 differentially expressed genes).

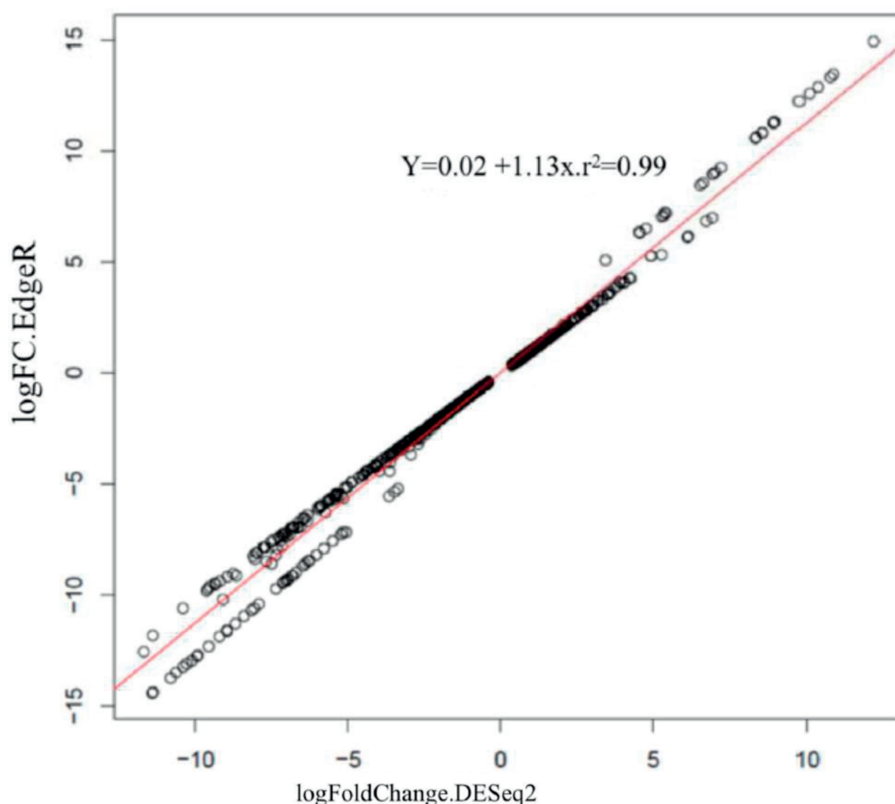

Figure 2S. Comparison of the LogFoldChange identified by EdgeR and DESeq2 for each of the differentially expressed genes.

Supplementary material chapter V

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mqsR2       AGAAATCATAAAAAATTTATTTGCTTTGTGAGCG--------------------------------GATAACAATTTCACACAGA 557
mqsR3       AGAAATCATAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCACACAGA 559
mqsR4       AGAAATCATAAAAAATTTATTTGCTTTGTGAGCG--------------------------------GATAACAATTTCACACAGA 554
mqsR5       AGAAATCATAAAAAATTTATTTGCTTTGTGAGCG--------------------------------GATAACAATTTCACACAGA 557
            **********************************                                *******************

pCA24N-mqsR ATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCGAAAAACGC 475
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mqsR2       ATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCGAAAAACGC 642
mqsR3       ATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCGAAAAACGC 644
mqsR4       ATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCGAAAAACGC 639
mqsR5       ATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCGAAAAACGC 642
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mqsR2       ACACCACATACACGTTTGAGTCAGGTTAAAAAACTTGTCAATGCCGGGCAAGTTCGTACAACACGTAGTGCCCTGTTAAATGCAG 727
mqsR3       ACACCACATACACGTTTGAGTCAGGTTAAAAAACTTGTCAATGCCGGGCAAGTTCGTACAACACGTAGTGCCCTGTTAAATGCAG 729
mqsR4       ACACCACATACACGTTTGAGTCAGGTTAAAAAACTTGTCAATGCCGGGCAAGTTCGTACAACACGTAGTGCCCTGTTAAATGCAG 724
mqsR5       ACACCACATACACGTTTGAGTCAGGTTAAAAAACTTGTCAATGCCGGGCAAGTTCGTACAACACGTAGTGCCCTGTTAAATGCAG 727
            *************************************************************************************

pCA24N-mqsR ATGAGTTAGGTTTGGATTTTGATGGTATGTGTAATGTTATCATTGGATTATCAGAGAGCGACTTTTATAAAAGCATGACCACCTA 645
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mqsR3       ATGAGTTAGGTTTGGATTTTGATGGTATGTGTAATGTTATCATTGGATTATCAGAGAGCGACTTTTATAAAAGCATGACCACCTA 814
mqsR4       ATGAGTTAGGTTTGGATTTTGATGGTATGTGTAATGTTATCATTGGATTATCAGAGAGCGACTTTTATAAAAGCATGACCACCTA 809
mqsR5       ATGAGTTAGGTTTGGATTTTGATGGTATGTGTAATGTTATCATTGGATTATCAGAGAGCGACTTTTATAAAAGCATGACCACCTA 812
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mqsR1       CTCTGATCATACTATCTGGCAGGATGTTTACAGACCCAGGCTTGTTACAGGCCAGGTTTATCTTAAAATTACGGTAATTCATGAC 898
mqsR2       CTCTGATCATACTATCTGGCAGGATGTTTACAGACCCAGGCTTGTTACAGGCCAGGTTTATCTTAAAATTACGGTAATTCATGAC 897
mqsR3       CTCTGATCATACTATCTGGCAGGATGTTTACAGACCCAGGCTTGTTACAGGCCAGGTTTATCTTAAAATTACGGTAATTCATGAC 899
mqsR4       CTCTGATCATACTATCTGGCAGGATGTTTACAGACCCAGGCTTGTTACAGGCCAGGTTTATCTTAAAATTACGGTAATTCATGAC 894
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mqsR3       GTACTGATCGTCTCGTTTAAGGAGAAAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT 974
mqsR4       GTACTGATCGTCTCGTTTAAGGAGAAAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT 969
mqsR5       GTACTGATCGTCTCGTTTAAGGAGAAAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT 972
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Fig. S1. Plasmid sequences for inactivated MqsR. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the p*t5-lac* promoter, bold and underlined letters indicate the RBS, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5' end contains the 6x His tag, italic bold green highlight at the 5' and 3'ends indicates *SfiI* restriction sites, and pink highlight indicates the start and stop codons.

```
pCA24N-ghoT     GAGAAATCATAAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCA   384
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ghoT1           GAGAAATCATAAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCA   674
ghoT4           CGGAAATCATAAAAAAATTTATTTGCTTTGTGAGC--------------------------------GGATAACAATTTCA   674
ghoT3           GAGAAATCATAAAAAAATTTATTTGCTTTGTGAGC--------------------------------GGATAACAATTTCA   674
ghoT5           GAGAAATCATAAAAAAATTTATTTGCTTTGTGAGC--------------------------------GGATAACAATTTCA   673
                  ********************************                                **************

pCA24N-ghoT     CACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGG   464
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ghoT1           CACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGG   754
ghoT4           CACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGG   754
ghoT3           CACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGG   754
ghoT5           CACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGG   753
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pCA24N-ghoT     CCGCACTATTCTCTAAAATATTAATTTTTTATGTGATTGGTGTGAACATATCCTTTGTCATTATCTGGTTTATCTCACAT   544
ghoT2           CCGCACTATTCTCTAAAATATTAATTTTTTATGTGATTGGTGTGAACATATCCTTTGTCATTATCTGGTTTATCTCACAT   813
ghoT1           CCGCACTATTCTCTAAAATATTAATTTTTTATGTGATTGGTGTGAACATATCCTTTGTCATTATCTGGTTTATCTCACAT   814
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ghoT3           CCGCACTATTCTCTAAAATATTAATTTTTTATGTGATTGGTGTGAACATATCCTTTGTCATTATCTGGTTTATCTCACAT   814
ghoT5           CCGCACTATTCTCTAAAATATTAATTTTTTATGTGATTGGTGTGAACATATCCTTTGTCATTATCTGGTTTATCTCACAT   813
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ghoT4           TTCTCTCTTTGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCTGA          974
ghoT3           TTCTCTCTTTGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCTGA          974
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                ************************************************************
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Fig. S2. Plasmid sequences for inactivated GhoT. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the p*t5-lac* promoter, bold and underlined letters indicate the RBS, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5' end contains the 6x His tag, italic bold green highlight at the 5' and 3'ends indicates *SfiI* restriction sites, and pink highlight indicates the start and stop codons.

```
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ralR2           CGAGAAATCATAAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTC  652
ralR3           TCGGAAATCATAAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTC  653
ralR4           CGAGAAATCATAAAAAAATTTATTTGCTTTGTGAGCGGATAACAATT--------------------------------TC  653
ralR5           CGAGAAATCATAAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTC  655
                   ********************************************                                **

pCA24N-ralR     ACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGG  463
ralR1           ACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGG  732
ralR2           ACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGG  732
ralR3           ACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGG  733
ralR4           ACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGG  733
ralR5           ACACAGAATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGG  734
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pCA24N-ralR     GCCAGATATGACAATGTTAAACCATGTCCATTTTGTGGTTGTCCATCAGTAACGGTGAAAGCCATTTCAGGATATTACCG  533
ralR1           GCCAGATATGACAATGTTAAACCATGTCCATTTTGTGGTTGTCCATCAGTAACGGTGAAAGCCATTTCAGGATATTACCG  812
ralR2           GCCAGATATGACAATGTTAAACCATGTCCATTTTGTGGTTGTCCATCAGTAACGGTGAAAGCCATTTCAGGATATTACCG  812
ralR3           GCCAGATATGACAATGTTAAACCATGTCCATTTTGTGGTTGTCCATCAGTAACGGTGAAAGCCATTTCAGGATATTACCG  813
ralR4           GCCAGATATGACAATGTTAAACCATGTCCATTTTGTGGTTGTCCATCAGTAACGGTGAAAGCCATTTCAGGATATTACCG  813
ralR5           GCCAGATATGACAATGTTAAACCATGTCCATTTTGTGGTTGTCCATCAGTAACGGTGAAAGCCATTTCAGGATATTACCG  814
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ralR2           AGCGAAGTGTAACGGATGCGAATCCCGAACCGGTTATGGTGGAAGTGAAAAAGAAGCACTCGAAAGATGGAATAAACGAA  892
ralR3           AGCGAAGTGTAACGGATGCGAATCCCGAACCGGTTATGGTGGAAGTGAAAAAGAAGCACTCGAAAGATGGAATAAACGAA  893
ralR4           AGCGAAGTGTAACGGATGCGAATCCCGAACCGGTTATGGTGGAAGTGAAAAAGAAGCACTCGAAAGATGGAATAAACGAA  893
ralR5           AGCGAAGTGTAACGGATGCGAATCCCGAACCGGTTATGGTGGAAGTGAAAAAGAAGCACTCGAAAGATGGAATAAACGAA  894
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pCA24N-ralR     CCACTGGAAATAATAATGGAGGTGTTCATGTAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT  693
ralR1           CCACTGGAAATAATAATGGAGGTGTTCATGTAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT  972
ralR2           CCACTGGAAATAATAATGGAGGTGTTCATGTAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT  972
ralR3           CCACTGGAAATAATAATGGAGGTGTTCATGTAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT  973
ralR4           CCACTGGAAATAATAATGGAGGTGTTCATGTAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT  973
ralR5           CCACTGGAAATAATAATGGAGGTGTTCATGTAGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCT  974
                ********************************************************************************
```

Fig. S3. Plasmid sequences for inactivated RalR. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the p*t5-lac* promoter, bold and underlined letters indicate the RBS, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5' end contains the 6x His tag, italic bold green highlight at the 5' and 3'ends indicates *SfiI* restriction sites, and pink highlight indicates the start and stop codons.

```
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hha2          GAGAAATCATAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCACACAG 630
hha3          GAGAAATCATAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCACACAG 634
hha4          GAGAAATCATAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCACACAG 620
hha5          GAGAAATCATAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATAATAGATTCAATTGTGAGCGGATAACAATTTCACACAG 628
              *************************************************************************************

pCA24N-hha    AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCTCCGAAAA 474
hha1          AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCTCCGAAAA 720
hha2          AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCTCCGAAAA 715
hha3          AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCTCCGAAAA 719
hha4          AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCTCCGAAAA 705
hha5          AATTCATTAAAGAGGAGAAATTAACTATGAGAGGATCTCACCATCACCATCACCATACGGATCCGGCCCTGAGGGCCTCCGAAAA 713
              *************************************************************************************

pCA24N-hha    ACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTATCCAGACAATTGACACGCTGGAGCGTGTTATCGAGAAAAATAAA 559
hha1          ACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTATCCAGACAATTGACACGCTGGAGCGTGTTATCGAGAAAAATAAA 805
hha2          ACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTATCCAGACAATTGACACGCTGGAGCGTGTTATCGAGAAAAATAAA 800
hha3          ACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTATCCAGACAATTGACACGCTGGAGCGTGTTATCGAGAAAAATAAA 804
hha4          ACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTATCCAGACAATTGACACGCTGGAGCGTGTTATCGAGAAAAATAAA 790
hha5          ACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTATCCAGACAATTGACACGCTGGAGCGTGTTATCGAGAAAAATAAA 798
              *************************************************************************************

pCA24N-hha    TACGAATTATCAGATAATGAACTGGCGGTATTTTACTCAGCCGCAGATCACCGCCTCGCCGAATTGACCATGAATAAACTGTACG 644
hha1          TACGAATTATCAGATAATGAACTGGCGGTATTTTACTCAGCCGCAGATCACCGCCTCGCCGAATTGACCATGAATAAACTGTACG 890
hha2          TACGAATTATCAGATAATGAACTGGCGGTATTTTACTCAGCCGCAGATCACCGCCTCGCCGAATTGACCATGAATAAACTGTACG 885
hha3          TACGAATTATCAGATAATGAACTGGCGGTATTTTACTCAGCCGCAGATCACCGCCTCGCCGAATTGACCATGAATAAACTGTACG 889
hha4          TACGAATTATCAGATAATGAACTGGCGGTATTTTACTCAGCCGCAGATCACCGCCTCGCCGAATTGACCATGAATAAACTGTACG 875
hha5          TACGAATTATCAGATAATGAACTGGCGGTATTTTACTCAGCCGCAGATCACCGCCTCGCCGAATTGACCATGAATAAACTGTACG 883
              *************************************************************************************

pCA24N-hha    ACAAGATCCCTTCCTCAGTATGGAAATTTATTCGCGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCTGA 729
hha1          ACAAGATCCCTTCCTCAGTATGGAAATTTATTCGCGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCTGA 975
hha2          ACAAGATCCCTTCCTCAGTATGGAAATTTATTCGCGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCTGA 970
hha3          ACAAGATCCCTTCCTCAGTATGGAAATTTATTCGCGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCTGA 974
hha4          ACAAGATCCCTTCCTCAGTATGGAAATTTATTCGCGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCTGA 960
hha5          ACAAGATCCCTTCCTCAGTATGGAAATTTATTCGCGGCCTATGCGGCCGCTAAGGGTCGACCTGCAGCCAAGCTTAATTAGCTGA 968
              *************************************************************************************
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Fig. S4. Plasmid sequences for inactivated Hha. Red highlight indicates the -10 and -35 promoter regions, yellow highlight indicates promoter deletions, yellow letters indicate the p*t5-lac* promoter, bold and underlined letters indicate the RBS, green highlight indicates the coding portion of the gene sequence, bold green highlight at the 5’ end contains the 6x His tag, italic bold green highlight at the 5’ and 3’ends indicates *SfiI* restriction sites, and pink highlight indicates the start and stop codons.

Summary

Desde su descubrimiento las bacterias han sido encontradas en prácticamente cualquier ambiente por muy adverso que pudiera parecer. Sin embargo, la atmósfera no tiene una microbiota autóctona, aunque es un medio para la dispersión de muchos tipos de microorganismos (esporas, bacterias, virus y hongos) de otros ambientes. Algunos han creado adaptaciones especializadas que favorecen su supervivencia y permanencia. Los microorganismos dispersados por el aire tienen una gran importancia biológica y económica; produciendo enfermedades en plantas, animales y humanos, alterando los alimentos y materiales orgánicos, además de contribuir al deterioro y corrosión de monumentos y metales. La microbiología del aire comenzó en el siglo XIX, con Pasteur y Miquel, quienes diseñaron métodos para estudiar los microorganismos en el aire y descubrieron la causa de algunas enfermedades. Desde entonces, numerosos investigadores han trabajado en este campo enfocándose en el aire tanto en espacios abiertos como en ambientes cerrados. Las enfermedades transmitidas por el aire, causadas por bacterias, virus y hongos, son respiratorias (neumonía, tos ferina, tuberculosis, legionelosis, resfriado, gripe), sistémicas (meningitis, sarampión, varicela, micosis) y alérgicas.

Múltiples autores han estudiado la supervivencia de los microorganismos en aerosoles*: Bacillus, Escherichia coli, Pseudomonas, Corynebacterium, Micrococcus, Serratia and Mycobacterium, Staphylococcus*; así como hongos o virus (*influenza*). Miembros del género *Acinetobacter* puede encontrarse en casi cualquier ambiente, razón por la cual en la década pasada, los investigadores empezaron a especular sobre la capacidad de las especies del género *Acinetobacter* para transmitirse por el aire. Aunque aún se está discutiendo si la presencia de estas bacterias en el aire debiera ser considerada como una contaminación causada por la presencia de pacientes enfermos o bien como un medio de transmisión.

Las técnicas de secuenciación facilitan no sólo los análisis filogenéticos, sino que también permiten unos análisis epigenéticos más sencillos. Estos análisis proporcionan un mayor conocimiento de los genomas, ayudando a encontrar proteínas que podrían estar implicadas en el desarrollo de tolerancia, persistencia y resistencia.

Una de las mayores amenazas para la salud pública en nuestros días es el incremento de bacterias resistentes a los antimicrobianos. Dicho incremento se

debe a múltiples factores, entre los que comienzan a destacar la existencia de poblaciones bacterianas tolerantes y/o persistentes a las condiciones de estrés, incluyendo los tratamientos antibióticos, así como la exposición a antisépticos.

Esta es la razón por la cual el conocimiento de los mecanismos de persistencia y tolerancia es la clave para identificar los factores que favorecen la supervivencia de patógenos aéreos, con el objetivo de desarrollar estrategias para el control de la infección y aplicaciones para la biodefensa. Actualmente, hay múltiples mecanismos de persistencia y tolerancia descritos en la bibliografía, como son: La respuesta general al estrés (a través del RpoS), la respuesta a las especies reactivas de oxígeno (ROS), el metabolismo energético (mediante el citocromo bd y el metabolismo Tau), las bombas de expulsión (relacionadas en los últimos años con los sistemas de secreción tanto tipo 3 como tipo 6), la respuesta SOS, el ppGpp, los compuestos volátiles bacterianos, la red del Quorum (*sensing*/*quenching*) y los sistemas toxina-antitoxina.

La red de Quorum incluye tanto la comunicación bacteriana (*Quorum sensing*- QS) como su inhibición (*Quorum quenching*- QQ). El QS se caracteriza por moléculas difusibles (autoinductores) que se unen a receptores específicos provocando efectos en múltiples mecanismos moleculares, estas moléculas pueden producir su propia activación. Estos autoinductores pueden ser usados como probióticos, aprovechando la alteración que producen en la microbiota, su interacción con los factores de virulencia y su habilidad para estimular la fase de dispersión de las biopelículas. Por otra parte, el QQ se produce mediante la degradación de los autoinductores, inhibiendo su salida de la célula, evitando su acumulación o compitiendo por su receptor. La red del quorum está relacionada con múltiples mecanismos bacterianos incluyendo la generación de células persistentes y/o tolerantes.

Otro importante mecanismo relacionado tanto con la red del quorum como con la tolerancia y/o persistencia es el metabolismo de los compuestos volátiles bacterianos. Estos compuestos son producidos como metabolitos secundarios y son secretados por las bacterias como "armas" para el control de sus competidores, así como para favorecer su propia adaptación al ambiente. Uno de los metabolismos de

compuestos volátiles bacterianos más estudiados es el metabolismo de la acetoína, que permite a las bacterias sobrevivir a medios ácidos.

La relación entre la formación de células persistentes y tolerantes y los sistemas toxina-antitoxina (TA) ha sido ampliamente estudiada en las últimas décadas. Los sistemas TA son sistemas de dos componentes en los que la toxina (proteína estable) actúa como inhibidor del crecimiento celular y una antitoxina (proteína inestable) que bloquea la acción de la primera. Actualmente hay siete tipos de sistemas TA, clasificados en función de la interacción entre la toxina y la antitoxina. Estos sistemas están presentes en prácticamente todas las bacterias, encontrándose en la mayoría de los casos varios sistemas en el mismo genoma, pudiendo llegar incluso a ser redundantes; un claro ejemplo de esto es el caso de la *P. aeruginosa* en la que se han encontrado hasta 88 sistemas TA diferentes.

A lo largo de la presente tesis doctoral se han analizado diversos mecanismos moleculares de persistencia y/o tolerancia bacteriana, destacando la red del Quorum *(Sensing/Quenching*) y los sistemas Toxina-Antitoxina (TA) en cepas ambientales y clínicas.

En el capítulo 1 llevamos a cabo el análisis del genoma de una cepa aislada del ambiente aéreo hospitalario, concretamente del aire de una Unidad de Cuidados Intensivos (UCI) de Río de Janeiro (Brasil), llamada *Acinetobacter sp*. 5-2Ac02 (que resultó ser una nueva especie cercana a *A. towneri*). Esta cepa presentaba un genoma pequeño, de tan solo 2.951.447 pares de bases (el cromosoma de *A. baumannii* ATCC 17978 contiene 4.004.792 pares de bases), con un contenido de G+C del 40,9% y una previsión de 2.795 secuencias codificantes. Gracias a este análisis genómico descubrimos la presencia de múltiples genes de resistencia a metales pesados (proteínas de la familia TerC pertenecientes al operón ter, genes *kilA* y *kilB* pertenecientes al operón kil, genes del operón del arsénico, ...), un gen que codifica para una β-lactamasa que estaba bloqueado por una transposasa tipo ISAba3, así como cuatro sistemas toxina-antitoxina conocidos y dos posibles sistemas. Sorprendentemente, el genoma de esta bacteria contiene un 5% de secuencias de inserción, un porcentaje bastante más alto que en las especies clínicas de *Acinetobacter*.

A lo largo del capítulo 2 se realizaron estudios de resistencia a diferentes antibióticos y metales pesados que mostraron la alta sensibilidad de esta cepa a los antimicrobianos, siendo sin embargo resistente a varios de los metales pesados, indicando su elevada capacidad de adaptación al ambiente. Posteriormente llevamos a cabo estudios de expresión génica ante diferentes condiciones de estrés como fueron la presencia de mitomicina y enzimas de QQ (Acil-homoserina lactonas). Ante la presencia de mitomicina C observamos la expresión de los 6 sistemas TA, además de la expresión de genes del sistema SOS, de la respuesta ROS, de resistencia a metales pesados, la proteína colicina V y elementos móviles; todo ello relacionado con mecanismos de persistencia y/o tolerancia. Así mismo, hay que destacar que la combinación de mitomicina C junto con el antibiótico ciprofloxacino dió lugar a la generación de células tolerantes. En lo relativo a los estudios de expresión génica relacionados con la red del Quorum cabe destacar la sobreexpresión de genes encargados de la degradación de compuestos aromáticos, cluster de la acetoina. Este operón de genes fue encontrado en 18 cepas de *A. baumannii* procedentes del “II estudio español de *A. baumannii* GEIH-REIPI 2000-2010 (Bioproject PRJNA345289)” así como en *A. baumannii* ATCC 17978. Este cluster es conocido como un método bacteriano para prevenir la acidificación del medio, así como aumentar la resistencia a la polución.

Durante el estudio de este operón observamos que *Acinetobacter sp*. 5-2Ac02 carecía de uno de los genes con respecto al mismo operón en *A. baumannii* ATCC 17978, AcoN. Esta fue la razón que nos llevó a analizar el papel de este gen como regulador negativo del operón de la acetoína. Mediante un mutante isogénico de *A. baumannii* ATCC 17978 caracterizamos la función de este gen por medio de curvas de crecimiento en presencia de 5 mM de acetoína; esta curva mostró que el mutante isogénico crecía con un ratio significativamente mayor que su cepa salvaje (*A. baumannii* ATCC 17978), confirmando la función de AcoN como regulador negativo del cluster. Además, la proteína 2,3-butanediol deshidrogenasa del operón se sobreexpresaba en presencia de 3-Oxo-C12-HSL en el mutante *A. baumannii* ATCC 17978 *ΔacoN.*

A mayores observamos que el catabolismo de la acetoína es inducido por la luz y que el fotorreceptor BlsA interactua con este operón antagonizando la función de AcoN, viéndose afectado también por la temperatura. De hecho, se observó que el

crecimiento en presencia de acetoína era mejor bajo luz azul que en oscuridad, gracias a la acción de las proteínas BlsA y AcoN. Así mismo, los genes del catabolismo de la acetoína eran inducidos, en estas condiciones, de forma dependiente de BlsA y AcoN. Sin embargo, los mutantes de los genes *acoN* y *blsA* mostraron el comportamiento el opuesto, siendo BlsA necesario para la inducción del operón en luz azul, así como AcoN para su represión, indicando que BlsA efectivamente antagoniza el efecto de AcoN. Finalmente, ensayos con el sistema Y2H indicaron que BlsA interactúa con AcoN sólo en presencia de luz azul y no oscuridad. Estos datos corroboran el modelo en el cual BlsA interactúa con el operón y probablemente secuestra al represor del cluster, permitiendo un mejor crecimiento en condiciones de luz azul (de forma temperatura-dependiente). BlsA es por tanto un doble regulador, antagonizando la función de diferentes reguladores transcripcionales en oscuridad o bajo luz azul.

Posteriormente, en el capítulo 3 estudiamos la importancia de dicha red del Quorum en el desarrollo de bacteriemia secundaria a neumonía en aislamientos clínicos de *Acinetobacter baumannii* procedentes del “II estudio español de *A. baumannii* GEIH-REIPI 2000-2010 (Bioproject PRJNA345289)”.

El análisis de expresión de los genes *abaR* (gen del QS) y *aidA* (gen del QQ) mediante RT-qPCR, en 17 aislados procedentes de pacientes colonizados y 13 de pacientes con neumonía, no mostraron diferencias significativas. Sin embargo, sí fueron encontradas diferencias entre los pacientes con neumonía y bacteriemia (secundaria a la neumonía) y los pacientes que sólo presentaban neumonía. El gen *abaR* estaba sobreexpresado en las cepas procedentes de pacientes con neumonía y bacteriemia con respecto a las de los pacientes que sólo presentaban neumonía, así como el gen *aidA* que estaba sobreexpresado en los aislados de pacientes que sólo presentaban neumonía respecto a los pacientes con neumonía y bacteriemia. Además, el análisis de estos genes de la red del quorum bajo diferentes condiciones como las moléculas 3-Oxo-C12-HSL y el H2O2 en los aislados de cepas de pacientes que sólo presentaban neumonía mostró una regulación del gen *aidA* en presencia de ambas moléculas (en presencia de 3-Oxo-C12-HSL sobreexpresión y en presencia de H2O2 represión). Por otra parte, el gen *abaR* mostró una significativa disminución de su expresión en presencia de 3-Oxo-C12-HSL en estos pacientes. El análisis de estos genes en los aislados de pacientes con neumonía y

bacteriemia revelaron que el gen *aidA* no era regulado por ninguno de estos dos compuestos; sin embargo, el gen *abaR* sí que se sobreexpresaba en presencia de H2O2. Estos resultados indican que los aislados de pacientes con neumonía y bacteriemia no muestran un gen *aidA* funcional, en contraste con los aislados que no desarrollan una bacteriemia secundaria.

Por otra parte, el análisis de factores de riesgo asociados con el desarrollo de neumonía vs colonización demostró que el único factor significativo es la diabetes mellitus. Sin embargo, el único factor asociado con el desarrollo de bacteriemia secundaria a neumonía causada por *A. baumannii* es la ausencia de un gen *aidA* funcional. Estos resultados corroboran los obtenidos previamente en nuestro laboratorio, en los que se expone que el 67% de las cepas clínicas de *A. baumannii* respiratorias son no-motiles y expresan el gen *aidA*; mientras que el único aislado motil que además carecía de dicho gen era de origen sanguíneo.

Por último, los estudios *in vivo* en *Galleria mellonella* mostraron un 100% de mortalidad en las primeras 24h cuando eran infectadas por *A. baumannii* ATCC 17978, mientras *A. baumannii* ATCC 17978 Δ*abaI* solo producía un 70% de mortalidad.

Continuando con el estudio de los mecanismos de persistencia y/o tolerancia, en el capítulo 4 se estudió el efecto de la clorhexidina (un antiséptico comúnmente usados en los hospitales para desinfectar superficies incluido en el grupo de las biocidas), causante de un estrés bacteriano que debe ser añadido al de los antibióticos, en una cepa clínica de *A. baumannii* (procedente del “II estudio español de *A. baumannii* denominada Ab-2_clon_2010 GEIH-REIPI 2000-2010 (Bioproject PRJNA345289)”) que había sido previamente caracterizada.

El estudio transcriptómico de la cepa Ab-2_clon_2010-CHLX (tolerante a la clorhexidina) en presencia de clorhexidina, mostró la sobreexpresión de: i) Los genes de las bombas de expulsión AdeABC, arsenito y AceI clorhexidina; ii) los genes del plásmido PMMCU3p, que incluyen la OXA 24/40 β-lactamasa, OriV y proteínas de replicación del ADN; iii) los genes de los operones de genes CsuA/BABCDE, taurina y CydAB; y iv) genes del quorum *sensing*. Este análisis también mostró la represión de algunos genes de rutas metabólicas, que fueron analizados mediante la base de datos “kyoto Encyclopedia of Genes and Genomes

(KEGG)”: i) La red ppGpp, que es mediada por una variedad de proteínas homólogas a RelA/SpoT (RSH), precisamente la acumulación de ppGpp afecta a los procesos celulares de consumo de recursos y la producción de arresto celular en casos de inanición; y ii) la fosforilación oxidativa, en la que están implicadas las subunidades α/β de la ATP sintasa así como el factor rho (un factor implicado en la terminación de la transcripción), que estaban reprimidos en nuestro análisis.

Posteriormente, realizamos curvas de muerte celular de las cepas Ab-2_clon_2010 (cepa salvaje), Ab-2_clon_2010-CHLX (cepa adaptada), mostrando que la cepa tolerante a la clorhexidina sufría una muerte masiva en presencia de imipenem; confirmado por los bajos niveles de expresión de la OXA 24/40 β-lactamasa, la antitoxina AbkA y la sobreexpresión de la toxina AbkB. Además, se observó un recrecimiento de las células persistentes generadas en el aislado Ab-2_clon_2010-CHLX, en presencia de la combinación de imipenem y clorhexidina. Para completar los experimentos, usamos dos cepas tipo de *A. baumannii* como controles (ambas susceptibles a las carbapenemasas) y sorprendentemente, encontramos que la cepa *A. baumannii* ATCC 17978 (que contiene el sistema AbkB/AbkA en su genoma) también sufría una reactivación del crecimiento en presencia de la combinación imipenem con clorhexidina, al contrario que la cepa *A. baumannii* ATCC 19606 (que carece de dicho sistema). Todos estos datos fueron también confirmados por el método enzimático WST-1 de supervivencia celular.

Tras demostrar la importancia de los sistemas TA, así como su estrecha relación con la formación de células persistentes y/o tolerantes, en el capítulo 5 decidimos analizar lo que ocurre en una bacteria al sobreexpresar una toxina de un sistema TA.

Los sistemas TA están ampliamente distribuidos no solo en bacterias, sino también en los reinos Archea y Fungi; su amplia distribución y variedad implica que confieren una ventaja evolutiva a aquellas células que los poseen. Sin embargo, es sabido que estos sistemas TA requieren una estricta regulación, para prevenir reacciones indeseadas en el metabolismo celular.

Nosotros proporcionamos claves sobre cómo las bacterias afrontan la actividad de una toxina (procedente de un sistema TA) al identificar qué mutaciones tienen lugar en las bacterias para inactivar dichas toxinas, en los casos en los que las antitoxinas

no funcionan o no están presentes. Usando una cepa de *E. coli* con el plásmido de expresión pCA24N, que llevaba integrado un gen de una toxina; concretamente genes de cuatro sistemas TA diferentes (RalR- tipo I, MqsR- tipo II, GhoT- tipo V y Hha- tipo VII), llevamos a cabo curvas de crecimiento en presencia de IPTG para la sobreexpresión de los genes previamente mencionados. A través de estas curvas pudimos observar cómo a pesar de la clara inhibición del crecimiento producida por estas toxinas en las primeras horas, al cabo de 12h las bacterias habían recuperado su crecimiento, llegando a presentar un cultivo en fase estacionaria. Sorprendentemente, una segunda curva de crecimiento usando las bacterias que habían crecido en el experimento anterior, mostró que estas células ya no se veían afectadas por la sobreexpresión de la toxina con IPTG, permitiendo un crecimiento normal de las mismas, lo que implicaba la presencia de mutaciones estables en el ADN de estas bacterias.

Con el objetivo de determinar qué modificaciones habían ocurrido en estas células, se procedió a la secuenciación del plásmido integrado observando la deleción del promotor en algunos de los aislados estudiados, pero no en todos. De entre todos los aislados que no mostraron modificaciones en el plásmido se seleccionaron 5 para la realización de un análisis de polimorfismos de un solo nucleótido (SNPs) en su genoma. Este análisis mostró la presencia de una serie de SNPs que estaban presentes en todas las cepas y que afectaban a varios genes, destacando los genes que codifican para las proteínas LacI (regulador negativo del operon Lac), IraM (factor anti-RssB, implicado en la estabilización del RpoS) y MhpR (regulador transcripcional).

Las curvas de crecimiento usando mutantes que portaban estos genes delecionados confirmaron que la ausencia de estos genes, efectívamente afecta a la acción de la toxina, bloqueando su acción. También es importante destacar que los genes de estas cuatro toxinas están altamente conservados en una población de 1000 genomas de *E. coli*, a excepción del caso del gen ralR que muestra una mayor variabilidad en su secuencia.

A lo largo de toda esta tesis doctoral se han estudiado principalmente la red del quorum y los sistemas toxina-antitoxina (dos importantes mecanismos de persistencia y tolerancia), así como sus efectos en cepas clínicas bajo condiciones

de estrés. Gracias a este estudio se encontró que *Acinetobacter sp*. 5-2Ac02, cepa aislada del aire de una unidad de cuidados intensivos en Brasil, tenía un genoma de 2,95MB con un contenido de G+C del 40,9%; en este genoma encontramos un 5% de secuencias de inserción, seis sistemas TA, múltiples genes de resistencia a metales pesados y una β-lactamasa OXA-48 interrumpida por una transposasa tipo ISAba3. Esta cepa fue determinada como una nueva especie próxima a *A. towneri*.

Siguiendo con el estudio de esta cepa en el capítulo 2 encontramos además de múltiples mecanismos de persistencia y tolerancia activados bajo condiciones de estrés, el regulador negativo del cluster de la actetoína en *A. baumannii* y la regulación de este operón de genes por el quorum *sensing* y la luz.

Por otra parte, en el capítulo 3 encontramos que la reducción de la expresión de la enzima de QQ *aidA* o su ausencia en cepas clínicas de *A. baumannii* es un factor clave en el desarrollo de bacteriemia secundaria a neumonía; y que la falta del gen *abaI* (sintasa del QS) reduce la mortalidad de la infección por *A. baumannii* ATCC 17978 en un modelo animal.

También describimos una importante conexión entre la presencia de imipenem y clorhexidina y la generación de células persistentes en una cepa clínica de *A. baumannii* perteneciente al clon ST-2 que porta un plásmido con una β-lactamasa OXA 24/40 y el sistema TA AbkB/AbkA.

Por último, observamos que *E. coli* es capaz de inactivar una toxina sobreexpresada, en ausencia de su antitoxina, inactivando el promotor de la misma o modificando su propio cromosoma, pero no modificando la propia toxina; demostrando la importancia no solo de la conservación de los genes procedentes de los sistemas TA, sino de la regulación de estos sistemas.

Esta tesis es el estudio de los mecanismos globales de tolerancia y persistencia en patógenos clínicos, aportando datos que pueden llegar a ser la clave para el desarrollo de nuevos tratamientos antiinfecciosos por medio del uso de nuevas dianas moleculares.

Curriculum Vitae

Curriculum Vitae

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- PRIS-II, Phagic endolisins: clinical aplication (2017-2018). Agency of health knowledge (ACIS). Principal Investigator **María del Mar Tomás Carmona.**

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CONGRESS COMMUNICATIONS:

Oral communication

- "Expression of mechanisms of bacterial tolerance under chlorhexidine pressure in *A. baumannii* ST-2 clinical strain". ECCMID 2017. ePoster session.

Poster communication

- Two Poster communications to the European Congress ECCMID 2019.

- Four Poster communications to the European Congress ECCMID 2018.

- One Poster communications to the European Congress ECCMID 2017.

- One Poster communication to the American Microbiology Society Congress 2017.

- Two Poster communications to the XXI National Congress SEIMC 2017.

- One Poster communication to the 11th International Symposium on the Biology of *Acinetobacter* 2017.

LANGUAGES:

- English (B2 level). Cambridge English: First Certificate.

- Spanish. Native language.