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Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*

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Acinetobacter baumannii **is an opportunistic pathogen, causing infections mainly in patients in intensive care units where the extensive use of antimicrobial agents can select for the emergence of multiresistant strains. In fact, since strains resistant to all antimicrobial agents have been reported, *A. baumannii* is considered the paradigm of multiresistant bacteria. Both acquired and intrinsic resistance can contribute to multiresistance. The ability to acquire multidrug resistance can be due to either the acquisition of genetic elements carrying multiple resistant determinants or mutations affecting the expression of porins and/or efflux pump(s), which can affect unrelated antimicrobial agents. Meanwhile, intrinsic resistance can be generated by the interplay of decreased permeability and constitutive expression of active efflux systems and it too can affect unrelated antimicrobial agents. This review is focused on the current knowledge of porins and efflux pump(s) in this microorganism.**

Keywords: permeability, intrinsic resistance, *A. baumannii*

Introduction

Thirty-two different genomic species are currently accepted in the *Acinetobacter* genus and *Acinetobacter baumannii* is, undoubtedly, the most frequently isolated species of greatest clinical interest.[1] Since isolates resistant to all antimicrobial agents have been described,[2] this species can be considered the paradigm of multiresistant bacteria. Several factors can favour the acquisition of multiresistance: one is the ability to survive in environmental and human reservoirs. Numerous publications have reported the presence of *Acinetobacter* spp. in different hospital environments, either as the source of an outbreak or in metastatic locations.[3,4] *Acinetobacter* spp. may survive on dry surfaces longer than reported for *Staphylococcus aureus* and *Pseudomonas aeruginosa*[5] and there is no difference between the survival times of sporadic and outbreak strains of *A. baumannii*.[6] Survival is probably due to the minimal nutritional requirements needed by *Acinetobacter* spp. to grow and its ability to grow at different temperatures and pH values.[7] *A. baumannii* may also contribute to the bacterial flora of the skin, particularly in regions such as the axilla and groin.[8] *Acinetobacter* spp. have also occasionally been found in the oral cavity and respiratory tract of healthy individuals.[9] However, the carrier state in these zones is more common in hospitalized patients, particularly during an epidemic outbreak. Colonization of the intestinal tract by *Acinetobacter* spp. is controversial. While some authors suggest that it is an unusual event,[10] others report that the gastrointestinal tract is the most important reservoir of resistant strains.[11] The difference is probably due to the epidemiological situation, i.e. whether there is an epidemic outbreak or not. The second factor widely influencing the acquisition of multiresistance is the acquisition of genetic elements. Among these elements, plasmids, transposons and integrons have been reported. In the early 1980s, Goldstein and colleagues[12] demonstrated the presence of a plasmid containing three resistance genes, one gene encoding a β-lactamase TEM-1 and two genes encoding aminoglycoside-modifying enzymes [APH(3′)(5′)I and ADD(3″)(9)]. Transposons may also play an important role in ensuring the establishment of new resistance genes. Ribera *et al.*[13] partially characterized a transposon carrying the *tetR* and *tet*(A) genes, encoding a regulatory protein and a tetracycline resistance determinant. In the last 5 years, a plethora of papers has been published reporting the implication of the integrons in *A. baumannii* as genetic elements that carry different antibiotic resistance genes.[14–20] On comparing the genome of a multiresistant *A. baumannii* strain versus a fully susceptible strain, Fournier *et al.*[21] recently found that the resistant strain carried a 86 kb resistance island in which 45 resistance genes were clustered. This island also contained two operons associated with arsenic and mercury resistance, respectively and four *qacE1* genes encoding small multidrug resistance (SMR) efflux pumps, which confer low-level resistance to ammonium antiseptics.

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The third factor favouring the acquisition of multiresistance is the intrinsic resistance of these microorganisms, which can be explained by the low permeability of certain antibiotics through the outer membrane, the constitutive expression of some efflux pumps or the interplay between the two processes. This review is focused on the current knowledge of outer membrane permeability as well as outer membrane proteins (OMPs) and efflux pumps associated with antibacterial agent resistance characterized to date.

OMPs

Porins are proteins able to form channels allowing the transport of molecules across lipid bilayer membranes, that show little permeability for hydrophilic solutes. They provide membranes with multiple functions. Porins can act as potential targets for adhesion to other cells and binding of bactericidal compounds to the surface of Gram-negative bacteria. Variations in their structure as a mechanism to escape from antibacterial pressure or regulation of porin expression in response to the presence of antibiotics are survival strategies that have been developed by many bacteria. Porins may play a significant role in mechanisms of resistance.

One of the limitations of our knowledge of *A. baumannii* is the lack of information concerning its OMPs and the permeability properties of this outer membrane. Until now, only a few OMPs have been reported and their functions remain unclear.[22] The small number and size of porins could explain the decrease in *A. baumannii* outer membrane permeability (less than 5%) when compared with other Gram-negative organisms.[23] The outer membrane in *A. baumannii* is less permeable to antimicrobial agents than that in *Escherichia coli*. In accordance with Sato and Nakae[24] the coefficient of permeability to cephalosporins is between 2- and 7-fold larger in *P. aeruginosa* than in *Acinetobacter* spp. They therefore suggested that the intrinsic cause of the resistance to antimicrobial agents could be attributed to the small number of porins as well as their small size. However, another possibility to maintain this intrinsic resistance to antimicrobials could be the low level of constitutive expression of one or several active efflux systems in *A. baumannii* or to the interplay between both low permeability and constitutive expression of efflux pump(s).[7]

In several reports the decreased expression of some OMPs has been shown to be associated with antimicrobial resistance in *A. baumannii*.[25–27] The major OMP of *A. baumannii* described to date is the heat-modifiable protein HMP-AB.[28] These porins show a different mobility following SDS–PAGE without heating and after 10 min at 95°C.[29] The HMP-AB gene encodes a protein of 346 amino acids with a molecular mass of 35 636 Da and is assembled in the membrane in a similar manner to monomeric porins.[28] Sequence comparison of HMP-AB with other OMPs revealed a clear homology with the monomeric OMP A (OmpA) of Enterobacteriaceae and the OMP F (OprF) of *P. aeruginosa*. Secondary structure analysis indicated that HMP-AB has a 172-amino-acid N-terminal domain that spans the outer membrane by eight amphiphilic beta strands and a C-terminal domain that apparently serves as an anchoring protein to the peptidoglycan layer. Analysis of the amino acid sequence reveals the typical structure of Gram-negative bacterial porins: a highly negative hydropathy index, absence of hydrophobic residue stretches, a slightly negative total charge, low instability index, high glycine content and an absence of cysteine residues. This porin belongs to the OmpA family. Porins of this family are known as slow porins that allow the penetration of β-lactams and saccharides up to approximately 800 Da.[28,29] Slow porins belonging to this family allow a much slower diffusion of small solutes but allow the diffusion of much larger solutes that cannot penetrate through the OmpF channel of *E. coli*.[30] Therefore, in organisms that lack the classical trimeric porin, the protein of this family functions as the major porin and contributes to the high levels of intrinsic resistance.[30]

The OmpA from different species of *Acinetobacter* has recently been described and characterized.[31] The sequenced fragment was found to be homologous among *A. baumannii*, *Acinetobacter radioresistens* and *Acinetobacter junii*. However, the authors did not mention the similarity between this OmpA and the above-mentioned HMP-AB. In the *A. radioresistens* (KA53) strain, the OmpA was found to be a secreted emulsifier. It is known that bio-emulsifiers play an important role in bacterial pathogenesis, quorum sensing and biofilm formation, regulating adhesion to surfaces.[31]

Three other OMPs have been reported to be missing in the imipenem-resistant strains of *A. baumannii*: one is a 33–36 kDa protein,[32] another is a 29 kDa protein, designated CarO[27,33] and, finally, a 43 kDa protein, which shows significant peptide homology with OprD from *P. aeruginosa*.[34] On studying CarO by mass spectrometry Siroy *et al.*[33] detected another 25 kDa protein that they called Omp25, together with CarO. Both 25/29 kDa proteins adopted a typical β-barrel conformation, however, only one of these proteins (CarO) displayed pore-forming properties. No binding site for imipenem could be detected in CarO, suggesting an unspecific monomeric channel function rather than a specific function.[33] It is important to mention that the protein OprD of *P. aeruginosa* has been demonstrated to be involved in the uptake of basic amino acids, small peptides and of imipenem and meropenem.[30] Therefore, CarO may function as a carbapenem-unspecific channel and the OprD-like protein may function as a carbapenem-specific channel.

Another OMP in *A. baumannii* is OmpW, which shows high homology with OmpW found in *E. coli* and *P. aeruginosa*. Its function in *A. baumannii* remains unclear, however, we have recently found that the expression of this OMP was decreased in an '*in vitro*' colistin-resistant *A. baumannii* mutant (Sara Martí, unpublished data). However, based on the observation that OmpW expression was dramatically decreased in a ceftriaxone-resistant strain of *Salmonella* Typhimurium, it was recently proposed that OmpW might also be involved in the uptake of this antibiotic.[35]

More in-depth studies are necessary to elucidate the role of these OMPs in multidrug resistance and to fully characterize the complex structure of the outer membrane of *A. baumannii* that confers its special permeability features.

Efflux-pump-mediated resistance

In Gram-negative bacteria, the outer membrane limits the rate of antimicrobials entering the cell and the multidrug efflux pumps actively export multiple, structurally-distinct classes of antimicrobials out of the bacteria.[36] Efflux transporters are expressed in all living cells, protecting them from the toxic effects of

organic chemicals. Bacterial multidrug resistance has often been associated with overexpression of these transporters. The antimicrobials expelled out of the cell have to cross the low permeability outer membrane in order to enter again; therefore the efflux pumps work synergistically with the low permeability of the outer membrane.[36] An increased efflux of antibiotic from the bacterium produces a reduction in drug accumulation and an increment in the MIC. The most common antimicrobials expelled by the efflux pumps are macrolides, tetracyclines and quinolones.[37] In all the metabolic processes there is generally a high degree of specificity in the transport of proteins and enzymes, although multidrug efflux pumps recognize a broad range of structural and chemically different substrates.[38]

The multidrug efflux systems have been grouped into six families:[36] the ATP binding cassette (ABC) family, the major facilitator superfamily (MFS), the resistance-nodulation-division (RND) family, the multidrug and toxic compound extrusion (MATE) family, the SMR family and the drug/metabolite transporter (DMT) superfamily.

ABC-type efflux pumps are ATP-dependent multidrug transporters and use ATP as a source of energy to expel the antimicrobials out of the cell. The members of this family are rarely involved in acquisition of resistance to antimicrobials in Gram-negative bacteria. The other types of efflux pumps are drug-proton antiporters. Antimicrobial expulsion is accomplished utilizing the proton motive force as the driving force for efflux.[36,39] The major efflux pumps involved in multidrug resistance belong to this group of proton-motive-force-dependent exporters, with the most important group being the RND family, as well as the MFS and SMR families.[36]

In *A. baumannii*, efflux-pump-mediated resistance to antimicrobials is generally associated with the MFS and RND family (Table 1). Using a comparative genomic approach Fournier *et al.*[21] recently attempted to identify all the resistance genes present in the *A. baumannii* multidrug-resistant strain AYE, which was epidemic in France. Most of the resistance genes found in this strain had been acquired from other bacteria such as *Pseudomonas*, *Salmonella* or *E. coli* and were clustered in an 86 kb region or island. This resistance island (AbaR1) contained 45 genes predicted to be associated with resistance to antimicrobial drugs, heavy metals and antiseptics. Outside of this island, 46 ORFs were putatively associated with resistance to antimicrobials of which 32 ORFs were associated with the RND family, seven with the MFS, two with the MATE family and one with the SMR family. Moreover, one gene was associated with the ABC superfamily and another with the DMT superfamily.[21]

Major facilitator superfamily (MFS)

The MFS efflux pumps are not normally multidrug transporters, but more usually function as specific exporters for certain classes of antimicrobial agents.

Tet efflux pumps

The two main mechanisms of resistance to tetracycline in Gram-negative bacteria are the expression of an efflux pump or a ribosomal protection system. The Gram-negative *tet* efflux genes can be present on transposons that are inserted into plasmids from a variety of incompatibility groups, most of which are conjugative. Ribera *et al.*[13] identified the *tet*(A) gene in a Tn*1721*-like transposon. Their results suggested that there is a horizontal transfer among different genera of Gram-negative bacteria that share the same ecological niche. This gene encodes a membrane-associated efflux protein that confers resistance to tetracyclines. These efflux pumps belong to the MFS and exchange a proton for a tetracycline–cation complex.[40] In Gram-negative bacteria, there is one gene encoding for an efflux protein and there is another gene encoding for a repressor protein. The system is regulated by the presence of tetracycline. In the absence of tetracycline, the repressor protein blocks the transcription of the structural genes. The process starts when a tetracycline–Mg^{2+} complex

Table 1. Efflux pumps described in *Acinetobacter baumannii* and their activity in front of several antimicrobial agents

Efflux pump	Family	Antibiotics	NCBI accession number(s)
Tet(A)	MFS	tetracycline	AAO38186
Tet(B)	MFS	tetracycline, minocycline	
CmlA	MFS	chloramphenicol	CAJ77032
AdeABC	RND	aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracyclines and ethidium bromide; reduced susceptibility to fluoroquinolones	AAL14439, AAL14440, AAL14441
AbeM	MATE	norfloxacin, ofloxacin, ciprofloxacin, gentamicin, 4′,6-diamino-2-phenylindole (DAPI), triclosan, acriflavine, Hoechst 33342, daunorubicin, doxorubicin, rhodamine 6G and ethidium bromide	BAD89844

MFS, major facilitator superfamily; RND, resistance-nodulation-division; MATE, multidrug and toxic compound extrusion.

binds to the repressor protein, changing the conformation of this repressor and allowing the transcription of the efflux structural and repressor genes.[40]

In *A. baumannii* the main efflux pumps in this category are: Tet(A) and Tet(B). The efflux determinant Tet(A) confers resistance to tetracycline and Tet(B) confers resistance to tetracycline and minocycline.[41] These efflux pumps do not affect the new tetracyclines such as glycylcyclines. Recently, Martí and colleagues[41] analysed the prevalence of the *tet*(A) and *tet*(B) genes in a collection of 79 tetracycline-resistant *A. baumannii* strains that were not epidemiologically related. They found that 66% of the strains carried the *tet*(B) gene and 13.6% the *tet*(A) gene. None of the strains analysed had both genes. Guardabassi *et al.*[42] suggested that these two efflux pumps were infrequently found among *Acinetobacter* spp. from an aquatic environment.

CmlA and MdfA efflux pumps

The chloramphenicol resistance gene (*cmlA*) encodes for an efflux pump that confers resistance to chloramphenicol and it has recently been described by Fournier *et al.*[21] as forming part of a 86 kb resistance island in *A. baumannii* strain AYE. MdfA is a transporter described in several Enterobacteriaceae, we have recently identified an MdfA orthologue (42.7%) in an *A. baumannii* clinical isolate (data not shown). Bacteria expressing MdfA exhibit multidrug resistance, affecting among others ciprofloxacin and chloramphenicol.

Resistance-nodulation-division (RND) family

AdeABC efflux pump

This family of efflux pumps expels the antimicrobial by utilizing the proton motive force as the driving force for efflux.[36] Overexpression of this normally cryptic, antimicrobial efflux pump confers resistance to aminoglycosides, β-lactams, chloramphenicol, erythromycin, tetracyclines and ethidium bromide.[39,43] In addition, AdeB has been associated with acquisition of reduced susceptibility to fluoroquinolones.[44] Most of the multidrug transporters belonging to this family interact with a membrane fusion protein (MFP) and an OMP. This interaction allows the antimicrobial agent to pass across the inner and the outer membranes of the bacteria without accumulating in the periplasm. Therefore, AdeABC is a three-component efflux pump where AdeA is the MFP, AdeB is the multidrug transporter and AdeC is the OMP.[39] The three genes that encode for these three-component efflux pumps are contiguous in the genome and directly oriented which suggests that they form an operon.[43] The regulator gene appears next to the gene encoding the MFP, followed by the gene encoding the transporter protein and, finally, the gene encoding the OMP; the MFP and the transporter protein are generally co-transcribed.[45] This efflux pump is regulated by a two-component regulatory system (AdeRS): AdeS is a sensor kinase and AdeR is a response regulator.[43] The genes that encode these two proteins are located in front of the *adeABC* genes and are transcribed in the opposite direction. The sensor protein monitors the environmental conditions and activates or inactivates the response regulator protein which controls the expression of the efflux pump.[43] The presence of the OMP AdeC is not essential for resistance because this efflux pump may be associated with other OMPs such as AdeK. This OMP belongs to a new efflux pump identified in *A. baumannii* but which is still being characterized.[43]

Multidrug and toxic compound extrusion (MATE) family

Efflux pump AbeM

This is a multidrug efflux pump that belongs to the MATE family of transporters and has recently been identified by Su *et al.*[46] The AbeM protein shows homology with NorM, but also shows similarities with PmpM in *P. aeruginosa*, VcmA in *Vibrio parahaemolyticus*, YdhE in *E. coli* and HmrH in *Haemophilus influenzae*. The presence of this efflux pump confers more than a 4-fold increase in the MICs of norfloxacin, ofloxacin, ciprofloxacin, gentamicin, 4′,6-diamino-2-phenylindole (DAPI), triclosan, acriflavine, Hoechst 33342, daunorubicin, doxorubicin, rhodamine 6G and ethidium bromide. Moreover, it also produces a reproducible 2-fold increase in the MICs of kanamycin, erythromycin, chloramphenicol, tetraphenylphosphonium chloride (TPPCl) and trimethoprim.[46] This family of efflux pumps is associated with two energy sources: the proton motive force and the sodium ion gradient.[45] AbeM utilizes the proton motive force to expel the antimicrobial out of the cell.[46]

The prevalence of the overexpression of the efflux pumps in *A. baumannii* clinical isolates has only been studied using some efflux pump inhibitors, such as reserpine and MC 207,110.[47,48] These inhibitors are notoriously non-specific and may affect multiple efflux systems disproportionately, however, this does not preclude their utility for detecting the compounded efflux effects of multiple systems. Ribera *et al.*[48] found that in 45% of the *A. baumannii* epidemiologically unrelated clinical isolates, the MIC of nalidixic acid decreased at least 8-fold in the presence of MC 207,110. In contrast, when the MIC was determined in the presence of reserpine, the MIC of ciprofloxacin decreased at least 4-fold in 33% of the *A. baumannii* clinical isolates without affecting nalidixic acid.[47] These disparities in the proportion and degree of changes in the MICs of these two antimicrobial agents implies the functioning of multiple efflux pumps.

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Transparency declarations

None to declare.

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