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MINIREVIEW

Antibiotic resistance of pathogenic *Acinetobacter* species and emerging combination therapy

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The increasing antibiotic resistance of *Acinetobacter* species in both natural and hospital environments has become a serious problem worldwide in recent decades. Because of both intrinsic and acquired antimicrobial resistance (AMR) against last-resort antibiotics such as carbapenems, novel therapeutics are urgently required to treat *Acinetobacter*-associated infectious diseases. Among the many pathogenic *Acinetobacter* species, *A. baumannii* has been reported to be resistant to all classes of antibiotics and contains many AMR genes, such as bla_{ADC} (*Acinetobacter*-derived cephalosporinase). The AMR of pathogenic *Acinetobacter* species is the result of several different mechanisms, including active efflux pumps, mutations in antibiotic targets, antibiotic modification, and low antibiotic membrane permeability. To overcome the limitations of existing drugs, combination theraphy that can increase the activity of antibiotics should be considered in the treatment of *Acinetobacter* infections. Understanding the molecular mechanisms behind *Acinetobacter* AMR resistance will provide vital information for drug development and therapeutic strategies using combination treatment. Here, we summarize the classic mechanisms of *Acinetobacter* AMR, along with newly-discovered genetic AMR factors and currently available antimicrobial adjuvants that can enhance drug efficacy in the treatment of *A. baumannii* infections.

Keywords: *Acinetobacter*, multidrug resistance, biofilm, membrane permeability, natural compounds, adjuvants

Introduction

The increasing concentration of antibiotics in the natural environment is of great concern because it can exert a selective pressure, which facilitates the horizontal gene transfer (HGT) of antimicrobial resistance (AMR) among microorganisms, thus contributing to the spread of multidrug-resistant (MDR) bacteria. The number of infectious diseases caused by MDR bacteria has risen worldwide and become a major public health concern (Blair *et al.*, 2015). Many pathogenic *Acinetobacter* species, particularly *A. baumannii*, have emerged as serious human health risks and are associated with many human diseases such as pneumonia, wound infections, meningitis, and sepsis. Their intrinsic genetic makeup and rapid evolution through induced mutagenesis and HGT may contribute to the ecological success of *Acinetobacter* species as opportunistic pathogens (Poirel *et al.*, 2011; Stogios *et al.*, 2017). The genus *Acinetobacter* currently comprises 51 genomic species, of which 20 are both categorized as pathogens and found in humans (Table 1). *Acinetobacter* species are a common causative agents of human infections in hospitals which include *A. baumannii*, *A. pitti*, and *A. nosocomialis*, the most frequently isolated species in hospital patients; as a result, these have been widely examined in recent years (Jung and Park, 2015) (Table 1). *A. baumannii* forms an *A. baumannii* complex with *A. nosocomialis* and *A. pittii*, which accounts for most (90–95%) clinically significant infections (Nemec *et al.*, 2011). Over the past 5 years, several novel *Acinetobacter* species of human origin have been described: *A. seifertii*, *A. variabilis*, *A. proteolyticus*, *A. vivianii*, and *A. modestus* (Table 1), which indicates possible discovery of more disease-causing *Acinetobacter* species in the future.

Acinetobacter baumannii possesses a range of intrinsic AMR genetic elements and shows high genome plasticity. In addition, recent data has demonstrated that this species has acquired and accumulated AMR determinants from other clinically important Gram-negative species such as *Escherichia*, *Salmonella*, and *Pseudomonas* species (Poirel *et al.*, 2011). *A. baumannii* uses several intrinsic and acquired AMR mechanisms such as (i) increased production of antibiotic efflux pumps, (ii) adaptive evolution that generates point mutations in target proteins to avoid antibiotic action, (iii) enzymatic modification, and degradation of antibiotics, and (iv) reduction of membrane permeability. In many *A. baumannii* isolates, drug-resistant genes are clustered together into antibiotic resistance islands known as *A. baumannii* Resistant Islands (AbaRs) which accumulate in specific genetic regions such as *comM* (Pagano *et al.*, 2016). Many variants of AbaRs have been documented, each differing in the number and nature of the genes involved in antibiotic resistance and transposons events. Many antibiotic resistance genes such as *tetA*(A), *aphA1b*, *aacC1*, and *aadA1* can be found in these AbaR regions (Post *et al.*, 2010). These AbaR-type genome islands

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Table 1. Focus of research on clinically isolated *Acinetobacter* species

Clinical isolate species	Number of papers*				Total*
	1948–1999	2000–2005	2006–2011	2012–2017	
Acinetobacter baumannii	376	579	1,919	3,532	6,406
Acinetobacter calcoaceticus	1,057	169	188	223	1,637
Acinetobacter lwoffii	74	47	65	78	264
Acinetobacter baylyi	-	4	97	87	188
Acinetobacter haemolyticus	67	24	30	52	173
Acinetobacter johnsonii	47	34	26	50	157
Acinetobacter junii	35	26	37	46	144
Acinetobacter radioresistens	25	30	22	33	110
Acinetobacter pittii	-	-	6	99	105
Acinetobacter nosocomialis	-	-	7	96	103
Acinetobacter ursingii	-	2	10	12	24
Acinetobacter guillouiae	-	-	2	18	20
Acinetobacter bereziniae	-	-	2	17	19
Acinetobacter schindleri	-	1	5	10	16
Acinetobacter parvus	-	1	-	9	10
Acinetobacter seifertii	-	-	-	10	10
Acinetobacter variabilis	-	-	-	5	7
Acinetobacter proteolyticus	-	-	-	1	1
Acinetobacter vivianii	-	-	-	1	1
Acinetobacter modestus	-	-	-	1	1

* Number of papers in PubMed. Papers might be overlapped when different species are studied in the same paper.

have also been reported in other *Acinetobacter* strains including *A. nosocomialis* and *A. seiffertii* (Pagano *et al.*, 2016). These AbaR regions are thought to have emerged from the integration of plasmids or other mobile elements (Post *et al.*, 2010). This HGT may result in the generation of AMR hotspot regions. As described above, the resistance of pathogenic *Acinetobacter* species to broad-spectrum antibiotics has received increased attention; thus, studies examining *Acinetobacter* species-specific AMR mechanisms and new therapeutics for patient treatment are needed (Poirel *et al.*, 2011). This review discusses AMR mechanisms within pathogenic *Acinetobacter* species and describes several studies that have used adjuvants to overcome AMR and increase the efficacy of existing antibiotics.

Multidrug efflux pumps within pathogenic *Acinetobacter* species

Cellular efflux-mediated extrusion, which reduces susceptibility to many antibiotics and other biocides, is a well-known mechanism in many pathogenic bacteria, including *A. baumannii* (Coyne *et al.*, 2010; Beceiro *et al.*, 2013). Bioinformatics analysis using whole genome sequencing has found that numerous multidrug transporters within the core genome of *A. baumannii* can eliminate many antibiotics with low specificity. *A. baumannii* may contain more than six different transporter superfamilies capable of actively pumping out a broad range of antimicrobial and toxic compounds from the cell (Hassan *et al.*, 2015) (Fig. 1). Active drug transporters can be divided into two groups on the basis of their energy requirements: primary and secondary. Primary active transporters belong to the ATP-binding cassette (ABC) transporter superfamily which uses the energy from ATP hydrolysis to transport molecules across a membrane against the gradient. Secondary active transporters are ion-coupled transporters (typically Na^+, H^+, or metabolites) and include the 12–14 TM of major facilitator superfamily (MFS) efflux pump functioning as a component of a tripartite system, the 4 TM and proton-coupled small multidrug resistance (SMR) family, the proteobacterial antimicrobial compound efflux (PACE) protein family, and the resistance-nodulation-cell division (RND) superfamily. Members of the multidrug and toxic compound extrusion (MATE) family function as drug/sodium or proton antiporters (Fig. 1A). RND-type transporters in particular are known to play a dominant role in the MDR of many *Acinetobacter* species (Yoon *et al.*, 2013).

In *A. baumannii*, all Ade transporters belong to the RND family and confer AMR on various antibiotics such as aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, macrolides, chloramphenicol, and trimethoprim (Damier-Piolle *et al.*, 2008; Coyne *et al.*, 2010). Interestingly, transcriptomic analysis has also revealed the upregulation of many RND family efflux pumps under ampicillin in soil-borne *A. oleivorans* DR1; this change in the expression of many RND family proteins may be linked to ampicillin resistance (Heo *et al.*, 2014). Each Ade system has a different affinity for each antibiotic; for example, AdeFGH cannot transport aminoglycosides (Coyne *et al.*, 2010). Many *A. baumannii* clinical isolates overexpress Ade transporters, which is thought to contribute to AMR (Chu *et al.*, 2006; Damier-Piolle *et al.*, 2008; Coyne *et al.*, 2010; Yoon *et al.*, 2016). While the overexpression of Ade transporters is often beneficial to bacteria, this is not always the case; some Ade transporters, such as AdeABC, AdeFGH, and AdeIJK, can be toxic to cells when overexpressed, presumably because of changes to membrane

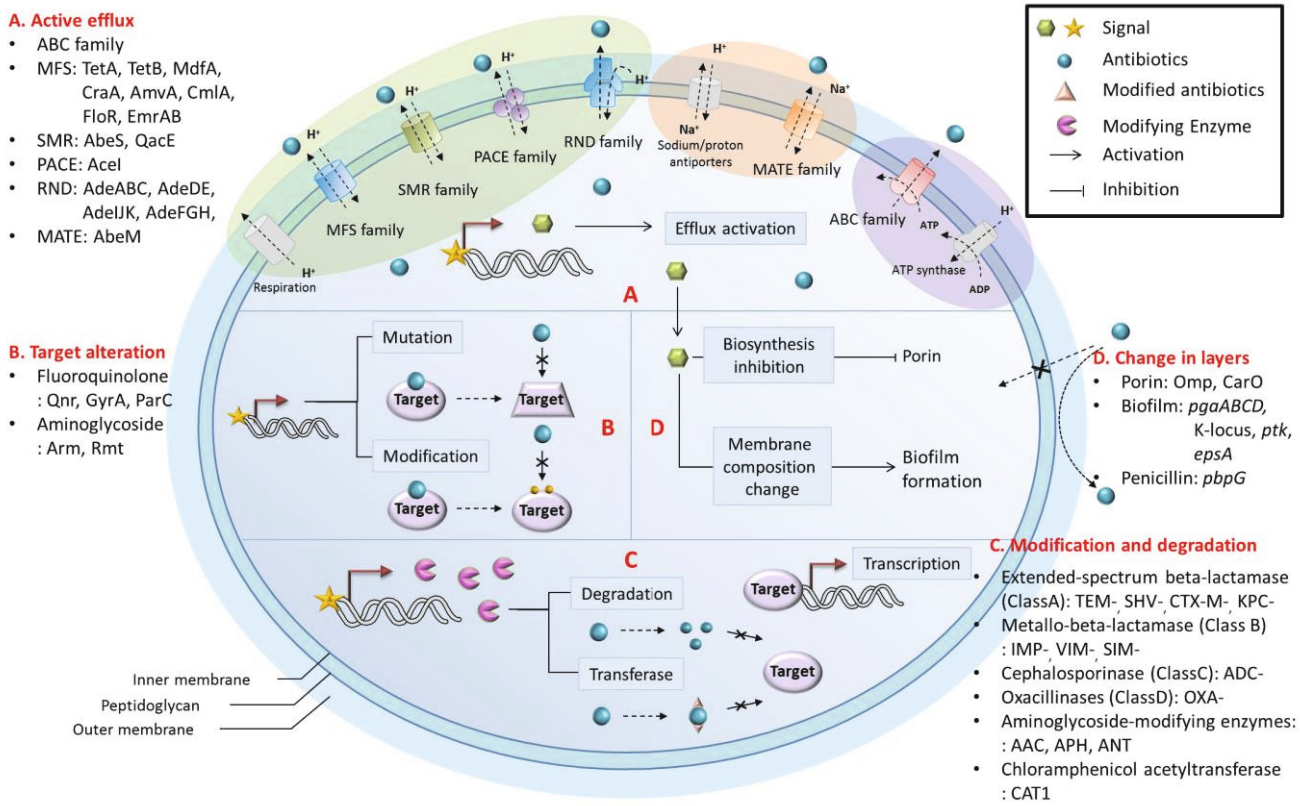

Fig. 1. Mechanisms of antibiotic resistance in *Acinetobacter* species. Resistance to antibiotics can be mediated by the cooperation of numerous different mechanisms. These include active efflux of the drug from the cell (A); mutation and modification of the target, thereby lowering the affinity of the drug for the target (B); modification or degradation of the drug (C); and impaired influx because of low membrane permeability (D). The large oval shape represents the bacterial cell. An electrochemical H^+ gradient across the cytoplasmic membrane is generated by respiration (gray). Energy from the proton-motive-force is used to power the transport of the secondary active transport systems, such as those within the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, proteobacterial antimicrobial compound efflux (PACE) family, and resistance/nodulation/division (RND) family. ATP production by ATP-synthase (gray) is also powered by the proton-motive-force, and ATP is used to power transport by the primary active transporters of the ATP-binding cassette (ABC) superfamily. Sodium/proton antiporters (gray) harness the proton-motive-force to generate an Na^+ gradient that powers transport by other multidrug efflux pumps, including those in the multidrug and toxic compound extrusion (MATE) family.

integrity caused by transporter overexpression (Chu *et al.*, 2006; Damier-Piolle *et al.*, 2008; Coyne *et al.*, 2010). Genomic DNA Group 3 (GDG3) of *Acinetobacter* species expresses the AdeXYZ and AdeDE transporters, which are similar to the AdeIJK and AdeAB transporters in *A. baumannii* (Chau *et al.*, 2004; Chu *et al.*, 2006). Contribution of AdeDE to multidrug resistance was experimentally proven in the GDG3 of *Acinetobacter* species. Downstream sequence of AdeDE is not the ORF encoding an outer membrane protein, which implied that AdeDE might be associated with other unidentified outer membrane proteins (Chau *et al.*, 2004).

Several MFS efflux pumps have been identified in *A. baumannii*, including those for tetracycline (TetA and TetB) (Ribera *et al.*, 2003), chloramphenicol (CraA, CmlA, and FloR) (Roca *et al.*, 2009; Coyne *et al.*, 2011), and erythromycin (AmvA) (Rajamohan *et al.*, 2010). Tetracycline efflux pumps, referred to as Tet proteins, are members of MFS and are often found on plasmids in pathogenic *Acinetobacter* strains (Nikaido, 2009). The inactivation of the CraA efflux pump, a homolog of *E. coli* MdfA, results in chloramphenicol susceptibility in *A. baumannii* (Roca *et al.*, 2009). The homolog of the *E. coli* EmrAB efflux pump in *A. baumannii* has been shown to be involved in colistin resistance (Lin *et al.*, 2017). The SMR family of pumps, which belong to the drug/metabolite transporter superfamily, are composed of four TM α-helices of around 100–140 amino acids in length. In *A. baumannii*, the SMR family of proteins are chromosomally encoded. AbeS, which is located in the inner membrane, confers tolerance on antibiotics such as erythromycin and novobiocin as well as detergents and dyes (Srinivasan *et al.*, 2009). The QacE protein, another well-known SMR family member, removes quaternary ammonium compounds which are important ingredients of disinfectants used in the disinfection of surfaces and medical instruments (Coyne *et al.*, 2011). Previous research has shown that class 1 integron incidence is significantly higher for populations that are preexposed to quaternary ammonium compounds (Gaze *et al.*, 2005). Expression analysis revealed that *qacE* is also upregulated in the presence of kanamycin in soil-borne *A. oleivorans* DR1 cells (Heo *et al.*, 2014). Within the MATE family of proteins, AbeM is an H^+-drug antiporter protein that allows for the efflux of aminoglycosides, quinolones, and chloramphenicol

(Su *et al.*, 2005).

The AceI (*Acinetobacter* chlorhexidine efflux) transporter, which is a part of the newly discovered PACE family, is proven to be important for resistance to many different biocides, antibiotics, and antimicrobial dyes (Hassan *et al.*, 2015). Homologs of AceI are found in many proteobacteria including *Pseudomonas*, *Bukholderia*, and *Vibrio* species. Interestingly, AceI in *A. baumannii* is up-regulated 10-fold by chlorhexidine (Hassan *et al.*, 2015). The RND family protein AdeAB also appears to be involved in adaptive resistance to chlorhexidine in *A. baumannii*. The common laboratory strains *A. baumannii* AYE and ATCC 17978 contain 46 and 73 genes, respectively, that are marked as putative drug efflux pumps, suggesting that there are more novel efflux pump proteins capable of contributing to MDR in *A. baumannii* (Fournier *et al.*, 2006; Hood *et al.*, 2010). Also, previous research has suggested that human serum can promote the expression of unknown efflux pumps which extrude multiple antibiotics, including aminoglycosides, quinolones, and carbapenems (Jacobs *et al.*, 2012). Understanding the role of those efflux pump proteins in modulating antibiotic tolerance is important because the development of efflux pump inhibitors can be a new way to treat infectious diseases.

Mutational alteration of antibiotic targets

The modification of antibiotic-binding sites in targets such as 16S rRNA, the beta subunit of RNA polymerase, DNA gyrase, and inner and outer cell membranes is another major mechanism in pathogenic *Acinetobacter* species to achieve AMR (Fig. 1B). In this mechanism, genetically diverse microorganisms produced by the activity of error-prone polymerases can be selected for in the presence of a given antibiotic and then proliferate under continued antibiotic pressure. Other mechanisms for generating an adaptive bacterial population also exist. Reactive oxygen species, produced as a result of antibiotic treatment, are thought to aid in the development of adaptive populations (Dwyer *et al.*, 2009). Intrinsic or acquired genes that encode proteins capable of modifying a drug target can also produce adaptive populations in the presence of antibiotics (Blair *et al.*, 2015). Resistance to aminoglycoside is often conferred by aminoglycoside-modifying enzymes, including acetyltransferases, nucleotidyl transferases and phosphotransferases (Karthikeyan *et al.*, 2010). Many genes encoding 16S rRNA methylases, such as *armA*, *rmtA-rmtH*, and *npm*, have been reported in *Acinetobacter* species (Karthikeyan *et al.*, 2010). Of these, only *armA* is routinely associated with clinically isolated *Acinetobacter* species (Yu *et al.*, 2007). Notably, the ArmA protein found in *Acinetobacter* is identical to that found in *Salmonella enterica.* Interestingly, the *armA* in some *A. baumannii* species is present on plasmids between *tnpU* and *tnpD* as part of the composite Tn*1548* transposon (Karah *et al.*, 2016). *RmtB* was also found to be present in some clinical *A. baumannii* strains (Beceiro *et al.*, 2013). Because *rmt* genes have only recently been reported, the presence of other *rmt* genes in pathogenic *Acinetobacter* strains should be investigated.

Mutations in *gyrA* and *parC*, which encode DNA gyrase and topoisomerase IV, respectively, decrease ciprofloxacin susceptibility (Vila *et al.*, 1997). Many high-frequency heritable *gyrA* mutations have been observed to occur not only in pathogens but also in the soil *Acinetobacter* strain (Kim *et al.*, 2013). Enzymes such as DNA gyrase and type IV topoisomerase can be protected by Qnr proteins (QnrA-D, QnrS, and QnrVC), although few studies have examined these AAC- or Qnr-based resistance mechanisms (Tran and Jacoby, 2002; Doi *et al.*, 2004; Jiang *et al.*, 2014). Colistin resistance occurs when lipopolysaccharide (LPS) is altered due to mutations in the *pmrAB* two-component system and *lpxACD* lipid A biosynthesis genes. PmrB-regulated NaxD modifies lipid A by deacetylating *N*-acetylgalactosamine to galatosamine, which affects surface charge, thus lowering the affinity for cationic polymyxin antibiotics in *A. baumanii* (Chin *et al.*, 2015). Many clinical isolates of *Acinetobacter* species contain mutations in *lpxACD*, which lead to the loss of LPS production or a reduction in LPS net charge (Beceiro *et al.*, 2013; Chin *et al.*, 2015).

Antibiotic modification and degradation

The enzymatic modification of antibiotics has been extensively reported for cases of AMR in *Acinetobacter*. Several mechanisms are involved, including β-lactam hydrolysis by different β-lactamases and antibiotic modification by nucleotidyltransferases, acetyltransferases, and phosphotransferases (Fig. 1C) (Rodríguez-Martínez *et al.*, 2010). β-Lactamases that degrade carbapenems, cephalosporin, and monobactam are encoded on the chromosome and on plasmids. β-Lactamases are grouped into four classes based on their amino acid sequences (A, B, C, and D) (Rodríguez-Martínez *et al.*, 2010). Class A β-lactamases include major extended-spectrum β-lactamases (ESBLs), such as TEM-1, TEM-2, or SHV-1, which possess a serine residue at the active site and are generally inhibited by the common β-lactamase inhibitor clavulanic acid. They are derived from narrow-spectrum beta-lactamases through mutations that alter the amino acid configuration around the enzyme active site (Naas *et al.*, 2011; Bajpai *et al.*, 2017). Clinical isolates of *A. baumannii* contain many different ESBLs, including SHV-12, TEM-116, CTX-M-2, KPC-2 (*Klebsiella pneumoniae* carbapenemase-2), GES-1, and VEB-1 (Naas *et al.*, 2011; Farajnia *et al.*, 2013).

The occurrence of class B β-lactamases, referred to as metallo-β-lactamases (MBLs), has increased with the increasing global emergence of AMR *A. baumannii* (Anwar *et al.*, 2016). Four MBL enzymes have been reported to be expressed in *A. baumannii*: imipenemase (IMP), Verona imipenemase (VIM), Seoul imipenemase (SIM), and New Delhi metallo-β-lactamase (NDM-1-type) (Higgins *et al.*, 2010). Unlike class A and class D β-lactamases, MBLs contain a metal ion, typically zinc, in the active site (Anwar *et al.*, 2016). IMP-1, -2, -4, -5, -6, -11, and -55, VIM-2, SIM-1, and NDM-1 and -2 MBLs are commonly found as part of a class 1 integron in *A. baumannii* (Karthikeyan *et al.*, 2010; Naas *et al.*, 2011; Roca *et al.*, 2012; Azizi *et al.*, 2016; Chatterjee *et al.*, 2016). NDM-1 has been detected in many other pathogenic *Acinetobacter* species, including *A. johnsonnii*, *A. pitti*, *A calcoaceticus*, *A. lwoffii*, *A. nosocomialis*, *A. junni*, *A. variabilis*, and

A. haemolyticus (Chatterjee *et al.*, 2016; Pagano *et al.*, 2016).

Class C β-lactamases, including *ampC*, have been found in the chromosomal DNA of certain *Acinetobacter* species and may represent a distinct family or *Acinetobacter*-derived cephalosporinases (ADCs) (Tian *et al.*, 2011). To date, 16 bla_{ADC} genes have been reported in *A. baumannii*: ADC-1, -3, -4, -6, -7, -11, -26, -30, -33, -56, -57, -68, -73, -76, -77, and -81 (Rodríguez-Martínez *et al.*, 2010; Tian *et al.*, 2011; Karah *et al.*, 2016). These ADCs are commonly observed in clinical isolates. The β-lactamase genes encoded by these class C cephalosporinases can hydrolyze narrow-spectrum and extended-spectrum cephalosporins, but not cefepime and carbapenems. ADC-33 and ADC-56 are an exception, however, as these can hydrolyze ceftazidime and cefepime (Rodríguez-Martínez *et al.*, 2010; Tian *et al.*, 2011).

Class D oxacillin-hydrolyzing β-lactamases (OXA β-lactamases) are often present on plasmids and confer resistance to penicillins; however, some OXAs can also hydrolyze cephalosporins (Benjamin and Sevastian, 2014; Liao *et al.*, 2015). Clinical isolates of carbapenem-resistant *A. baumannii* express the main oxacillinases (OXA-23, OXA-40, and OXA-58; Benjamin and Sevastian, 2014). Interestingly, the outer membrane vesicles originally identified as OmpA secretion platforms include OXA-24 and OXA-58, which might contribute to increased antibiotic resistance and virulence in *A. baumannii* (Liao *et al.*, 2015; Weber *et al.*, 2017) (Fig. 1D).

Other oxacillinases, such as OXA-51, have been shown to be chromosomally encoded in some *A. baumannii* strains (Benjamin and Sevastian, 2014). Several OXA-23-like genes (OXA-23, -24, -102, -103, -105, -133, and -134) and OXA-51/69-like genes have been found to be encoded in the chromosomes of *A. radioresistens* and *A. baumannii* isolates, respectively (Boo and Crowley, 2009; Poirel *et al.*, 2011). Their presence in different *Acinetobacter* species indicates that OXA β-lactamases are essential components in the genetic makeup of the *Acinetobacter* genus (Benjamin and Sevastian, 2014). Outbreaks of *A. baumannii* harboring OXA β-lactamases have been reported worldwide, reflecting the rapid dissemination of these particular β-lactamases (Benjamin and Sevastian, 2014; Liao *et al.*, 2015).

Aminoglycoside-modifying enzymes (nucleotidyltransferases, acetyltransferases, and/or phosphotransferases) have also been detected in most aminoglycoside-resistant clinical *Acinetobacter* species (Doi *et al.*, 2004; Atasoy *et al.*, 2015). Genes encoding aminoglycoside-modifying enzymes can be divided into the phosphotransferases (*aph* (*3′*)*-Ia*, *aph* (*3′*)*-VIa*, *aph* (*3′*)*-II)*, acetyltransferases *(aac* (*3*)*-Ia, aac* (*3*)*-IIa*, *aac* (*6′*)*-Ib, aac* (*6′*)*-Ih*, *aac* (*6′*)*-Iad*, *aac* (*6′*)*-Im*, and *aac* (*6′*)*-II)*, and nucleotidyltransferases (*ant* (*2′′*)*-Ia*, *ant* (*3′′*)*-Ia*, and *ant* (*3′′*)*-Id*) (Atasoy *et al.*, 2015; Stogios *et al.*, 2017). In some *Acinetobacter* species, quinolone drugs can be modified by acetyltransferases (AAC), commonly AAC(6′)-Ib-cr. Although the involvement of chloramphenicol acetyltransferase (CAT) in AMR in *Acinetobacter* has not been fully explained, most CAT genes have been found either chromosomally or on plasmids in clinical *Acinetobacter* isolates such as *catI* of *A. baumannii* in Tn2670; *cat* of *A. calcoaceticus* in Tn2670-like transposon; and *catB3* of *A. baumannii* in chromosomes (Schwarz *et al.*, 2004).

Non-enzymatic antibiotic resistance

Non-enzymatic AMR to antibiotics is linked to biofilm formation, changes in the affinity or composition of the cell membrane, and decreased expression of influx proteins such as porins (Fig. 1). The common structural components of most microbial biofilms are exopolysaccharides, proteins, and nucleic acids. Biofilm formation on biotic or abiotic surfaces play an important role in the ability of AMR microorganisms to persist in the environment by reducing the effective concentration of antibacterial compounds. Surface colonization of hospital equipment and medical devices by pathogenic *Acinetobacter* species can cause nosocomial infections (Djeribi *et al.*, 2012). On polystyrene, a medically relevant surface, the production of pili by clinical strains is essential for biofilm formation. Csu proteins are responsible for type I pilus production, which has proven to be important for biofilm formation and virulence in *Acinetobacter* species. The role of pili produced by the *csuA/BABCDE* six open reading frames in biofilm formation on glass and polystyrene has been reported in *A. baumannii* ATCC19606 strain (Tomaras *et al.*, 2003).

For an antibacterial drug to be effective against Gram-negative bacteria, the antibiotic must penetrate the extracellular biofilm matrix, whose main components are acidic exopolysaccharides (EPSs). Poly-*N*-acetylglucosamine (PNAG), which is produced in a biofilm-dependent manner, is synthesized from the *pgaABCD* locus and is an important EPS component for emerging pathogens such as *A. baumannii* (Choi *et al.*, 2009) (Fig. 1D). However, few studies have examined the regulation and synthesis of PNAG in *Acinetobacter*. In addition, the detailed mechanisms by which secondary messengers such as ppGpp and c-di-GMP are linked to biofilm formation remain undefined for *A. baumannii*. Transcriptomic and enzymatic studies have suggested that PNAG production under static conditions is specific to pellicle formation (Nait Chabane *et al.*, 2014). Another study, using a soil *Acinetobacter* strain, showed that oxidative stress causes the *pga*ABCD locus to become active, resulting in increased biofilm formation (Jang *et al.*, 2016). However, the link between PNAG and resistance to various antibiotics remains unclear.

In addition, the production of other defensive surface polysaccharides, referred to as K-locus polysaccharides, is known to protect against killing by host serum and increases bacterial virulence in animal models (Geisinger and Isberg, 2015). This K-locus-dependent capsule production, which occurs upon exposure to antibiotics, is controlled by the two-component regulatory system, *bfmRS* (Geisinger and Isberg, 2015). BfmR is negatively regulated via phosphorylation by BfmS. When the phosphoregulation of BfmR is relieved under antibiotic stress, K-locus expression increases (Geisinger and Isberg, 2015). The capsular polysaccharide appears to play an important role in protecting bacteria and is a critical virulence factor that enables immune evasion (Wong *et al.*, 2017), though how the K-locus contributes to intrinsic antibiotic resistance is poorly understood. The outer membrane also offers a much higher level of defense against antimicrobial agents because of its LPS-coated surface, which significantly limits the diffusion of many compounds, thereby lowering its permeability for hydrophobic agents (Nikaido, 2003).

Table 2. **Antibiotic and non-antibiotic combination therapy producing synergistic effects against pathogenic *Acinetobacter* since 2010**

Name	Combination antibiotics	Type of infection	Methods	References
Antibiotics				
Colistin	chloramphenicol	XDR	*in vitro*	Wei and Yang (2017)
	rifampicin, meropenem, azithromycin, doxycycline	MDR	*in vitro*	Timurkaynak *et al.* (2006), Tangden *et al.* (2017)
	rifampicin	CI, CR	*in vivo* (patients)	Durante-Mangoni *et al.* (2013), Aydemir *et al.* (2013)
	rifampin, teicoplanin	CO	*in vitro*	Bae *et al.* (2016)
	carbapenem	XDR	*in vivo* (patients)	Shields *et al.* (2012)
	meropenem, tigecycline, fosfomycin, fusidic acid, rifampin, sulbactam	XDR	*in vivo* (mouse)	Fan *et al.* (2016)
	doripenem	XDR	*in vitro*	Shields *et al.* (2011) Maifiah *et al.* (2017)
	carbapenem, piperacillin-tazobactam, sulbactam or other	MDR	*in vivo* (patients)	Falagas *et al.* (2010)
	vancomycin	MDR	*in vitro*	Gordon *et al.* (2010)
	fosfomycin	CI	*in vivo* (patients)	Petrosillo *et al.* (2014)
	carbapenem, sulbactam, tigecycline or other	XDR	*in vivo* (patients)	Batirel *et al.* (2014)
	teicoplanin, vancomycin	MDR	*in vitro*	Hornsey and Wareham (2011)
	trimethoprim-sulfamethoxazole	CR	*in vitro*	Nepka *et al.* (2016)
Polymyxin B	doripenem and/or rifampin	CR	*in vitro*	Urban *et al.* (2010)
	minocycline	CI	*in vivo* (mouse)	Bowers *et al.* (2015)
	rifampicin, tigecycline	CR	*in vitro*	Lim *et al.* (2011)
	tigecycline	CR	*in vitro*	Hagihara *et al.* (2014)
	minocycline, fosfomycin	MDR	*in vitro*	Zhang *et al.* (2013)
Meropenem	rifampicin	MDR	*in vitro*	Sun *et al.* (2014)
	novel monosulfactam BAL30072	CR	*in vitro*	Hornsey *et al.* (2013)
Doripenem	levofloxacin, amikacin, colistin	CI	*in vitro*	Pankuch *et al.* (2010)
	sulbactam, amikacin, colistin, tigecycline	CR	*In vitro*	Dinc *et al.* (2015)
Minocycline	amikacin, rifampicin	MDR	*in vitro*	He *et al.* (2015)
	rifampicin, colistin, imipenem	MDR	*in vitro*	Rodríguez *et al.* (2015)
	meropenem	XDR	*in vitro*	Liang *et al.* (2011)
	colistin	MR	in virto	Yang *et al.* (2016)
Tigecycline	ceftazidime, ceftriaxone, piperacillin/tazobactam, carbapenem	MDR	*in vivo* (patients)	Lee *et al.* (2013)
	amikacin, colistin	TR	*in vitro*	Li *et al.* (2017)
	Meropenem, ampicillin, sulbactam	CI (PR)	*in vitro*	Lenhard *et al.* (2017)
	colistin	XDR, CI	*in vitro*	Yilmaz *et al.* (2015), Cai *et al.* (2017)
	imipenem, sulbactam, colistin	MDR	*in vitro*	Pachon-Ibanez *et al.* (2010)
Sulbactam	colistin (tigecycline shows a weak effect)	CR	*in vivo* (mouse)	Dinc *et al.* (2013)
	ampicillin, carbapenem, cefoperazone	CI	*in vivo* (patients)	Chu *et al.* (2013)
	meropenem, ciprofloxacin, amikacin, tigecycline, colistin	CR	*in vitro*	Temocin *et al.* (2015)
	colistin	MDR, XDR	*in vivo* (patients)	Yilmaz *et al.* (2015)
Non-antibiotics				
Polyamine	aztreonam	CR	*in vitro*	Malone and Kwon (2013)
Epigallocatechin-3-Gallate (EGCG)	meropenem, carbenicillin, chloramphenicol, tetracycline	CI	*in vitro*	Lee *et al.* (2017)
Essential oils	iprofloxacin, gentamicin, polymyxin B	MDR	*in vitro*	Knezevic *et al.* (2016)
Essential oils	polymixin B, ciprofloxacine	MDR	*in vitro*	Aleksic *et al.* (2014)
Essential oils (Aniba rosaeodora and Pelargonium graveolens)	gentamicin	RS	*in vitro*	Rosato *et al.* (2010)
Coriander oil	ciprofloxacin, gentamicin, chloramphenicol, tetracycline	RS	*in vitro*	Duarte *et al.* (2012)
Carvacrol (oregano oil), eugenol (clove oil), and cinnamaldehyde (cinnamon oil).	doxycycline	CI	*in vitro*	Valcourt *et al.* (2016)
Holarrhena antidysenterica extracts	rifampicin, cefazolin	XDR, MDR	*in vitro*	Chusri *et al.* (2014a)

Table 2. Continued

Name	Combination antibiotics	Type of infection	Methods	References
Apocynaceae extracts	rifampicin, cefazolin	XDR, MDR	*in vitro*	Chusri *et al.* (2014b)
Silver nanoparticles (AgNPs)	polymixin B, rifampicin	CR	*in vivo* (mouse)	Wan *et al.* (2016)
	oregano essential oil*	MDR	*in vitro*	Scandorieiro *et al.* (2016)
	piperacillin, eryth-romycin	MDR	*in vitro*	Ghosh *et al.* (2012)
	chitosan acetate*	MDR	*in vivo* (mouse)	Huang *et al.* (2011)
Zinc oxide nanoparticles (ZnO-NPs)	ciprofloxacin and ceftazidime	MDR	*in vitro*	Ghasemi and Jalal (2016)
Nanoscaled titania (nano TiO2)	new isomeric carborane derivatives (a pair of geometrical isomers ferrocene-carborane derivatives)	MDR	*in vitro*	Li *et al.* (2013)
Light (635 nm, 600 J/cm^2)	chitosan	RS	*in vitro*	Tsai *et al.* (2011)
Light (400–1,000 nm, 20,000–40,000 J/cm^2)	colistin	CR	*in vitro*	Boluki *et al.* (2017)
φkm18p	φTZ1 and φ314	XDR	*in vitro*	Shen *et al.* (2012)
LysABP-01 from ØABP-01	colistin	RS	*in vitro*	Thummeepak *et al.* (2016)
Artilysins	polycationic nonapeptide	CI	*in vitro*	Briers *et al.* (2014)

* Double combination between non-antibiotic compounds.
MR, meropenem-resistant; CO, Colistin-Resistant; IR, imipenem-resistant; CI, Clinical isolate; CR, Carbapenem-resistant; TR, Tigecycline-resistant; PR, Polymyxin B-resistant; MDR, Multidrug-resistant; XDR, Extremely drug-resistant

Porins are β-barrel membrane proteins that cross cell membranes and act as pores through which molecules such as nutrients, toxins, and antibiotics can pass. The alteration, modification, and reduction of the expression of porins are related to antibiotic resistance (Sugawara and Nikaido, 2012). Using *in vivo* protein interaction network analysis, a recent report on outer membrane porins from a clinical isolate of *A. baumannii* revealed that a significant amounts of oxacilinases interact with outer membrane porins including OmpA, CarO, and OmpW (Wu *et al.*, 2016). The *A. baumannii* OmpA protein was recently reported to be associated with resistance to cephalothin and cephaloridine in *A. baumannii* (Sugawara and Nikaido, 2012). It has been also reported that the inactivation of CarO causes increased resistance to carbapenems in *A. baumannii* strains (Hood *et al.*, 2010). The overall results indicate that CarO participates in the selective uptake of L-ornithine, carbapenems, and other basic amino acids in *A. baumannii* (Mussi *et al.*, 2007). The interaction of these functional porins with protective enzymes can be understood as a general advantageous strategy against antibacterial compounds (Wu *et al.*, 2016).

In vitro-selected mutants resistant to cefoxitin, cefoperazone, or ceftazidime also exhibit reduced expression of porins and changes in PBP expression and/or the affinity for β-lactams in *A. calcoaceticus* (Obara and Nakae, 1991). Similarly, proteome analysis was performed in an imipenem-resistant *A. baumannii* strain under imipenem treatment conditions (Yun *et al.*,. 2011). When imipenem-resistant *A. baumannii* was treated with imipenem, the induction of RND family transporters (AdeABC and AdeJIK), a protein kinase (BfmS), and PBPs, as well as reduction of the OMPs OmpA and OmpW was observed. These results suggest that these mechanisms together contribute to imipenem resistance in *A. baumannii*.

Treatment of *Acinetobacter* infections

To treat infection of MDR pathogens, a series of new quinolones such as levofloxacin, ciprofloxacin, moxifloxacin, ofloxacin, and gemifloxacin have been developed as antibacterial drugs (Jiang *et al.*, 2014; Blair *et al.*, 2015). However, resistance to new antibiotics arises quickly. Thus, new therapeutic methods are needed to combat MDR pathogens. The development of a new single drug is costly and time-consuming, thus studies have focused on combination treatments. Despite the prevalence of antibiotic resistance, the current body of research is too small to allow clinicians to select an optimal treatment for *A. baumannii* infections. To combat serious *A. baumannii*-associated infections, various trials have been conducted using antibiotics, bacteriophages, plant extracts, and nanoparticles (Djeribi *et al.*, 2012) (Table 2). *In vitro* and *in vivo* studies have shown that combinations of drugs may be synergic and highly bactericidal when used against clinical isolates of drug-resistant *A. baumannii* (Table 2). Such synergic combinations typically include two or three different classes of antibiotics. It has already been shown in many studies that the effect of antibiotics is increased by the addition of other antimicrobial agents such as chimeric peptides (Gopal *et al.*, 2014). Advantages of combination therapies include a broad spectrum of antibacterial coverage, synergistic effects between different antimicrobial compounds, and the prevention of resistance (Lutsar *et al.*, 2014). To summarize the extensive *in vitro* and *in vivo* information shown in Table 2, recent combination experiments have primarily focused on polymyxins, sulbactam, tigecycline, and rifampin or carbapenems.

Polymyxins have been successfully used as an aerosol to treat ventilator-associated pneumonia caused by *A. baumannii*, with lower nephrotoxicity than predicted (Urban *et al.*, 2001; Gales *et al.*, 2006). Polymyxin E (colistin) is present in two forms, colistin sulfate for oral and topical use and colistin sulfomethate sodium for parenteral use, with the latter being a non-active prodrug used for parenteral administration because of its lower toxicity. Polymyxins are cationic polypeptides that interact with LPS molecules in the outer cell membranes of Gram-negative bacteria. *A. baumannii* strains

are susceptible to polymyxins, which are toxic to humans, and therefore therapies that combine polymyxins with other antibiotics to decrease their side effects by reducing the dose have already been used to treat *A. baumannii* infections in humans (Falagas *et al.*, 2010). The *in vitro* synergism of colistin with carbapenem, rifampicin, tigecycline and other antibiotics against *A. baumannii* has been reported (Table 2). In addition, the *in vivo* results reported for solid organ transplant recipients who were colonized by or infected with extremely drug-resistant (XDR) *A. baumannii* suggest that a combination of colistin and carbapenem improves clinical responses and survival compared to other therapies such as colistin-tigecycline and may also limit the emergence of colistin resistance (Shields *et al.*, 2012) (Table 2). The combination colisin and rifampicin in the treatment of ventilator-associated pneumonia has also demonstrated improved clinical outcomes (Aydemir *et al.*, 2013) (Table 2). However, 30-day mortality was not reduced by the addition of rifampicin to colistin despite the increased mortality rates of *A. baumannii* cells (Durante-Mangoni *et al.*, 2013) (Table 2).

Of the β-lactamase inhibitors, sulbactam has shown the strongest intrinsic bactericidal activity against *A. baumannii* isolates. The results of clinical investigations have documented the efficacy of sulbactam in mild-to-severe *A. baumannii* infections, and sulbactam has been successfully used to treat infections associated with MDR *A. baumannii* such as meningitis, ventilator-associated pneumonia, and catheter-related bacteremia (Temocin *et al.*, 2015). In most patients, sulbactam has been used in combination with ampicillin and carbapenems but its usefulness is increasingly compromised by bacterial resistance. A recent study on the use of tigecycline against XDR *A. baumannii* infections analyzed 120 patients who were treated with imipenem and sulbactam (Lee *et al.*, 2013) (Table 2). Even if the dosage is reduced in combination therapies, the prolonged use of antibiotics can disturb the normal gastrointestinal microflora, reducing the natural defense mechanisms provided by the microbial exosystem in the colon and making the host susceptible to infection by symbiotic microorganisms or nosocomial pathogens (Rafii *et al.*, 2008). Despite conflicting evidence of their efficiency due to each toxicity, complex antibiotics with different mechanisms of action are commonly used in severe infections.

Other possible approaches to treat pathogenic *Acinetobacter* infection

To overcome the risks inherent to the use of antibiotics, the number of other available combination treatments needs to be expanded. The extracts of plants used in traditional medicine have been used to reduce the side effects of antibiotic resistance and combination therapy (Tiwari *et al.*, 2015). The ineffectiveness of synthetic antibiotics against drug-resistant bacteria has led to the reemergence of interest in silver and other non-antibiotics that have a long history as antibacterial agents. Oleanolic acid and ursolic acid can act synergistically in combination with aminoglycosides to increase energy and membrane permeability (Shin and Park, 2015). The antimicrobial activity of essential oils, polyamines and nanoparticles has been intensively explored, mainly in the course of developing new antimicrobial agents to overcome microbial resistance (Malone and Kwon, 2013; Wan *et al.*, 2016; Chaib *et al.*, 2017) (Table 2). Some compounds in plants are potently antimicrobial when used against *A. baumannii*. For examples, traditional medical herb extracts from *L. salicaria* exhibit antibacterial activity against *A. baumannii* (Guclu *et al.*, 2014). In addition, essential oils from *Syzygium aromaticum*, *Cinnamomum zeylanicum*, and *Thymus* are effective as nanomedicines against multidrug-resistant *A. baumannii* (Tiwari *et al.*, 2015). Compounds from *Scutellaria baicalensis* such as baicalin and baicalein have also been reported to have antibacterial properties (Tiwari *et al.*, 2015). Studies have shown that many phenolic compounds derived from plant extracts enhance the effectiveness of synthetic antibiotics against *A. baumannii in vitro* (Tiwari *et al.*, 2015).

The antibacterial effects of polyamines (spermine and spermidine), which are small molecular compounds with positively charged amine groups existing at millimolar levels in all living cells, against carbapenem-resistant *A. baumannii* increase significantly when used in conjunction with aztreonam (Malone and Kwon, 2013). The activity of many natural substances against *A. baumannii* has been tested, but the exact mechanisms by which certain compounds act in synergy with each other have not been defined and further research is needed. A combination therapy approach to the development of chemically modified synthetic substances based on the structure of natural active compounds derived from medicinal herbs may be a novel form of treatment for the MDR and XDR strains of *A. baumannii*.

Bacteriophage has been considered as an possible option for infectious disease, but research on *Acinetobacter* was mainly about transduction, phage typing or phage classification (Shen *et al.*, 2012). Despite the large number of publications on phage therapy, little is known about its antibiotic combination therapy against *A. baumanii*. Recently, bacteriophages and their endolysins have been recognized as alternative therapeutic agents that can combat drug-resistant bacterial infections. For example, in *A. baumannii*, the synergistic antibacterial effect of φkm18p has been only observed in combination with other phages such as φTZ1 and φ314 (Shen *et al.*, 2012) (Table 2). Some *Acinetobacter* genus are known to sense and respond to light, which can modulate metabolic pathways including phenylacetic acid, trehalose, acetoin, and also change lipid metabolism, virulence, and resistance to antibiotics (Ramírez *et al.*, 2015). It was revealed that light can reduce susceptibility to minocycline and tigecycline and modulate motility, biofilm formation, and virulence through the blue-light-sensing-using flavin photoreceptor BlsA (Muller *et al.*, 2017). However, photodynamic inactivation (PDI) which uses nontoxic chemicals with harmless visible light was shown to be a promising treatment method against *A. baumannii* (Tsai *et al.*, 2011). Several studies also suggested that effectiveness of PDI for killing Gram-negative bacteria could be promoted in the presence of some antibiotics including polymyxin B (Tsai *et al.*, 2011). Interestingly, chitosan alone has no significant antimicrobial activity, but the PDI treatment with chitosan effectively killed many Gram-negative pathogenic bacteria. The PDI appeared to have a synergistic effect with colistin for killing a pan-drug resistant strain of *A. baumannii* isolated from burn patients (Boluki

et al., 2017). To date, little is known about the PDI combination treatment antibacterial compounds against *A. baumannii* (Table 2). Antimicrobial combination therapy including the PDI appears to be a reasonable alternative in the treatment of MDR *A. baumannii*. However, clinical experience with combination therapy is also limited. Developing a sufficient volume of usable natural compounds may be an obstacle to the development of new medicines as the supply should be large enough to first support initial *in vitro*/*in vivo* studies.

Concluding Remarks

More than half a century has passed since the first antibiotics were introduced commercially. Microbes quickly developed drug resistance to these original antibacterial drugs. Multidrug resistance is now a worldwide problem that does not recognize international borders. Although conventional antibiotics have proven efficacious in the treatment of bacterial infections, non-antibiotics such as nanoparticles and herbal medicines may offer alternative treatment options. Because MDR is increasing, clinicians require a novel form of treatment for resistant bacteria such as *A. baumannii*. There is a need to develop new drugs that can potentially be used to treat infections caused by currently resistant *Acinetobacter* pathogens (Lutsar *et al.*, 2014). Non-antibiotics such as silver, ethanol extracts of something such as plants, and tea tree oil are currently available as topical formulations (Dai *et al.*, 2010), and these products may in the future play a role in reducing the toxicity of antibiotics to human organs and other serious side effects.

Some herbal extracts and compounds, such as those from green tea, are known to be less toxic to native gastrointestinal flora (Cabrera *et al.*, 2006). If a synergistic combination of compounds removes an infection more efficiently, the duration of treatment may be shortened and associated antibiotic toxicity may be reduced. Thus, the more efficient killing of microbes may decrease the risk of progressively increasing antibiotic resistance. In the future, a detailed understanding of both the conditions under which resistance occurs and the synergy arising from various combinations of promising compounds will help clinicians to develop more efficient dosing regimens that minimize resistance to current and new antibiotics. As interest in antibiotic resistance increases, members of the genus *Acinetobacter,* which are ubiquitous in hospitals and natural environments, have emerged as model bacteria to study of sharing genes horizontally through conjugation, transformation, and transduction (Jung and Park, 2015). The transfer of virulence genes from a non-pathogenic bacterial strain to a pathogenic strain may result in the emergence of potentially powerful new pathogens which can gain antibiotic resistance from environmental bacteria (Beceiro *et al.*, 2013). The increase in MDR and pan-drug resistant *A. baumannii* is a growing concern, and the production of new antibiotics, including host-defense peptides, is therefore urgently required. The molecular mechanisms of drug resistance need to be described so that this information can be used in the development of new drugs and subsequent clinical use.

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