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Antibiotic Resistance in *Burkholderia* Species

Katherine A. Rhodes[1,2] and **Herbert P. Schweizer**[1,*]

[1]Department of Molecular Genetics and Microbiology, College of Medicine, Emerging Pathogens Institute and Institute for Therapeutic Innovation, University of Florida, Gainesville, FL, USA

[2]Department of Microbiology Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

Abstract

The genus *Burkholderia* comprises metabolically diverse and adaptable Gram-negative bacteria, which thrive in often adversarial environments. A few members of the genus are prominent opportunistic pathogens. These include *B. mallei* and *B. pseudomallei* of the *B. pseudomallei* complex, which cause glanders and melioidosis, respectively. *B. cenocepacia*, *B. multivorans*, and *B. vietnamiensis* belong to the *B. cepacia* complex and affect mostly cystic fibrosis patients. Infections caused by these bacteria are difficult to treat because of significant antibiotic resistance. The first line of defense against antimicrobials in *Burkholderia* species is the outer membrane penetration barrier. Most *Burkholderia* contain a modified lipopolysaccharide that causes intrinsic polymyxin resistance. Contributing to reduced drug penetration are restrictive porin proteins. Efflux pumps of the resistance nodulation cell division family are major players in *Burkholderia* multidrug resistance. Third and fourth generation β-lactam antibiotics are seminal for treatment of *Burkholderia* infections, but therapeutic efficacy is compromised by expression of several β-lactamases and ceftazidime target mutations. Altered DNA gyrase and dihydrofolate reductase targets cause fluoroquinolone and trimethoprim resistance, respectively. Although antibiotic resistance hampers therapy of *Burkholderia* infections, the characterization of resistance mechanisms lags behind other non-enteric Gram-negative pathogens, especially ESKAPE bacteria such as *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*.

Keywords

Burkholderia; *Burkholderia cepacia* complex; glanders; melioidosis; antibiotics resistance

1. Introduction

Antimicrobial resistance is rapidly becoming an unavoidable public health crisis with the potential to radically alter the standard of medical care across the globe (Brown and Wright,

*Corresponding author: Department of Molecular Genetics and Microbiology, College of Medicine, Emerging Pathogens Institute, University of Florida, 2055 Mowry Road, Gainesville, FL 32610, USA, hschweizer@ufl.edu, Tel. 352-273-9402.

2016; Lushniak, 2014; Michael et al., 2014). Without an increased understanding of the mechanisms that drive resistance to therapy for bacterial infection, especially those considered drugs of last resort, the treatment obstacles posed by these factors could ultimately prove insurmountable. If current resistance trends continue and the pipeline for new drugs with activity against Gram-negative remains stagnant, infections caused by these organisms possess the most potential for complete emergence into a post-antibiotic state. Species within this group have been established as organisms of concern in both environmental and nosocomial settings, with members of the *Pseudomonas*, *Acinetobacter*, *Klebsiella* and *Burkholderia* genera already known to be intransigent to standard first-line therapy as a result of both acquired and intrinsic resistance factors (Boucher et al., 2009; Michael et al., 2014). Many of these factors are found in all four clades and some members, including *P. aeruginosa*, *A. baumannii* and *K. pneumonia*, are ESKAPE bacteria (Boucher et al., 2009).

The *Burkholderia* genus is large clade within the β-proteobacteriaceae class, containing over 70 species (Sawana et al., 2014). Of these, only *B. mallei* is considered an obligate parasite of eukaryotic hosts, while the rest are found as environmental saprophytes (Galyov et al., 2010). Despite this, many species other than *B. mallei* are opportunistic pathogens and capable of causing disease. *B. pseudomallei* is the etiologic agent of a serious and often fatal syndrome known as melioidosis (Cheng and Currie, 2005; Limmathurotsakul and Peacock, 2011; Peacock, 2006; Wiersinga et al., 2012; Wiersinga et al., 2006). The *B. cepacia* complex (BCC) consists of a group of at least 17 species (Mahenthiralingam et al., 2005; Mahenthiralingam and Vandamme, 2005; Vandamme and Dawyndt, 2011; Vanlaere et al., 2009). Several members of this complex including *B. cenocepacia, B. multivorans*, and *B. vietnamiensis*, are opportunistic pathogens. These organisms are particularly problematic in patients suffering from cystic fibrosis, and are responsible for high mortality rates within this patient cohort (Drevinek and Mahenthiralingam, 2010; Jassem et al., 2011). An underlying theme within the genus is the ability to evade the action of multiple classes of antimicrobials, a capacity that is partially responsible for the seriousness of infections caused by its member species (Dance, 2014; Golini et al., 2004; Jassem et al., 2011; Peeters et al., 2009; Rajendran et al., 2010; Schweizer, 2012a; Wuthiekanun and Peacock, 2006).

2. Bacterial antibiotic resistance factors

Before we review antibiotic resistance in *Burkholderia* species, it is prudent to present a brief overview of the factors that govern bacterial resistance. This will provide a perspective of the commonalities and differences of the types of resistance determinants that *Burkholderia* species employ when compared to other bacteria, especially other Gram-negatives.

Three types of mechanisms contribute to antimicrobial resistance in bacteria. Intrinsic antibiotic resistance is caused by physicochemical properties of a bacterium that are not subject to genetic change in response to antibiotic exposure. Possibly the best recognized example of intrinsic resistance is exclusion of drug molecules from Gram-negative bacteria by constraints of the cell envelope, mostly the outer membrane and its lipopolysaccharide and porin constituents. Acquired resistance is caused by acquisition of a previously absent

resistance trait, for instance mutation of a chromosomally encoded target, transfer of foreign resistance genes via mobile plasmids, phage-mediated transduction, and transformation using free DNA (Fig. 1). Mutations of a regulatory element for a normally not expressed resistance trait such as an enzyme, an efflux pump, etc., also contribute to acquired resistance.

Bacteria can exhibit tolerance to antimicrobials that is either dependent or independent of genetic change. This includes bacterial lifestyle adaptations, for instance planktonic versus biofilm growth, intracellular anaerobiosis or persister cell formation (Balaban et al., 2013; Mah, 2012; Monroe, 2007).

An overview of mechanisms that bacteria use to resist antibiotics is presented in Fig. 2. (Blair et al., 2015; Walsh and Wencewicsz, 2016). They include the following: 1) Exclusion from or reduced penetration into the cell by constraints mediated by the cell envelope; 2) Active efflux from the cell; 3) Enzymatic inactivation, either by substrate cleavage or chemical modification, for instance acetylation, adenylation, glucosylation and phosphorylation; 4) Target alteration by point mutations or, rarely, deletion. Targets can also be enzymatically modified, for instance by methylation of ribosomal RNA; 5) Metabolic bypass by substitution of a susceptible target with a resistant target; 6) Target overproduction by either increased transcription or gene multiplication; and 7) Drug sequestration by specific binding proteins (Schweizer, 2012a; Walsh and Wencewicsz, 2016).

Bacteria frequently employ disparate mechanisms that act synergistically to achieve elevated resistance. The often high-level acquired or intrinsic resistance of non-enteric bacteria such as *P. aeruginosa* and *Burkholderia* species is in no small part attributable to synergy between reduced penetration into and efflux from the cell (Schweizer, 2012b).

In this review we will provide an overview of major resistance mechanisms described in select pathogenic members of the genera, specifically the *Burkholderia pseudomallei* complex (Bpc) and the *Burkholderia cepacia* complex (Bcc).

3. Burkholderia pseudomallei complex organisms

Members of the Bpc consist of *B. pseudomallei, B. mallei, B. humptydoensis* and *B. thailandensis* and are characterized by their similarities to *B. pseudomallei* at a genetic and phenotypic level (Brett et al., 1998; Ginther et al., 2015; Holden et al., 2004; Nierman et al., 2004; Yu et al., 2006). These organisms are thought to share a common progenitor, most likely very similar to *B. pseudomallei* itself. *B. mallei* is considered a clone of *B. pseudomallei* having diverged from the latter in an animal host approximately 3.5 million years ago and developed into an obligate pathogen by in-host evolution (Losada et al., 2010; Song et al., 2010). Both *B. pseudomallei* and *B. mallei* are capable of causing serious disease (Galyov et al., 2010). *B. thailandensis*, although generally considered non-pathogenic, is known to cause sporadic human disease (Glass et al., 2006).

Mortality rates of *B. pseudomallei* infections still can exceed 50% without initiation of rapid and appropriate antibiotic therapy, especially in patients with underlying risk factors such as diabetes (Cheng and Currie, 2005; Limmathurotsakul and Peacock, 2011; Peacock, 2006;

Wiersinga et al., 2012; Wiersinga et al., 2006). The majority of the resistance factors encoded by *B. pseudomallei* are also found in *B. mallei*, and *B. thailandensis.* However, *B. mallei* is generally more susceptible to antibiotics than *B. pseudomallei*, presumably because ongoing in-host evolution leads to genome reduction, including loss of antibiotic resistance determinants (Nierman et al., 2004).

Glanders, caused by *B. mallei*, is a rare but frequently fatal infection mostly affecting solipeds but occasionally also humans (Whitlock et al., 2007). The organism is known to have been weaponized during both modern and ancient conflicts, most likely because of its potentially devastating effect on cavalry horses and pack animals (Cheng et al., 2005; Dance, 2005; Van Zandt et al., 2013). Few modern human cases have been documented, but symptoms in both animals and humans range from suppurating abscess of the mucosa and solid organs, to sepsis, and pneumonia, depending on the route of infection (Van Zandt et al., 2013).

Melioidosis, caused by *B. pseudomallei*, is characterized by a variety of symptoms ranging from self-limiting abscess, to sepsis, necrotizing pneumonia, osteomyelitis, and dissemination to the solid organs and brain (Bartley et al., 1999; Caldera et al., 2013; Currie et al., 2010; Jane et al., 2012; Maguire et al., 1998; McLeod et al., 2015; Morse et al., 2013; St John et al., 2014; Wiersinga et al., 2012). Current recommended therapy for melioidosis consists of two weeks of intravenous ceftazidime or a carbapenem, followed by up to six months of oral trimethoprim+sulfamethoxazole (co-trimoxazole)(Chetchotisakd et al., 2014; Dance, 2014; Lipsitz et al., 2012; Pitman et al., 2015). In either phase of therapy, the administration of amoxicillin+clavulanate (co-amoxiclav) is used in instances where other drugs are contraindicated (Dance, 2014; Lipsitz et al., 2012; McLeod et al., 2015). Based largely on the similarity of the *in vitro* antibiotic susceptibility patterns of *B. mallei* and *B. pseudomallei* treatment of glanders in humans follows a similar scheme (Kenny et al., 1999; Lipsitz et al., 2012; Peacock et al., 2008).

3.1 β-lactam resistance in Bpc bacteria

Treatment failure during the administration of ceftazidime occurs in approximately 11–17 percent of clinical cases, although it was found that only a small minority of these cases were due to primary resistance when isolates are assessed using *in vitro* methods (Chierakul et al., 2005; Wuthiekanun and Peacock, 2006). A survey of over 4,000 *B. pseudomallei* patient isolates obtained over a period spanning 20 years showed that primary resistance to β-lactam antibiotics is rare, but does exist. For instance, 24 of 4,021 (or 0.6%) of isolates were resistant to ceftazidime (n=8), amoxicillin+clavulanic acid (n=8) or both drugs (n=13). None of the isolates was resistant to carbapenems.

A Class A β-lactamase encoded by *penA* located on chromosome 2 is responsible for primary resistance to β-lactam antibiotics in the majority of clinical *B. pseudomallei* isolates (Godfrey et al., 1991)(Fig. 3). Two types of mutations have been implicated in PenA-mediated ceftazidime resistance in clinical *B. pseudomallei* isolates. The majority of these cause changes to or near conserved β-lactamase domains, the so-called Ambler motifs (Ambler et al., 1991). Notable amino acid substitutions implicated in β-lactam resistance in clinical isolates include Cys69Tyr, Ser72Phe and Pro167Ser, which cause ceftazidime

(Cys69Tyr and Pro167Ser) and clavulanic acid (Ser72Phe) resistance, respectively (Rholl et al., 2011; Sam et al., 2009; Sarovich et al., 2012a; Sarovich et al., 2012b; Tribuddharat et al., 2003). Several strains have been identified that contain amino acid substitutions leading to simultaneous ceftazidime and clavulanate resistance, thereby rendering β-lactamase inhibitors of the clavulanate family ineffective in their presence. Some ceftazidime resistant clinical *B. pseudomallei* isolates also contain a point mutation in the *penA* upstream region, which probably causes an increased expression of PenA via increased *penA* transcription (Sarovich et al., 2012b). This notion is supported by results of a recent study with *in vitro* selected ceftazidime resistant *B. thailandensis* mutants (Yi et al., 2012a). Treatment of ceftazidime resistant *B. pseudomallei* is still possible as these isolates remain susceptible to carbapenems. A recent study characterizing *B. pseudomallei* PenA found that it is a membrane bound lipoprotein, secreted by the twin-arginine transport (TAT) system (Randall et al., 2015; Rholl et al., 2011). The unique membrane association of the enzyme could be a productive route of inquiry as investigative drugs capable of targeting lipoprotein synthesis, as well as TAT-mediated protein export may exhibit efficacy against ceftazidime resistant *penA* strains. Indeed, *tat* mutants are susceptible to β-lactam antibiotics and TAT inhibitors could potentially be used to sensitize *B. thailandensis* to β-lactams (Rholl et al., 2011; Vasil et al., 2012).

Both *B. mallei* and *B. thailandensis* express a conserved PenA Class A β-lactamase, and therefore can be anticipated to exhibit similar molecular properties and phenotypes (Winsor et al., 2008). Most observations made with *B. pseudomallei* β-lactamase have been corroborated by *in vitro* studies with *B. thailandensis* (Yi et al., 2012a; Yi et al., 2012b). Studies on PenA β-lactamase expression and its contribution to β-lactam resistance are hampered by the diversity of potential functionally important PenA structural features and factors governing *penA* transcription in sequenced *B. pseudomallei* strains. While we are beginning to understand the roles that structural features of PenA play in its function and substrate profile, virtually nothing is known about factors governing the enzyme's expression. Reliable information about the role of PenA in clinically significant β-lactam resistance continues to be obtained by analysis of isogenetic pre- and post-ceftazidime therapy *B. pseudomallei* patient isolates (Tribuddharat et al., 2003; Sam et al., 2009; Sarovich et al., 2012a; Sarovich et al., 2012b).

β-lactam resistance in this organism can also occur as the result of large scale rearrangements at the chromosomal level. In a 2011 study examining ceftazidime treatment failures in a Thai hospital, several *B. pseudomallei* isolates were found to have deleted large segments of chromosome 2. All isolates encompassed deletion of a common 71 kb segment (Chantratita et al., 2011). This segment contained three genes encoding putative penicillin-binding proteins (PBPs), which are known targets for β-lactam antibiotics. Of the two genes encoding PBP3s and one gene encoding a putative PBP6, one PBP3 homolog was shown to be responsible for the severe growth defect manifested as a filamentous appearance by microscopy, as well as high-level ceftazidime resistance (Chantratita et al., 2011). Because of the propagation of distinct populations arising *in vivo*, the highly-resistant subtype had initially been overlooked during diagnostic testing and was only discernable on specialized Ashdown's selective medium, which contained glycerol that supported growth of the otherwise unstable mutants (Ashdown, 1979). This may represent a common persistence

mechanism in cases where clinical ceftazidime resistance occurs, but cannot be corroborated by standard susceptibility analysis *ex vivo*.

3.2. Efflux pump mediated multidrug resistance in Bpc members

Efflux is a major resistance mechanism in Bpc organisms, more so in *B. pseudomallei* and *B. thailandensis* than in *B. mallei.* A recent review focused on efflux-mediated drug resistance in *Burkholderia*, which will be covered herein in an abbreviated manner (Podnecky et al., 2015).

Bacterial genomes encode at least six efflux pump families (Fernandez and Hancock, 2012ß; Hassan et al., 2015; Nikaido and Pages, 2012; Piddock, 2006). These include: 1) The major facilitator (MFS) superfamily; 2) The resistance nodulation cell division (RND) family; 3) The small multidrug resistance (SMR) family; 4) The multi-drug and toxic compound extrusion (MATE) family; 5) The ATP-binding cassette (ABC) family; and 6) The proteobacterial antimicrobial compound efflux (PACE) family. Most bacteria encode several members of each of these efflux pump families, and *Burkholderia* species are no exception. In Gram-negative bacteria, efflux pumps of the RND family are of major significance because of their unique ability to span the entire cell envelope, which can lead to high-level resistance by synergy between the outer membrane permeability barrier and efflux into the extracellular milieu (Fernandez and Hancock, 2012; Schweizer, 2012b).

All *B. pseudomallei* strains encode at least 10 RND systems, seven of which are encoded by chromosome 1 and three by chromosome 2 (Holden et al., 2004; Kumar et al., 2008; Podnecky et al., 2015). Three of these RND pumps have been characterized in *B. pseudomallei.* AmrAB-OprA is expressed in most *B. pseudomallei* strains and responsible for intrinsic resistance to aminoglycosides and macrolides, and also confers some resistance to tetracyclines (Moore et al., 1999; Trunck et al., 2009). AmrAB-OprA overexpression confers high-level resistance to cethromycin (Mima et al., 2011). Rare aminoglycoside-susceptible *B. pseudomallei* environmental or clinical isolates either do not express AmrAB-OprB due to regulatory mutations, or lack the *amrAB-oprA* operon as a result of a chromosomal deletion, or express a non-functional efflux pump due to mutations that affect the AmrB RND transporter (Podin et al., 2014; Trunck et al., 2009). Several strains of *B. mallei* lack AmrAB-OprA and are therefore aminoglycoside and macrolide susceptible (Nierman et al., 2004).

In regulatory mutants, BpeAB-OprB confers low-level resistance to chloramphenicol, fluoroquinolones, macrolides, and tetracyclines (Chan et al., 2004; Mima and Schweizer, 2010). In some isolates this pump has been implicated in additional functions in host adaptation and quorum sensing (Chan and Chua, 2005). The clinical significance of BpeAB-OprB remains unclear. BpeEF-OprC is expressed in regulatory mutants, and extrudes chloramphenicol, fluoroquinolones, sulfamethoxazole, tetracyclines, and trimethoprim (Hayden et al., 2012; Kumar et al., 2006; Podnecky et al., 2015; Podnecky et al., 2013). It was shown that the multidrug resistant phenotype in a clinical relapse isolate is likely due to constitutive BpeEF-OprC expression as a result of a regulatory mutation caused by a 800 kb chromosomal inversion (Hayden et al., 2012; Podnecky et al., 2015).

The pump repertoire, expression pattern and ensuing drug resistance profile of *B. thailandensis* parallels that of *B. pseudomallei*: AmrAB-OprA confers resistance to aminoglycosides, macrolides, and tetracyclines; BpeAB-OprB extrudes tetracyclines; and and BpeEF-OprC effluxes chloramphenicol, fluoroquinolones, sulfamethoxazole, tetracyclines, and trimethoprim (Biot et al., 2013; Biot et al., 2011).

Together, AmrAB-OprA, BperEF-OprB, BpeEF-OprC effectively render six entire classes of compounds at least partially inactive depending on expression level, and evidence obtained mainly with *B. thailandensis* suggests that they may act in concert to tightly control the intrusion of these compounds into the bacterial cell (Biot et al., 2013).

3.3. Bpc species outer membrane permeability barrier

Although it has been known for some time that the relative outer membrane permeation of non-enteric bacteria like *Burkholderia* is significantly lower that that of *E. coli*, the underlying mechanisms remain poorly understood (Hancock, 1998). However, alterations to membrane permeability and structure have been implicated as resistance determinants in these species. For instance, atypical lipopolysaccharide (LPS) structure plays a crucial role in intrinsic resistance of *Burkholderia* species to cationic peptides, notably polymyxin B (Loutet and Valvano, 2011). A common LPS modification leading to resistance to polymyxin B in Gram-negative bacteria is the modification of lipid A with a positively charged 4-amino-4-deoxy-arabinose (Ara4N) moiety that masks the negative charges of the two phosphate moieties attached to the lipid A (Loutet and Valvano, 2011; Olaitan et al., 2014). The major lipid A species of *B. pseudomallei* and *B. thailandensis* was reported to contain a biphosphorylated disaccharide backbone modified with Ara4N at both phosphate groups (Novem et al., 2009). The presence of Ara4N effectively reduces the net negative charge of the cell envelope, reducing the permeation of cationic antimicrobials like polymyxins. The LPS core structure also plays a role in *B. pseudomallei* polymyxin B resistance. One gene (*waaF*) encodes a protein required for LPS core oligosaccharide biosynthesis that when mutated increases polymyxin B susceptibility. However, resistance to polymyxins is not restricted to a single pathway but is highly complex. Other mutants exhibiting a polymyxin susceptible phenotype had mutations in a predicted UDP-glucose dehydrogenase and an enzyme called IspH (formerly LytB) necessary for the synthesis of isoprenoids (Rohdich et al., 2002).

Outer membrane porin proteins also play a role in antimicrobial resistance, especially in concert with resistance enzymes or efflux pump expression (Fernandez and Hancock, 2012; Pages et al., 2008). Little is known about porins and their possible roles in *B. pseudomallei* drug resistance. *In vitro* studies with *B. pseudomallei* Omp38 in reconstituted liposomes indicated that this protein functions as a diffusion porin for neutral sugars and charged antibiotics (Siritapetawee et al., 2004). Subsequent black lipid membrane reconstitution studies, liposome swelling assays and expression in a porin-deficient *E. coli* strain confirmed translocation of antibiotics through Omp38 and suggested a possible contribution of this porin in *B. pseudomallei* ceftazidime and carbapenem resistance (Aunkham et al., 2014; Suginta et al., 2011). However, assessment of the true contribution of this porin to *B.*

pseudomallei antibiotic resistance awaits genetic and functional analyses in the source organism.

3.4. Alterations in drug targets

Point mutations that alter drug targets are one of the most common means that bacteria utilize to resist antibiotics (Blair et al., 2015; Walsh and Wencewicsz, 2016). Aside from the aforementioned PenA mutations that increase affinity to β-lactams that were previously poor substrates and the PBP3 deletion causing ceftazidime resistance in *B. pseudomallei*, reports on target mutations in *B. pseudomallei* leading to resistance to other antibiotics are scarce. To date there is one report on target mutation-based fluoroquinolone resistance in *B. pseudomallei*. Fluoroquinolones selectively target bacterial topoisomerase type II enzymes, DNA gyrase and topoisomerase IV (Walsh and Wencewicsz, 2016). Each enzyme is an A_2B_2-type heterotetramer, consisting of two GyrA and GyrB monomers for DNA gyrase and two ParC and ParD monomers for topoisomerase IV. Fluoroquinolones inhibit the respective "A" subunit of the topoisomerase heterotetramer, e.g. GyrA and ParC. Resistance to fluoroquinolones is frequently due to mutations affecting the quinolone resistance-determining regions (QRDR) of GyrA or ParC. In *E. coli* the GyrA QRDR encompasses a relatively small region (amino acids 67 to 106) of the protein (Yoshida et al., 1990). Quinolone resistance in *E. coli* and other bacteria is caused most often by mutations affecting amino acids 81 through 87. In *E. coli* roughly 50% of mutations affect Ser83 (Yoshida et al., 1990). In *B. pseudomallei*, all *in vitro* selected fluoroquinolone resistant mutants contained a Thr83Ile mutation (Viktorov et al., 2008). This is consistent with the finding that most *Burkholderia* species contain a GyrA Thr83 instead of the Ser83 found in other bacteria (Winsor et al., 2008). Notably, *P. aeruginosa* also contains Thr83 in GyrA and a frequent amino acid change seen in fluoroquinolone resistant clinical isolates is a Thr83Ile mutation (Kureishi et al., 1994).

4. *Burkholderia cepacia* complex organisms

The *Burkholderia cepacia* complex (Bcc) organisms are opportunistic nosocomial pathogens capable of causing severe disease in immunocompromised individuals, especially those with cystic fibrosis (CF) (Mahenthiralingam et al., 2005). In these patients, infection with Bcc organisms causes "cepacia syndrome", typically characterized by necrotizing pneumonia, sepsis and an overall negative prognosis (Mahenthiralingam and Vandamme, 2005). Although several species from this group have been isolated from the lungs of CF patients, *B. cenocepacia* and *B. multivorans* appear to cause the most serious forms of disease in these individuals and account for 85% of all Bcc infections (Drevinek and Mahenthiralingam, 2010; Mahenthiralingam and Vandamme, 2005). Treatment of Bcc infections relies on ceftazidime and other extended-spectrum cephalosporins, as intrinsic resistance prevents the action of many other classes of antimicrobials. One study of several thousand clinical isolates isolated from CF patients found that resistance to many standard therapies was overwhelming; greater than 50 percent of isolates were resistant to chloramphenicol, co-trimoxazole, ciprofloxacin, tetracycline, rifampin, avibactam, and co-amoxiclav (Zhou et al., 2007). However, these data may overestimate the occurrence of resistance in Bcc organisms as the study was carried out on patient isolates solicited because they were in fact multidrug

resistant. Despite this caveat, resistance patterns, both intrinsic and acquired, must not be discounted in these organisms.

4.1 β-lactam resistance in Bcc bacteria

Resistance to β-lactam antibiotics such as ceftazidime is caused by class A β-lactamases encoded by Bcc organisms, first described in the PenA-PenR system of *B. cepacia* 249 (Trepanier et al., 1997), which are now named PenB and PenR (AmpR) (Hwang and Kim, 2015). A recent study in *B. cenocepacia* identified ceftazidime-driven mutations to the peptidoglycan recycling enzyme AmpD as a putative cause for up-regulation of the PenB and AmpC β-lactamases (Hwang and Kim, 2015). PenB is a Class A penicillinase with broad spectrum carbapenemase character that highly conserved across the Bcc and shares significant protein homology with PenA of Bpc organisms (Poirel et al., 2009; Hwang and Kim, 2015). Both *penB* and *ampC* promoters were associated with binding sites for LysR-type repressor PenR (AmpR). PenR is a bifunctional protein. It is a repressor when it binds the D-alanine-D-alanine pentapeptide stem terminus of the peptidoglycan precursor UDP-MurNAc-pentapeptide. This protein was previously found to bind 1,6-anhydro-MurNac peptides, which are produced in the presence of β-lactam antibiotics or after disruption of AmpD, an enzyme that normally degrades them. This binding causes PenR to become an activator and leads to transcription of its *penB* and *ampC* targets (Hwang and Kim, 2015; Vadlamani et al., 2015). As a result, susceptibilities to ceftazidime, cefotaxime, and meropenem were greatly reduced.

B. cenocepacia also contains a Class A PenA β-lactamase, which is 55% identical and 67% similar to *B. pseudomallei* strain 1026b PenA (Winsor et al., 2008). As in the Bpc bacteria, the *penA* gene is located on chromosome 2 and its genetic surroundings are very similar to that of *B. pseudomallei penA* (Fig. 3). However, unlike *B. pseudomallei* or *B. thailandensis* PenA (the *B. thailandensis* PenA is now also referred to as PenL in some publications (Hwang and Kim, 2015)) the *B. cenocepacia* enzyme has not yet been shown to be involved in β-lactam resistance. *B. thailandensis* mutants with *in vitro* selected ceftazidime resistance solely contain PenA (PenL) structural and/or regulatory mutations, whereas in *B. cenocepacia* the same selection leads to regulatory mutants that either overexpress PenB or AmpC (Hwang and Kim, 2015; Yi et al., 2012a). PenB and AmpC exhibit similar substrate spectra and both are under control of the LysR-type PenR regulatory protein (Fig. 3). PenB and AmpC are absent from Bpc bacteria. Of note is that the sequenced *B. cenocepacia* strain J2315 PenB β-lactamase contains a Ser72Tyr substitution, which may explain this strain's intrinsic clavulanate resistance (Hwang and Kim, 2015).

B. multivorans contains a PenA enzyme (Bmul_3689 in *B. multivorans* ATCC 17616), that is closely related to PenB in BCC bacteria (Poirel et al., 2009; Hwang and Kim, 2015). It is also similar to KPC-2, which is the most clinically significant serine carbapenemase (Papp-Wallace et al., 2013). *B. multivorans* PenA is closely related to *B. pseudomallei* PenA (also called PenI by Dr. Bonomo's group (Papp-Wallace et al., 2013)). However, the *B. multivorans* enzyme is an inhibitor-resistant carbapenemase, whereas the *B. pseudomallei* enzyme is an extended spectrum β-lactamase. The role of PenA in clinically significant *B. multivorans* β-lactam resistance compromising therapy is not well established.

4.2. Efflux pump mediated multidrug resistance in Bcc members

The roles of efflux pumps in antibiotic resistance of members of the Bcc have recently been reviewed (Podnecky et al., 2015). In *B. cenocepacia*, at least six efflux pumps of the RND family have been implicated in drug resistance – RND-1, RND-3, RND-4, RND-8, RND-9, and RND-10 (Bazzini et al., 2011; Buroni et al., 2014; Buroni et al., 2009; Coenye et al., 2011; Nair et al., 2004). Of these RND-3, RND-4, and RND-10 (also known as CeoAB-OpcM) correspond to *B. pseudomallei* AmrAB-OprA, BpeAB-OprB and BpeEF-OprC, respectively, although the resistance patterns bestowed by the respective pumps are somewhat different, with the exception of RND-10 and BpeEF-OprC whose resistance profiles are very similar (Podnecky et al., 2015). An inventory of *B. cenocepacia* resistance mechanisms showed that efflux pump activity is prevalent in this bacterium and that mutations in the RND-3 pump regulator are the major cause of efflux pump activity and RND-3 mediated antibiotic resistance (Tseng et al., 2014). In *B. vietnamiensis*, aminoglycoside resistance emerges during chronic infection or after *in vitro* exposure to aminoglycosides and is the result of AmrAB-OprM efflux pump expression, which is most likely a homolog of *B. pseudomallei* and *B. thailandensis* AmrAB-OprA (Jassem et al., 2014; Jassem et al., 2011).

The *B. vietnamiensis* NorM pump, a member of the multidrug and toxic compound extrusion family of efflux systems, was shown to contribute to polymyxin B resistance, but curiously only in the presence of exogenously added tetracycline (Fehlner-Gardiner and Valvano, 2002).

4.3. Bcc species outer membrane permeability barrier

Similar to Bpc organisms, Bcc bacteria are typically resistant to polymyxins (Loutet and Valvano, 2011; Olaitan et al., 2014). As described above, this is thought to be partially attributable to a unique LPS structure that inhibits polymyxin binding to the outer membrane (Loutet and Valvano, 2011). In *B. cenocepacia*, an amino arabinose biosynthesis operon is responsible for the synthesis of 4-amino-4-deoxy-L-arabinose (Ara4N) used for modifications of lipid A that alter the total charge of the LPS molecule, thereby decreasing susceptibility to cationic antimicrobial peptides and polymyxins (Isshiki et al., 1998; Loutet and Valvano, 2011; Olaitan et al., 2014; Ortega et al., 2007).

The alternative sigma factor RpoE, which controls the expression of a regulon of genes required for bacteria to respond to extracytoplasmic stress, plays a significant role in polymyxin B resistance in *B. cenocepacia* at 37°C but not at 30°C (Loutet and Valvano, 2011).

As in Bpc bacteria, polymyxin resistance in Bcc species is complex and the pathways found in the former are also found in Bcc bacteria, as reviewed in Loutet et al. (Loutet and Valvano, 2011).

In *B. multivorans*, genes needed for the synthesis of hopanoid compounds are also implicated in polymyxin resistance (Malott et al., 2012). Interestingly, the use of fosmidomycin may potentiate colistin activity against *B. multivorans* by interrupting this process. Through disruption of the isoprenoid synthesis pathway, fosmidomycin prevents

hopanoid synthesis and alters membrane composition, ultimately resulting in a 64 fold decrease in the colistin MIC (Malott et al., 2014).

The role of porins in decreased antibiotic susceptibility of Bcc species has long been established. Bcc complex bacteria such as *B. cenocepacia* contain general porins that exhibit a permeability that is similar of *P. aeruginosa*, and approximately 10 times less than *E. coli* (Parr et al., 1987). β-lactam resistant CF *B. cenocepacia* isolates and a resistant mutant were shown to have decreased porin content (Aronoff, 1988).

4.4. Alterations in Bcc bacteria drug targets

Drug target modification in these species has been mostly associated with resistance to fluoroquinolones. Depending on the selection scheme and drug concentration used, *in vitro* selection of ciprofloxacin resistant *B. cenocepacia* mutants yielded mostly Thr83Ile or Asp87Asn mutations in the GyrA QRDR (Pope et al., 2008). These resulted in a 12–64 fold increase in the ciprofloxacin minimal inhibitory concentration (MIC). High-level ciprofloxacin resistance (MIC>256 μg/ml) required an additional Ser80Leu mutation in the ParC QRDR (Pope et al., 2008). Similar *gyrA* mutations were identified in a majority of levofloxacin resistant isolates studied in a survey of resistance mechanisms in Bcc (Tseng et al., 2014). The resistant isolates contained Gly81Asp, Thr83Ile, and Asp87His mutations. None of the isolates contained mutations in the *parC* QRDR.

Dihydrofolate reductase is the target of trimethoprim. Purification of the enzyme from trimethoprim susceptible and trimethoprim resistant *B. cepacia* strains indicated that the protein from the resistant strain was indeed refractory to trimethoprim inhibition (Burns et al., 1989). While this finding is consistent with target alteration, the molecular and genetic basis for this reduced inhibition was not established.

5. Conclusions

Although the resilience of *Burkholderia* species to antimicrobials has been recognized for quite some time, a literature review quickly reveals that our overall understanding of resistance in these bacteria is still rather rudimentary. Fig. 4 summarizes the current state of knowledge of the various resistance determinants that have been observed in antibiotic resistant *Burkholderia* species. First, the outer membrane permeability barrier is an important contributor to *Burkholderia* drug resistance. The two major players involved are lipopolysaccharide (LPS) and outer membrane porins. LPS typically plays a general role in drug resistance, but in most *Burkholderia* species it plays a major role in resistance to cationic peptides. Lipid A modification by aminoarabinose and changes to the LPS core are major determinants for the widespread intrinsic polymyxin resistance in *Burkholderia*. Restrictive porin proteins are contributing factors to drug resistance, especially in combination with other determinants such as efflux. RND pump-mediated efflux makes major contributions to intrinsic and acquired multidrug resistance. Depending on species, either periplasmic or membrane-bound β-lactamases play important roles in intrinsic β-lactam resistance and acquired resistance to clinically significant β-lactam antibiotics. Resistance due to antibiotic target mutations has mostly been associated with fluoroquinolones, but has also been implicated with resistance to other antibiotics, e.g.

trimethoprim. Of note is that most, if not all, of the *Burkholderia* resistance determinants identified to date are encoded by the genome of the respective organisms. The genomes of *Burkholderia* species consist of at least two chromosomes, e.g. Bpc bacteria, but other species contain additional genetic elements, e.g. *B. cenocepacia*, whose sequenced prototype strain J2315 contains a third chromosome and a plasmid (Holden et al., 2009; Holden et al., 2004; Nierman et al., 2004). Plasmids have not yet been demonstrated in Bpc bacteria. The constellation of both intrinsic and acquired resistance mechanisms in this genus combines to create a unique and often difficult challenge for researchers and clinicians. Further study is necessary to understand the interplay of these factors and their effect on antimicrobial therapy.

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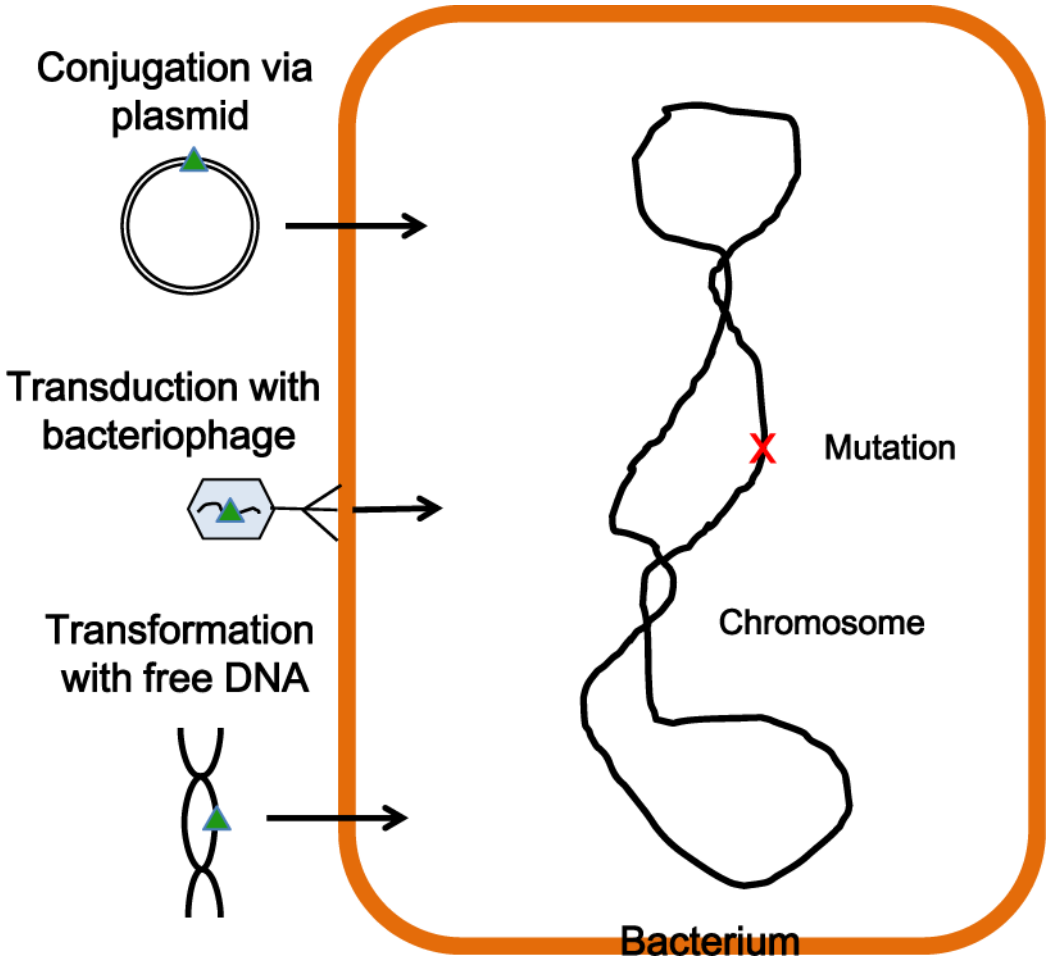

Figure 1. Genetic mechanisms contributing to acquired resistance in bacteria

Genetic mechanisms that lead to acquisition of a resistance trait (indicated by the green triangle) include mutations on the chromosome (indicated by the red x) that can either affect drug targets or regulatory factors for normally not expressed resistance proteins, conjugal transfer of a plasmid-encoded resistance marker, bacteriophage-mediated transduction of a resistance marker, or acquisition of a resistance trait via natural DNA transformation, e.g. with chromosomal DNA. This figure is adapted from (Walsh and Wencewicsz, 2016).

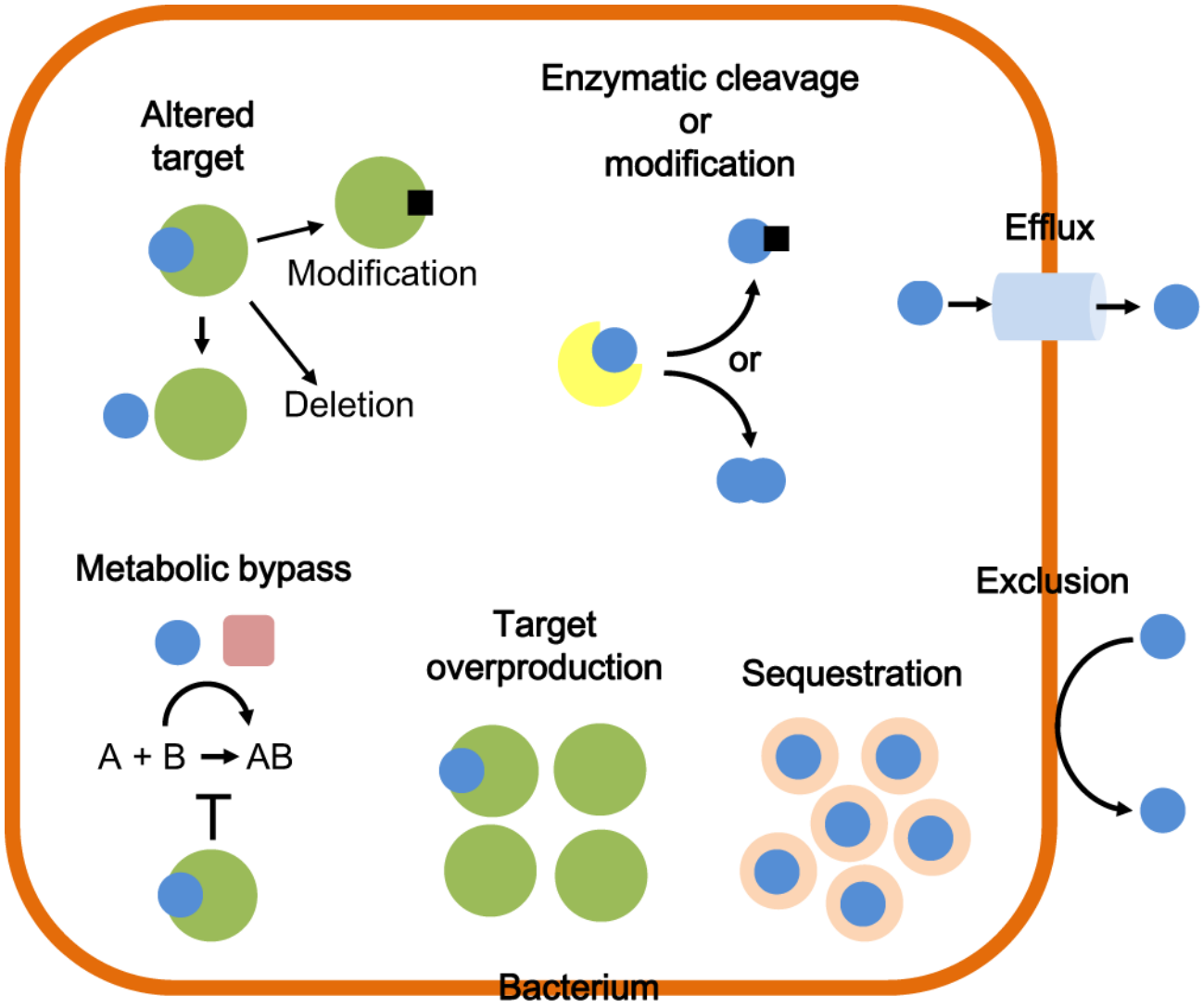

Figure 2. Bacterial mechanisms of resistance

Bacterial antimicrobial resistance mechanisms include – in counterclockwise order – drug (blue sphere) exclusion from or reduced penetration into the cell; active extrusion via efflux pumps; inactivation either by drug cleavage or chemical modification via group (black square) transfer catalyzed by a specific enzyme (indicated in yellow); target (indicated in green) alteration via mutation or enzymatic modification via group transfer (e.g. methylation, black square); metabolic bypass via substitution of a susceptible target with a resistant target; target overproduction via increased transcription or gene multiplication; and sequestration by a binding protein (indicated in rose color). Frequently, different resistance mechanisms act in concert or synergistically, e.g. exclusion and efflux. Resistance mechanisms are either intrinsic or can be acquired, e.g. by chromosomal regulatory mutations that can cause expression of a normally not expressed resistance trait such as an enzyme, an efflux pump, etc. This figure is adapted from (Schweizer, 2012a).

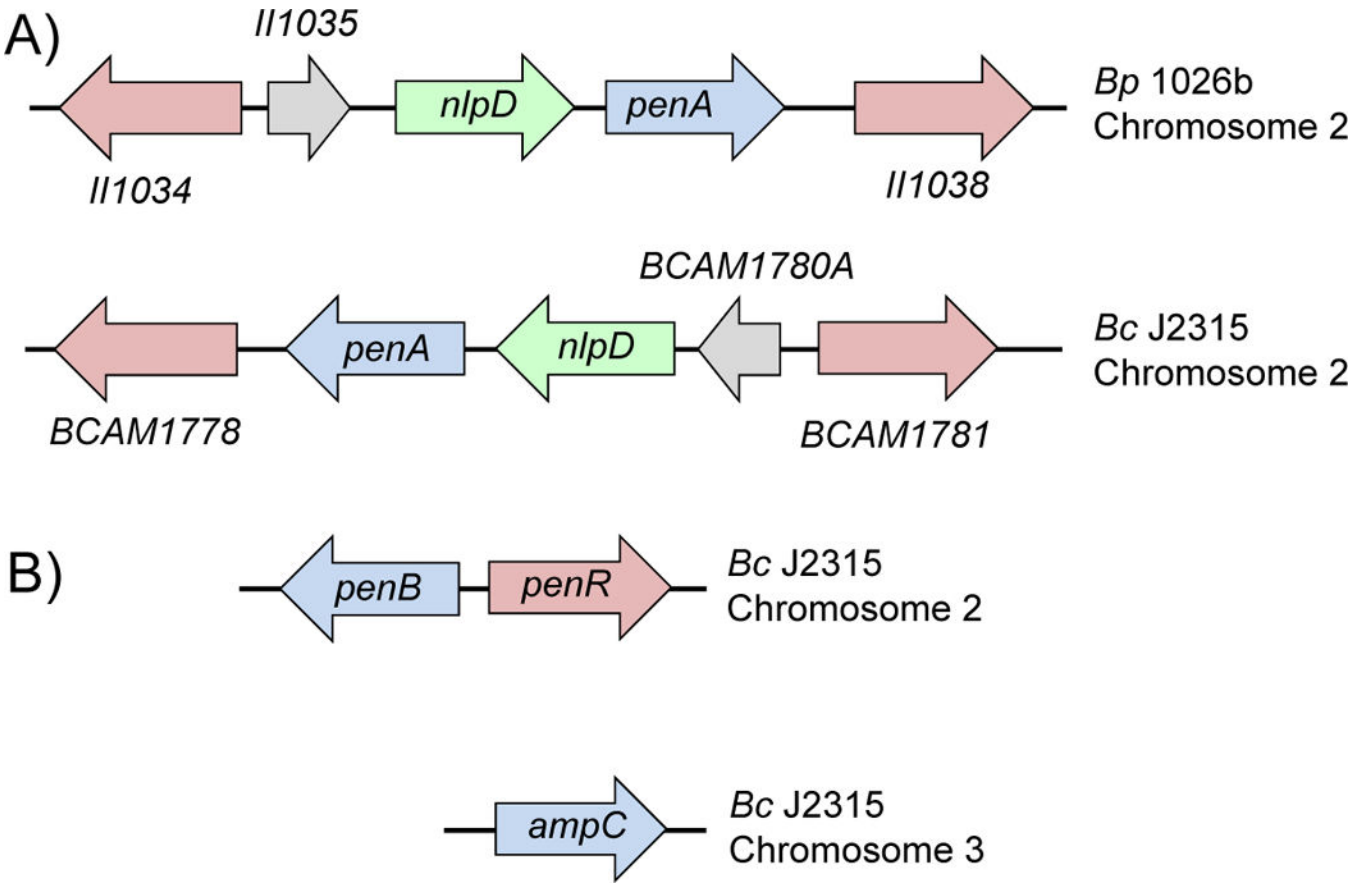

Figure 3. Genetic organization of Class A β-lactamase encoding genes implicated in β-lactam resistance in *B. pseudomallei* and *B. cenocepacia*

Chromosome location, gene organization and annotation are shown for representative *B. pseudomallei* (*Bp*) strain 1026b and *B. cenocepacia* (*Bc*) strain J2315. **A)** PenA confers β-lactam resistance in *B. pseudomallei* and *B. mallei.* The role of PenA in *B. cenocepacia* β-lactam resistance is unknown. **B)** PenB (originally known and annotated as PenA) and AmpC confer β-lactam resistance in *B. cenocepacia*. Genes encoding LysR regulatory proteins are indicated with magenta arrows. PenR (sometimes referred to as AmpR) regulates the expression of PenB and AmpC in *B. cenocepacia*. The *nlpD* gene encodes an outer membrane protein with peptidase and peptidoglycan-binding domains. Grey arrows indicate genes for hypothetical proteins. Annotations and coordinates are taken from (Winsor et al., 2008) and (Hwang and Kim, 2015). *II1034*, *II1035* and *II1038* are short for BP1026B_II1034, BP1026B_II1035, and BP1026B_II1038 used in annotation of the *B. pseudomallei* strain 1026b genome (Winsor et al., 2008).

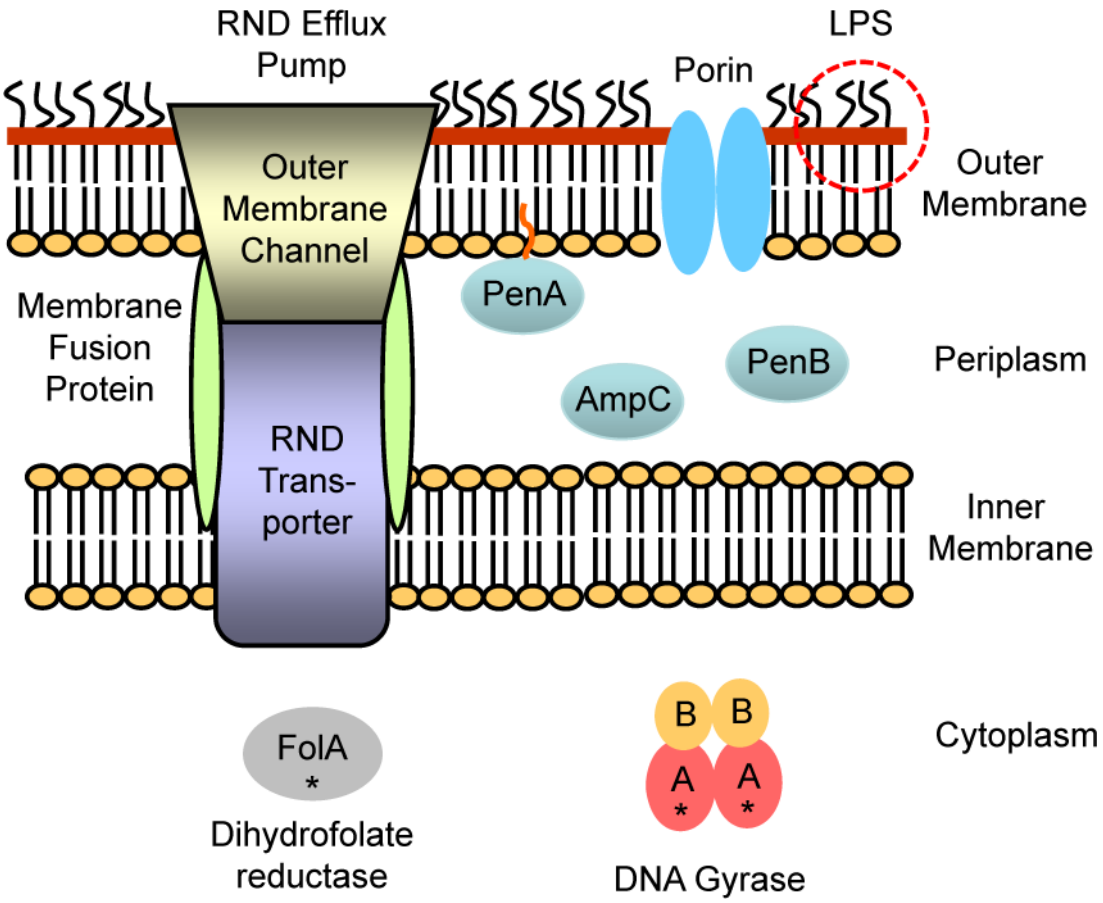

Figure 4. Summary of resistance determinants identified in *Burkholderia* species

The outer membrane provides a major penetration barrier, mainly because of the physicochemical properties of lipopolysaccharide (LPS) and restrictive porins. Efflux via tripartite RND pumps consisting of an RND transporter, a membrane fusion protein and an outer membrane channel plays a major role in multidrug resistance. Depending on species, acquired and intrinsic resistance to β-lactam antibiotics is provided by periplasmic β-lactamases, e.g. AmpC and PenB, or a membrane-bound β-lactamase, e.g. PenA. Target mutations (symbolized by black asterisks) in in the DNA gyrase A subunit and dihydrofolate reductase cause resistance to fluoroquinolones and trimethoprim, respectively.