Chapter 16
Antimicrobial Drug Efflux Pumps in *Burkholderia*

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Abstract The genus *Burkholderia* includes more than 90 species able to colonize different environments and characterized by a versatile metabolism. Some members of the *Burkholderia* genus are opportunistic pathogens, especially for immunocompromised and cystic fibrosis patients. Of note, they show a high level of intrinsic drug resistance, and many genes encoding virulence factors were identified in their genomes. Main contributors to antimicrobial resistance of these bacteria are efflux pump proteins which span the cytoplasmic and outer membranes. These systems are able to recognize and extrude very dissimilar compounds, thus rendering the antimicrobial therapy challenging. A detailed description of the resistance-nodulation-cell division (RND) transporter superfamily, which is the most represented in Gram-negative bacteria such as *Burkholderia* spp., is given. This includes the distribution of RND-encoding genes in the various *Burkholderia* spp. genomes and the list of the principal RND pumps in *B. cenocepacia*, *B. vietnamiensis*, *B. pseudomallei*, *B. mallei* and *B. thailandensis*. The clinical significance of RND efflux transporters in *Burkholderia* spp. and relevant existing efflux pump inhibitors is also discussed.

Keywords *Burkholderia* • *Burkholderia cepacia* complex • Antimicrobial resistance • Efflux pumps • RND

16.1 Introduction

Currently the genus *Burkholderia* consists of more than 90 formally described species and a large number of candidate species [1–4]. *Burkholderia* species are Gram-negative bacteria occurring in very different environments and possessing very

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diverse metabolisms; they can be found in pristine and contaminated soils, in plant rhizospheres and phytosphere, in invertebrate intestinal tracts and in the human respiratory tract [5–7]. The metabolic versatility of these species is partially due to their large genomes that are among the largest bacterial genomes known, with sizes spanning from 7 to 9 Mb [8]. These large genomes consist of two to three different chromosomes and, in some species such as *Burkholderia cenocepacia*, also of plasmids [9]. Most members of the *Burkholderia* genus are pathogens characterized by well-known drug resistance and virulence factors [10, 11]. Among various mechanisms of antimicrobial resistance, drug efflux pumps play an important role in *Burkholderia* spp. In this chapter, we first describe the major features of several species of *Burkholderia* of public health concern and then provide a review of the current status on the role of drug efflux pumps in drug resistance in these species.

16.2 The Genus *Burkholderia*

16.2.1 Burkholderia cepacia *Complex*

Members of the *Burkholderia cepacia* complex (Bcc) bacteria share a high level (>97.5 %) of 16S rRNA gene sequence similarity and moderate (30–60 %) DNA-DNA hybridization values. They are characterized by very different biological features making them both a friend and a foe to humans [12]. Bcc bacteria have large genomes (7.5–8.5 Mb) with a G+C base composition of approximately 67 %, and they are characterized by multiple replicons, providing them with exceptional metabolic capacities [13]. Currently, Bcc has been dissected into more than 20 species, but all available data demonstrated that there is still a large number of unnamed Bcc species. From old and new classification techniques, it is clear that Bcc bacteria constitute a genotypic continuum in which separated entities, called species, have only developed in the last years. New methods are better than the traditional 16S rRNA-based approach, as they analyse a larger part of the genome with a higher resolution in order to precisely discriminate closely related bacteria [13].

Presently, the complex consists of more than 20 genetically closely related species, isolated from human infections, as well as from natural environments [14–16]. Many members of the Bcc are opportunistic pathogens particularly dangerous for immunocompromised individuals and cystic fibrosis patients. Several Bcc species are transmissible from one cystic fibrosis patient to another, thus causing epidemic outbreaks [17–19]. Among Bcc bacteria, *B. cenocepacia* and *B. multivorans* predominate in cystic fibrosis, accounting for 85–97 % of all Bcc infections [14].

B. cenocepacia is one of the most dangerous pathogens in cystic fibrosis, and infection with this organism is associated with reduced survival and a high risk of developing fatal cepacia syndrome [20, 21]. Research on the pathogenicity of Bcc bacteria is focused on *B. cenocepacia* because of the preponderance of epidemic strains and because the first Bcc genome sequenced was that from *B. cenocepacia*

J2315 [9, 22]. The latter was isolated from a cystic fibrosis patient and is a member of the epidemic ET12 lineage, which is responsible for infecting many patients in Canada and the United Kingdom. The *B. cenocepacia* J2315 genome of 8.06 Mb consists of three circular chromosomes plus a plasmid [9]. It contains 14 genomic islands not found in other *Burkholderia* spp. [9]. In the evolution of the ET12 lineage, the exchange of genomic islands was shown as crucial, introducing features necessary for the survival and for the pathogenesis in the cystic fibrosis lung. In particular, J2315 strain has developed increased resistance to many antimicrobials [23], and the genome sequencing showed that it contains drug resistance determinants in genomic islands, underlining the important role of a horizontal transfer [9]. Comparative genomic studies highlighted that gain of functions through horizontal transfer and loss of functions via mutations were necessary for J2315 strain to sustain the growth and persistence in cystic fibrosis infections [9].

Pharmacological treatment of Bcc infections is very difficult due to the high intrinsic and acquired resistance of most strains to a broad range of antimicrobial drugs. Such resistance is due to various mechanisms, including reduced permeability, changes in lipopolysaccharide structure, the presence of numerous multidrug efflux pumps, inducible chromosomal β-lactamases and altered penicillin-binding proteins [24]. Furthermore, Bcc bacteria are able to form biofilms that contribute to increase the survival in the cystic fibrosis lung environment protecting bacteria from antimicrobials [24]. In this scenario, the treatment of Bcc-infected patients should be based on a combination therapy driven by antimicrobial susceptibility tests, with two or three antimicrobial agents that function synergistically.

16.2.2 Burkholderia pseudomallei

B. pseudomallei is a saprophytic intracellular opportunistic pathogen that multiplies within macrophages. It causes melioidosis, a disease characterized by sepsis, pneumonia and abscess formation in almost any organ. It is endemic in tropical and subtropical regions [25–27]. *B. pseudomallei* is a potential bioterrorism agent and should be manipulated in biosafety level 3 (BSL-3) laboratories only. The genome of *B. pseudomallei* has been sequenced and found to comprise two chromosomes of 4.07 Mb and 3.17 Mb, respectively [28]. The larger chromosome contains genes associated with core function such as cell growth and metabolism, while the smaller one carries genes for accessory functions and for adaptation and survival in different environments. Approximately 6 % of the genome is constituted by putative genomic islands, probably derived from horizontal gene transfer, but it is not known if these regions are involved in pathogenesis [29]. Using multilocus sequence typing to study the molecular epidemiology of *B. pseudomallei*, a high level of genetic recombination was hypothesized [30]. From the comparison of *B. pseudomallei* and *B. mallei* (see below), it seems that the latter derived from a single clone of the former through a “genomic down-sizing” [29].

The clinical symptoms of melioidosis are multifarious, ranging from acute sepsis to chronic recurrent infections as well as disease without clinical symptoms. These different aspects are due to a combination of infecting dose, type of infection, host risk factors and still unknown bacterial virulence determinants [27]. If the diagnosis is not rapid, and without appropriate antimicrobial treatment, the mortality rate is ~40 % and can increase to >90 % in subjects with septic shock [26]. The disease affects at-risk patients, like those suffering from cystic fibrosis [28, 29, 31, 32], non-cystic fibrosis bronchiectasis [33] and also diabetes.

Infections caused by *B. pseudomallei* are characterized by particular morbidity and mortality. Therapy is extensive and divided in many phases: parenteral (ceftazidime, amoxicillin-clavulanic acid or meropenem) and oral (trimethoprim-sulfamethoxazole).

The resistance mechanisms documented in *B. pseudomallei* are the modification of the cell envelope constituents to decrease the cell permeability, efflux pump activation and modification or deletion of target sites [34]. Moreover, other factors that contribute to antimicrobial resistance are the biofilm formation like in Bcc strains [35], the intracellular and non-replicative metabolic state [36] and growth under stress conditions [37].

16.2.3 Burkholderia mallei

B. mallei, an obligate mammalian pathogen, is a non-motile, facultative intracellular bacterium known as the etiologic agent of glanders. *B. mallei* infection can be chronic or acute: in the first case, the clinical symptoms are mucopurulent nasal discharge, lung lesions and nodules involving the liver and spleen, while the acute infection results in high fever and emaciation, with ulceration of the nasal septum, accompanied by haemorrhagic discharge [38]. Rarely, *B. mallei* can infect humans, including laboratory workers and those in contact with infected animals. Bacteria enter the body through the eyes, nose, mouth or wounds in the skin. Human symptoms are initial onset of fever, rigors and malaise and rapid onset of pneumonia, bacteraemia, pustules and abscesses, with death coming in 7–10 days without antimicrobial treatment. Ninety-five percent of untreated infections and 50 % of antimicrobial-treated cases are fatal [38]. *B. mallei* is highly infectious in the aerosol form, and only few bacteria are required to establish the infection, thus rendering it a potential biological threat agent. The use of this bacterium is confined to BSL-3 laboratories.

The genome of the *B. mallei* comprises two circular chromosomes (5.8 Mb) and a G+C content of 69 % [39]. The comparison with the closely related species *B. pseudomallei* and *B. thailandensis* reveals a significant similarity, with 99 % identity between the conserved genes in *B. pseudomallei*, even if *B. mallei* contains approximately 1.41 Mb less DNA than *B. pseudomallei* [39]. It is probable that *B. mallei* evolved from a single strain of *B. pseudomallei* after a colonization of an equine-like ancestral host [40].

The evolution was a result of intergenic sequence (IS)-mediated gene loss and genomic recombination [39, 41]. The IS intervention was found in diverse symbionts

and obligate pathogens, suggesting an elaborated genome transition during the initial bacterial evolution after establishing constant association with the host. The structural flexibility is the major feature of *B. mallei* genome in order to adapt to multiple distinct mammalian hosts and to increase the ability to escape the adaptive immune responses.

Many *B. mallei* strains show resistance to a high number of antimicrobial agents; in fact the genome contains at least 33 genes involved in the drug resistance [39]. *B. pseudomallei* is resistant to macrolide and aminoglycoside antibiotics because of the presence of multidrug efflux pumps, while *B. mallei* shows susceptibility to these drugs. In *B. mallei*, the 50 kb region where these genes are located in *B. pseudomallei* genome is absent [39].

16.2.4 Burkholderia thailandensis

B. thailandensis is a soil saprophyte common to tropical and subtropical regions, and it is used, as generally considered non-pathogenic, for antimicrobial and vaccine studies because it can be manipulated in BSL-2 laboratories. It is closely related to *B. pseudomallei*, and only occasionally it is reported to cause human disease in association with traumatic event or reduced immune competence [42].

B. thailandensis and *B. pseudomallei* diverged from a common ancestor about 47 million years ago, and the two species show a high level of 16S rRNA sequence similarity [43]. Their genomes are highly syntenic and approximately 85 % of their genes are conserved, with only four large inversions. It has been demonstrated that the use of live *B. thailandensis* expressing capsular polysaccharide on *B. pseudomallei* induces protective responses [44]. This result revealed the importance of capsular polysaccharides in the stimulation of immune response against *B. pseudomallei* and the efficacy of *B. thailandensis* E555 strain as potential vaccine in protecting against melioidosis [44].

16.3 Drug Efflux Pumps

Efflux pumps are considered among the three principal causes of drug resistance in bacteria, together with drug-modifying enzymes and alterations of the antimicrobial target [45]. Efflux pumps are able to extrude chemically very different compounds (including cationic dyes, detergents, solvents and antimicrobials) out of the cell, thus preventing these compounds from reaching their target [46].

Efflux pumps can be divided into five major families/superfamilies: ATP-binding cassette (ABC) superfamily, the resistance-nodulation-cell division (RND) superfamily [47], the multidrug and toxic compound extrusion (MATE) family [48], the major facilitator superfamily (MFS) [49] and the small multidrug resistance (SMR) family [50]. The ABC superfamily is the only one that uses ATP as the energy source [51], while the others obtain energy through the proton motive force. All the families are

found in bacteria, including pathogens [52]. Here we will focus on RND efflux transporters, as the members belonging to this super family are the principal mediators of multidrug resistance in Gram-negative bacteria, including *Burkholderia* spp. [53].

RND efflux transporters are good examples of the typical translocators of Gram-negatives, being composed of three proteins: an inner membrane protein; a membrane fusion protein, located in the periplasm; and an outer membrane protein [47, 54]. These components span the Gram-negative membranes, thus allowing the translocation of different kind of molecules from the outer leaflet of the inner membrane to the outside of the cell.

While the other families of efflux transporters can be composed of only one unit or form similar three-component complexes, it is thought that RND members cooperate with other transporters to deliver their substrates through their periplasmic and outer membrane proteins [55]. For example, in *Pseudomonas aeruginosa*, the TetA efflux pump has been shown to work in concert with MexAB-OprM and enable the resistance to tetracycline higher in the presence of both efflux pumps [56].

The regulation of the expression of these systems is at the transcriptional level and involves DNA-binding proteins acting as repressors or activators that, in turn, sense the presence of various compounds (including the ones that are translocated by the efflux pump). As an example, the crystal structures of AcrAB-TolC of *Escherichia coli* [57–59] and of its regulator AcrR [60] helped to shed light on its mechanism of extrusion and activation. Moreover, transcriptional regulation can be performed by global regulators belonging to the Mar, Sox and Rob families [61].

Additional information may be gained from studying kinetics. To this aim, both *in vitro* and *in vivo* experiments can be performed. The latter is interesting, but it is difficult to discriminate whether the data collected only come from one transporter or from the whole cellular environment. In this way, many knock-out mutants should be tested to properly assess the contribution of one protein in respect to the other ones. Examples of *in vivo* assays are the efflux of fluorescent dyes [62] and antimicrobial minimal inhibitory concentration (MIC) determination in wild type and mutant strains [63]. As regarding *in vitro* approaches, they allow to obtain kinetic constants of purified proteins [64]. The two assays can be combined due to the difficulties in designing a classical protein assay for efflux transporters. One useful method is the preparation of liposomes.

The *in vitro* approach presents limitations due to the hydrophobic properties of the substrate and its tendency to non-specifically bind to the membrane. Moreover, the proton gradient needed by the RND to perform the translocation is not easy to produce [64]. Different strategies to create the proton gradient and to perform liposome assays have been described by Verchère and collaborators [65]. Their conclusions are that the reconstitution of an efflux pump in a membrane-like environment and the vectorial substrate translocation represent the bottleneck step in developing functional *in vitro* assays, but they remain an excellent tool for the characterization of transport activity at the molecular level.

The importance of RND efflux pumps in different clinical isolates has been well described [66]. In clinical infections due to *E. coli*, about 50 % of the isolates exhibited an efflux pump overproduction [67]. Similarly, a serious increase in the prevalence of efflux-producing *Enterobacter aerogenes* strains was observed in a French

hospital [68]. Moreover, the involvement of active efflux in clinical isolates of *Klebsiella pneumoniae* has been reported to be responsible for the extrusion of 90 % of ciprofloxacin [69]. RND multidrug efflux pumps have been well described also in *P. aeruginosa*, another opportunistic pathogen of the respiratory tract. Examples are the MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM transporters [70–72]. A clear involvement of MexAB-OprM and MexXY-OprM in the expulsion of commonly used antimicrobial drugs has been described [73–77].

16.4 RND Efflux Pump Distribution in the Genus *Burkholderia*

The members of the RND superfamily are further classified into nine subfamilies: aryl polyene pigment exporter (APPE), eukaryotic (putative) sterol transporter (EST), hydrophobe/amphiphile efflux-1 (HAE1), hydrophobe/amphiphile efflux-2 (HAE2), hydrophobe/amphiphile efflux-3 (HAE3), heavy metal efflux (HME), putative nodulation factor exporter (NFE), secretion system DF family (SecDF) and hopanoid biosynthesis-associated RND (HpnN) [54, 78]. The names of the subfamilies depend on the substrate that they translocate: heavy metals (HME), multiple compounds (HAE), lipooligosaccharides (NFE) [79] or hopanoids (HpnN) [80]. APPE, HME, HAE1 and NFE are restricted to Gram-negative bacteria; HAE2 are typical for Gram-positives, while EST are found in eukaryotes. HAE3 are distributed among Archaea and Spirochaetes, while representatives of the SecDF family can be found in Gram-negatives, Gram-positives and Archaea [79].

In the *B. cenocepacia* J2315 genome, 16 operons encoding putative RND efflux pumps were described [9, 81], while in *B. pseudomallei* K96243, 10 operons encode RND transporters [82]. Most of these pumps consist of a polypeptide chain of 700–1,300 amino acids, with a characteristic topology of a transmembrane segment (TMS) at the N-terminus, an extracytoplasmic domain, six TMSs, another extracytoplasmic domain and five C-terminal TMSs.

In 2010, Perrin and collaborators analysed the 16 operons of *B. cenocepacia* and confirmed the presence of four highly conserved motifs [83] in all of them [84]. The 12 TMSs and the 2 large loops that are characteristic of RND proteins [79] were found in all of them. The organization of the operons was then studied, revealing three different arrays, based on the *ceoB* (the inner membrane portion) encoding gene position, while a phylogenetic analysis further splits the 16 sequences into 5 clusters [84]. Then, the distribution of the CeoB-like proteins was checked in the entire *Burkholderia* genus, and a variable number of proteins, ranging from 6 (in *B. mallei* strains) to 18 (in *B. cenocepacia* strains), were found. All the sequences identified in the *Burkholderia* genus were assigned to two RND subfamilies: HAE1 (further divided into three groups that likely transport unrelated substrates) and HME (split into two different groups, one for the export of monovalent and one for divalent cations) [84]. While no apparent relationship between bacterial lifestyle (in the environment, in the host or in both), pathogenicity or genome size and RND protein number was detected, a correlation between the number of proteins and

taxonomy could be found. In fact, a similar number of RND proteins are present in strains of the same species and/or related species [84]. As regarding the evolution of RND-encoding genes, they seem to derive from an ancestral *ceoB*-like sequence. In fact, the degree of sequence similarity is very high. Probably the ancestor was able to recognize different substrates, and then, through differentiation and duplication events, the transporters acquired the substrate specificity [84].

These analyses were subsequently deepened by performing a comprehensive comparative analysis of the RND superfamily efflux systems in 26 completely sequenced *Burkholderia* genomes [84, 85]. In this way, a new uncharacterized RND family was discovered, and the distribution of the other subfamilies was evaluated. In particular, at least one copy of the genes belonging to the HAE1 and SecDF families and to HpnN transporters was found in all the genomes analysed, indicating that these proteins are involved in the extrusion of different antimicrobial and/or toxic compounds in different microorganisms [86, 87], thus mediating the resistance.

16.5 RND Efflux Pumps in *B. cenocepacia*

The first evidence of the contribution of efflux transporters to *Burkholderia* spp. drug resistance came in 1989 when Burns and collaborators determined the mechanism of chloramphenicol resistance of a cystic fibrosis clinical isolate [88]. An outer membrane protein homologous to *P. aeruginosa* OprM was found to be responsible for that phenotype [89]. The entire efflux gene cluster was subsequently isolated and characterized and named "*ceo*" for "*cepacia* efflux operon" encoding CeoAB-OpcM [90]. It showed the ability to actively efflux chloramphenicol and salicylate out of the cell, and its involvement in the transport of trimethoprim and ciprofloxacin was assessed [90].

In 2006, a bioinformatic analysis allowed our group to identify 14 putative operons encoding RND efflux transporters in the genome of *B. cenocepacia* J2315 [81]. After the completion of the genome sequencing, two additional transporters were added to the list [9]. By reverse transcription-PCR experiments, *orf3*, *orf9*, *orf11* and *orf13* were shown to be expressed in *B. cenocepacia* J2315, and *orf3* expression was strongly induced in the presence of chloramphenicol [81]. One of the RND-encoding genes (*orf2*) was cloned into an inducible vector and transformed into an *E. coli* strain which lacks the *acrAB* genes. Orf2 was able to confer resistance to streptomycin, tetraphenylphosphonium, ethidium bromide, nalidixic acid, ciprofloxacin, ofloxacin and norfloxacin and was demonstrated to efflux ethidium bromide out of the cell [81].

Subsequently, to better understand the role of efflux pumps in the intrinsic drug resistance of *B. cenocepacia* J2315, we performed gene knock-out experiments. Firstly, we deleted three operons encoding RND-1 (*BCAS0591-BCAS0593*), RND-3 (*BCAL1674-BCAL1676*) and RND-4 (*BCAL2820-BCAL2822*). Then, the MICs of different compounds were determined for the deleted strains and compared to the wild type [91]. Strain D1 with inactivation of RND-1 did not show any increased

susceptibility to tested compounds, while an eightfold reduction in the MIC of nalidixic acid was observed in strain D3 with inactivation of RND-3. As regarding strain D4 with RND-4 disruption, it showed a 4- to 16-fold increase in drug susceptibility to aztreonam, chloramphenicol, ethidium bromide, gentamicin, tobramycin, nalidixic acid, ciprofloxacin, levofloxacin, norfloxacin and sparfloxacin, indicating that RND-4 plays a crucial role in the intrinsic resistance of *B. cenocepacia* [91]. RND-3 and RND-4 were also shown to be involved in *N*-acyl homoserine lactone export, an important trait which contributes to quorum sensing signalling and to the virulence of the bacterium [91].

In another work, the tolerance of *B. cenocepacia* to the disinfectant chlorhexidine was evaluated [92]. To verify whether efflux pumps contribute to this, chlorhexidine MIC was determined for the wild type and various mutant strains, both grown as biofilms or planktonically. The results indicated that RND-3 and RND-9 (*BCAM1945–BCAM1947*) are associated with chlorhexidine tolerance when cells are grown as a biofilm, while inactivation of RND-4 rendered *B. cenocepacia* planktonic cells more susceptible than wild type cells [92]. The double mutant D4-D9 was hypersusceptible, both in sessile and planktonic cultures. All these data suggested the presence of specific chlorhexidine tolerance mechanisms related to the bacterial lifestyle [92].

More features about RND-4 and RND-9 were elucidated by analysing the transcriptome of three mutants: the single mutants D4 and D9 and the double mutant D4-D9 [93]. Major classes of *B. cenocepacia* genes, with differential expression in the deleted strains as compared to the wild type, belonged to flagellum assembly, motility and chemotaxis. In particular, D4 and D4-D9 mutants shared 26 upregulated flagellum-related genes and 13 upregulated chemotaxis-related genes. Instead, the genes that showed a decreased expression profile in D4 and D4-D9 mutants belonged to many different functional classes. Exactly the contrary was true for D9 mutant. Microarray data were confirmed by quantitative reverse transcription-PCR and phenotypic experiments, as well as by phenotype microarrays. Together these results showed a phenotypic and molecular similarity between D4 and D4-D9 strains and suggested that the RND-4 and RND-9 pumps might have a biological role not only restricted to transport but also related to motility and/or chemotaxis [93].

RND-4 was further characterized by comparing the intracellular proteome of the deletion mutant to that of the wild type strain using two-dimensional electrophoresis [94]. The results pointed out 70 differentially expressed proteins, with 13 protein spots upregulated and 35 downregulated. Fifty percent of the 35 downregulated proteins belonged to the functional categories: “amino acids transport and metabolism”, “nucleotides transport and metabolism”, “lipid transport and metabolism”, “translation” and “ribosomal structure and biogenesis”. Conversely, 46 % of the 13 upregulated proteins belonged to the categories: “energy production and conversion”, “posttranslational modification” and “protein turnover, chaperones”. Together these results confirmed a wider role than just in drug resistance for RND-4 [94]. However, the prominent role in drug resistance of RND-4 was further highlighted when, in attempt to identify the cellular target of a new thiopyridine derivative effective against *B. cenocepacia*, a mechanism of resistance was characterized

which relied on RND-4 itself [95]. In addition, RND-9 has been very recently shown to contribute to resistance of *B. cenocepacia* J2315 against a new benzothiadiazole derivative [96].

To finally assess the role of each of the 16 RND efflux transporters of *B. cenocepacia*, we created knock-out mutants for all of them [97]. First of all, we checked if differences could be detected in the MICs of some drugs for the deleted strains in respect to the wild type strains. Strains D3 and D4 were more susceptible to ciprofloxacin, minocycline and tobramycin, while the behaviour of most mutants was identical to the one of the wild type. These data suggest that RND-3 and RND-4 efflux pumps are involved in resistance of planktonic *B. cenocepacia* cells, while the other RND systems do not play a major role. As regarding to the sessile cells, strain D3 showed the highest reductions in the number of cells in the presence of high concentrations of tobramycin and ciprofloxacin, thus indicating that this efflux system is important also for the protection of *B. cenocepacia* when grown as biofilm. RND-8 and RND-9 seem instead to protect sessile cells against tobramycin [97].

In summary, at present only a few RND efflux transporters out of 16 appear to play a role in drug resistance or to be involved in virulence (due to the transport of quorum sensing signal molecules) in *B. cenocepacia*. In particular, (a) RND-3 is involved in the efflux of nalidixic acid, ciprofloxacin, tobramycin and *N*-acyl homoserine lactone in planktonic cells and seems to have a role in the protection of sessile cells against ciprofloxacin, tobramycin and chlorhexidine; (b) RND-4 plays a role in the efflux of aztreonam, ethidium bromide, chloramphenicol, gentamicin, tobramycin, fluoroquinolones, chlorhexidine, a thiopyridine derivative and *N*-acyl homoserine lactone in planktonic cells; (c) RND-8 is important for the efflux of tobramycin in sessile cells; (d) RND-9 is involved in the transport of chlorhexidine and tobramycin in biofilm grown cells; it contributes to the resistance towards a new benzothiadiazole derivative; (e) RND-10 (Ceo) transports chloramphenicol, salicylate, trimethoprim and ciprofloxacin out of the cell.

In *B. cenocepacia*, only two non-RND efflux transporters have been described for their contribution to resistance, BcrA and a homolog to the *E. coli* Fsr, both belonging to the MFS. The former is able to confer resistance to tetracycline and nalidixic acid when overexpressed in *E. coli* [98] and the latter to fosmidomycin [99, 100].

16.6 RND Efflux Pumps in Other *Burkholderia* Species

16.6.1 Burkholderia pseudomallei *and* Burkholderia mallei

Ten operons encoding RND efflux pumps are present in the genome of *B. pseudomallei* [82], but their clinical importance is difficult to study. Only the role of AmrAB-OprA [101], BpeAB-OprB [102, 103] and BpeEF-OprC [104] was elucidated.

AmrAB-OprA is a multidrug efflux system required for both aminoglycoside and macrolide antibiotic extrusion. This efflux pump shows homology to multidrug

efflux systems studied in *E. coli*, *P. aeruginosa* and *Neisseria gonorrhoeae* [101]. *B. pseudomallei* strains susceptible to aminoglycosides and macrolides have single point mutations or deletions in the *amrAB-oprA* operon [105]. The presence of this efflux mechanism in *B. pseudomallei* explains the lack of therapeutic effect observed for aminoglycosides and macrolides. AmrAB-OprA is also able to reduce the activity of newer antimicrobials like cethromycin: indeed the exposure to cethromycin induces the selection of mutants overexpressing the operon and results in high resistance levels [106].

Another *B. pseudomallei* efflux pump is BpeAB-OprB which extrudes macrolides, fluoroquinolones, tetracyclines and chloramphenicol and contributes to the intrinsic resistance. However, except for macrolides, the resistance levels are low. Despite the relationship between BpeAB-OprB and the *P. aeruginosa* MexAB-OprM, they are quite different: the latter is broadly expressed and it is involved in the intrinsic resistance to many compounds [107], while BpeAB-OprB has lower expression levels and it plays a minor role in the resistance. Studies regarding the correlation between RND efflux pump and quorum sensing or virulence traits showed that BpeAB-OprB in *B. pseudomallei* KHW strain is used for the secretion of *N*-acyl homoserine lactones [108] and virulence-associated determinants, such as siderophores [109].

The last described efflux system in *B. pseudomallei* is BpeEF-OprC, the most important pump for antimicrobial resistance. Initially, it was identified as chloramphenicol and trimethoprim transporter. The *bpeEF-oprC* operon is expressed only in *B. pseudomallei* strains carrying mutations in the regulatory region. Its expression confers resistance to chloramphenicol, fluoroquinolones, tetracyclines and trimethoprim, and it is responsible for the spread of trimethoprim resistance in *B. pseudomallei* isolates [110]. In *P. aeruginosa*, there is a related efflux pump, MexEF-OprN, which is characterized by similar substrate efflux profile [111]. The clinical significance of BpeEF-OprC is described below in the section "RND efflux pumps in *Burkholderia* clinical isolates".

B. mallei, as already described above, is generally more susceptible than *B. pseudomallei* to antimicrobial agents. The ATCC 23344 strain is susceptible to aminoglycosides because of a chromosomal deletion which involves the *amrAB-oprA* operon [39]. In *B. mallei*, the genes coding for BpeAB-OprB and BpeEF-OprC efflux pumps are present, but it is not known if the corresponding efflux systems are functional or not.

16.6.2 Burkholderia thailandensis

B. thailandensis becomes multidrug resistant following chloramphenicol exposure due to the overexpression of two RND efflux systems very similar to the already studied BpeAB-OprB and BpeEF-OprC of *B. pseudomallei* [112]. In another work, Biot and colleagues [113] showed that doxycycline resistance was correlated with the overexpression of AmrAB-OprA or BpeEF-OprC efflux pumps. The expression

levels varied depending on the antimicrobial concentration, and this indicated a reversible multidrug resistance phenotype [113]. Moreover, analysis of mutants overexpressing the efflux pumps highlighted that BpeAB-OprB is able to partially substitute the absence of AmrAB-OprA or BpeEF-OprC [113]. Furthermore, another efflux pump of the MFS family, responsive only to urate, xanthine and hypoxanthine and controlled by a multiple antimicrobial resistance regulator MarR-like, has been described [114]. However, its contribution to drug resistance is still unclear.

16.6.3 Burkholderia vietnamiensis

In contrast to many other Bcc species, *B. vietnamiensis* is susceptible to aminoglycosides and to a broad range of other antimicrobials, while it remains highly resistant to other cationic agents [115]. The same study reported the acquisition of aminoglycoside resistance of *B. vietnamiensis* in cystic fibrosis chronic infection or during the *in vitro* exposure to the drugs [115]. This resistance was caused by an efflux pump homologous to the *B. pseudomallei* and *B. thailandensis* AmrAB-OprA. Mutations in *amrR*, the putative efflux pump regulator, influenced the expression of the *B. vietnamiensis* gene *amrB* [116]. Moreover, in *B. vietnamiensis*, the *norM* gene encoding a MATE-type efflux protein was described. The disruption of *norM* alone was shown to be insufficient to reduce high levels of norfloxacin resistance, because of the presence of other efflux systems [117]. Although the physiological role of NorM is yet unclear, it is probably involved in resistance to the cationic peptide polymyxin B, especially under stress conditions [117].

16.7 RND Efflux Pumps in *Burkholderia* Clinical Isolates

Due to the high degree of antimicrobial resistance among *Burkholderia* species, it is very important to evaluate the contribution of efflux transporters, especially in clinical isolates. In the last years, some papers describing this topic were published.

Recently, Tseng and collaborators [118] evaluated the role of efflux pumps in 66 clinical Bcc isolates recovered between 2009 and 2011 in Taiwan. In order to assess the presence of active efflux, resistance patterns were determined by measuring the MICs of antimicrobials in the presence of the efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and the efflux pump expression was evaluated through quantitative reverse transcription-PCR. The results showed that 78.6 % isolates (resistant to ceftazidime, chloramphenicol, levofloxacin, meropenem and trimethoprim-sulfamethoxazole) demonstrated presence of the efflux pump activity. Moreover, RND-3 and RND-9 transcripts were more abundant in all the tested strains compared to a strain without efflux pump activity. DNA sequences of the regulators of these two pumps were also sequenced, together with the promoter,

thus revealing five nucleotide deletions in RND-3 regulator which affected RND-3 efflux pump expression in *B. cenocepacia* clinical isolates causing antimicrobial resistance [118].

In *B. pseudomallei*, mutations in the *amrB* gene have been reported to be responsible for aminoglycoside sensitivity [119]. The whole-genome sequencing revealed non-synonymous mutations in a highly conserved region of *amrB* gene [119]. In another work, susceptibility of three isolates from Thailand was shown to be due to the lack of or greatly reduced expression of AmrAB-OprA, caused by deletions affecting the pump itself [105]. The role of AmrAB-OprA in the high-level cethromycin resistance of *B. pseudomallei* clinical isolates was further assessed by measuring the *amrB* transcript levels, the *amrR* repressor gene and the *amrR-amrA* intergenic region for presence of mutations and deleting the *amrAB-oprA* operon [106].

The clinical significance of BpeEF-OprC was corroborated by sequencing genomes of isolates from patients suffering from melioidosis with increased resistance to chloramphenicol, ofloxacin and trimethoprim-sulfamethoxazole [120]. As an example, a large inversion of 800 kb resulted in a deletion of the last 24 codons of *bpeT*, coding for the transcriptional regulator of the efflux pump. All these data sustained the hypothesis that BpeEF-OprC efflux pump has an important role in antimicrobial resistance of *B. pseudomallei* [34]. In another report by Podnecky and co-workers, the BpeEF-OprC efflux pump has been shown to contribute to trimethoprim resistance in *B. pseudomallei* clinical and environmental isolates from northeast Thailand and northern Australia [110].

The role of efflux transporters in intrinsic drug resistance of clinical isolates was also shown in the case of *B. vietnamiensis* strains [115, 116]. The authors demonstrated that strains that acquired aminoglycoside resistance during infection and after exposure to tobramycin or azithromycin overexpressed AmrAB-OprM and contained missense mutations in its repressor gene *amrR* [116].

16.8 Efflux Pump Inhibitors

A main concern for the treatment of *Burkholderia* infections is the inability to eradicate them with the available drugs. A possible new approach could be a combination of antimicrobial agents with efflux pump inhibitors (EPIs). This could potentially improve the antimicrobial therapy as the phenotype driven by efflux transporters frequently results in multidrug resistance. In this way, an EPI appears useful to block many pumps at a time, thus rendering bacteria more susceptible to drugs. Moreover, EPI administration should reduce the rates of resistance development [121].

Unfortunately, until now no EPIs entered the clinical trials because of toxicity, even if many have been developed [122, 123]. As an example, phenylalanine-arginine-β-naphthylamide (PAβN) exhibited a great activity in *P. aeruginosa* [124, 125], but it is nephrotoxic. Also, inhibitors of the MexAB-OprM efflux system were

developed and tested *in vivo* but subsequently abandoned [126, 127]. Moreover, some generic uncouplers (such as CCCP) are available. However, these compounds, causing the dissipation of the proton motive force and reducing the viability of the cells themselves, are cytotoxic, so this road is not feasible.

Moreover, EPIs, which have been shown to be effective in one microorganism, not always showed their activity in other species. As an example, PAβN seems to be ineffective in the genus *Burkholderia* [91, 102]. Also, not all the compounds extruded by a pump are potentiated by PAβN because it works by competing with antimicrobials for their binding site [124].

The unsuccessful development of EPI could be ascribed to the difficulties in understanding the features and mechanism of action of efflux pumps, in particular of RND. In fact, only a few crystal structures of individual components of these pumps are available, while a comprehensive knowledge of their assembly and mechanism of translocation is missing. In this way, it is very difficult to predict the specificity of the potential inhibitor and to study its pharmacokinetics. This is complicated also by the fact that the EPI has to be administered together with other antimicrobials, and their pharmacokinetics should be tailored. Even if the structure of the inhibitor D13-9001 bound to AcrB has been solved [128], another main problem for the development of Gram-negative EPIs is the uptake inside the cell (which is preferential for small hydrophilic molecules) and the specific binding to the inner membrane portion of RND pumps (usually hydrophobic molecules). The genetic and biochemical studies and the computational methods which could help to overcome these issues have been recently reviewed by Opperman and Nguyen [129].

16.9 Concluding Remarks

This chapter presents the genus *Burkholderia*, which includes more than 90 species. These species show a high level of intrinsic drug resistance and have many virulence factors. One of the contributors to resistance is the presence of efflux pump-encoding genes in their genomes. These pumps are able to recognize and extrude very dissimilar compounds, thus rendering the bacteria particularly difficult to eradicate. We described the distribution of RND pump-encoding genes in the various *Burkholderia* genomes and showed the role of the principal RND pumps in *B. cenocepacia* (especially studied in our laboratory), in *B. pseudomallei*, in *B. mallei*, in *B. thailandensis* and in *B. vietnamiensis* in drug resistance. A brief description of non-RND systems (MFS and MATE) has been added for each species in which they were studied. The clinical significance of RND efflux transporters in *Burkholderia* spp. has been demonstrated, thus confirming the importance of the research in this field and highlighting the need for new therapeutic solutions to be combined with the existing antimicrobial drugs to overcome the resistance problem of these infections.

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