Chapter 1

Introduction

The *Burkholderia* genus

General features

The genus *Burkholderia* (belonging to β-proteobacteria) owes its name to the American phytopathologist W. H. Burkholder, who, in 1942, described two species, *Phytomonas caryophylli* and *Phytomonas alliicola*, as pathogens of carnation and onion, respectively (Burkholder, 1942). After that, in 1950, Burkholder reported that the causal agent of sour skin in onion was *Pseudomonas cepacia* (Burkholder, 1950), and this species would then become the type species of the current genus, acquiring several names through the years (Suarez-Moreno *et al.*, 2012). Indeed, it is only in 1992 that Yabuuchi *et al.* (Yabuuchi *et al.*, 1992), defines the new genus *Burkholderia* to accommodate seven species belonging to the rRNA group II of the genus *Pseudomonas* [*P. cepacia, P. caryophylli, Pseudomonas gladioli, Pseudomonas mallei, Pseudomonas pseudomallei, Pseudomonas solanacearum* and *Pseudomonas picketti*. These two latter species were subsequently transferred to the genus *Ralstonia* (Yabuuchi *et al.*, 1995)].

Today the *Burkholderia* genus comprises more that seventy species (Prokaryotic Nomenclature Up-to-Date, http://old.dsmz.de/microorganisms/bacterial_nomenclature.php), isolated from a wide ranges of niches. Many members of the genus can cause infection in plants, animals and humans, and most studies have thus focused on these pathogenic species due to their clinical importance (Suarez-Moreno et al., 2012). However, recently, an increasing number of *Burkholderia* species associated with plants or with the environment, and able to fix nitrogen, to nodulate legume or to promote plant growth, were described (Suarez-Moreno et al., 2012).

The taxonomy of the *Burkholderia* genus has been continuously revised and different phylogenetic trees obtained from independent gene sequence analysis (16S rRNA, *recA*, *gyrB*, *rpoB*, *acdS*), suggest that two main clusters may be

distinguished within the genus (Suarez-Moreno et al., 2012) (Figure 1). One cluster comprises the human obligate and opportunistic pathogens, the animals and plants pathogens, as well as the endosymbiontic species from phytopathogenic fungi. The second cluster contains the non-pathogenic *Burkholderia* species associated with plants and/or with the environment (Suarez-Moreno et al., 2012). This clustering is consistent with groupings derived from multilocus sequences typing and whole genome analysis (Suarez-Moreno et al., 2012).

Recently, Estrada-de los Santos *et al*. (Estrada-de los Santos *et al.*, 2013), proposed that *Burkholderia andropogonis* and *Burkholderia rhizoxinica/Burkholderia endofungorum* may represent two other different lineages or even two other genus.

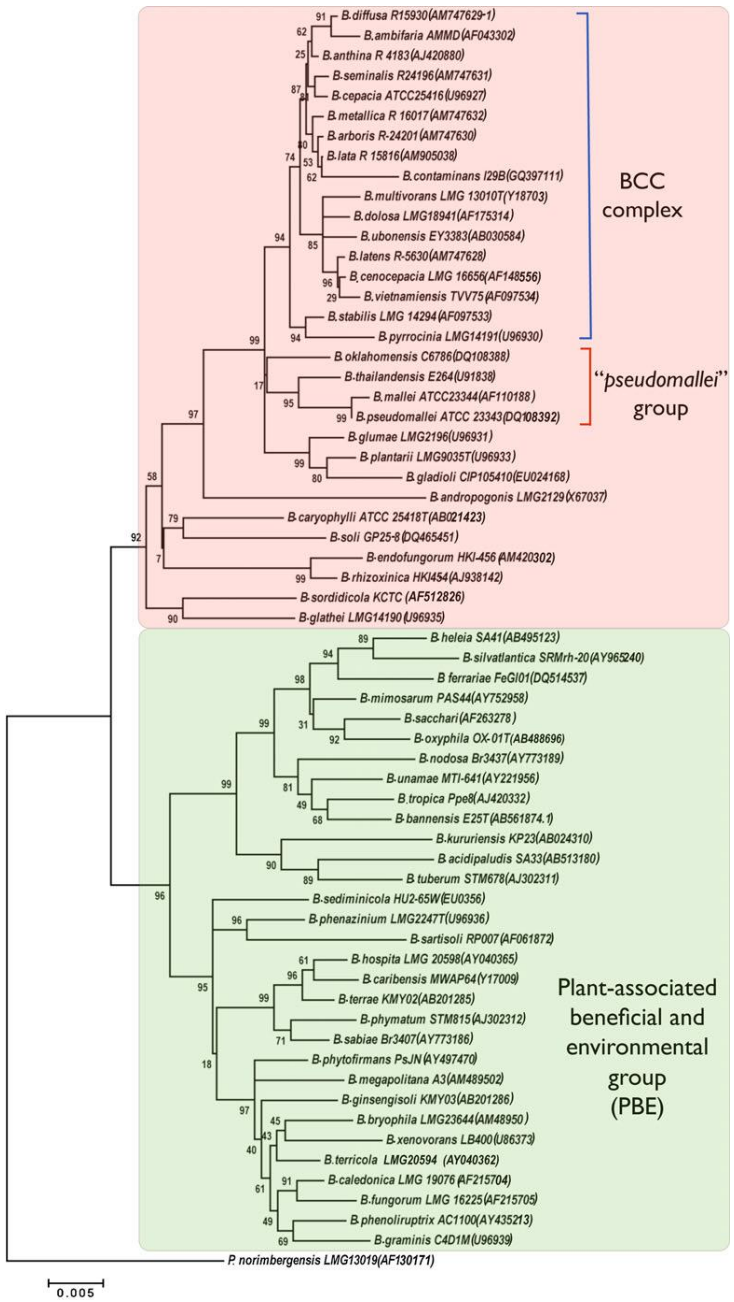


Figure 1: Phylogenetic tree based on 16S rRNA sequences of 62 species of the *Burkholderia* genus. Red—the pathogenic *Burkholderia* clade; green—plant-associated beneficial and environmental (PBE) group [from (Suarez-Moreno et al., 2012)].

The plant associated beneficial and environmental (PBE) *Burkholderia* group

The so-called "plant-beneficial-environmental (PBE) *Burkholderia* cluster" contains closely related species that have been isolated from all the continents. Many of them have been identified in association with plants, with which they may have epiphytic, endophytic, or endosymbiontic interactions, but species from this cluster may also be found free-living in the soil or even associated with fungi and insects. So far, species of this group have not been reported to cause a detrimental effect on plant (or animal) hosts (Suarez-Moreno et al., 2012), although a few reports exist of single strains of some of these species that were isolated from animal or human clinical samples (Estrada-de los Santos et al., 2013)

Several species of this group are able to convert atmospheric nitrogen to ammonia *via* biological nitrogen fixation (BNF), or have the ability to nodulate legumes or to degrade aromatic compounds. In addition most of them are catabolically versatile in that they are able to degrade recalcitrant compounds, and thus to survive in environments with limited nutrient availability. Some species are able to promote plant growth, while others are proposed for biotechnological uses, such as phytoremediation and biocontrol (Suarez-Moreno et al., 2012). Importantly, all of these species share a common *quorum sensing* (QS) system, which therefore appears to be part of the *core* genomes of this group of species. Details of their relationships, genome contents, and adaptations to specific niches will became clearer as more genomes of these species are sequenced and available (Suarez-Moreno et al., 2012).

Plants, animals and humans pathogens

Several species of the genus *Burkholderia* can induce plant disease. For example *B. carophylli* induces formation of bacterial wilt in various plant species while *Burkholderia plantarii* provokes seedling. *Burkholderia gladioli* induces bacterial soft rot in onions, leaf-sheath browning and grain rot in rice, and leaf and corm

disease in gladiolus ad iris species, *Burkholderia glumae* causes seedling and grain rot in rice and wilting symptoms in tomato, sesame, perilla, eggplant and hot pepper as well in other 20 plant species and *Burkholderia andropogonis* can infect more than 52 species of 15 families of unrelated monocotyledonous and dicotyledonous (Compant *et al.*, 2008).

Moreover, some phytopathogenic fungi can contain members of the genus *Burkholderia* as endosymbionts. For example *Burkholderia endofungorum* and *Burkholderia rhizoxinica* are two endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*, involved in the production of the causal agent of rice seedling blight, rhizoxin, and the toxic cyclopeptide rhizonin (Partida-Martinez *et al.*, 2007).

Interactions between burkholderias and humans or animals are traditionally known for *Burkholderia mallei* and *Burkholderia pseudomallei* that are the aetiological agents of glanders and mieloidosis respectively (Coenye & Vandamme, 2003).

B. pseudomallei is endemic to South-east Asia, Northern Australia and temperate regions that border the equator (Coenye & Vandamme, 2003). Melioidosis presents as a broad range of conditions from acute fulminant pneumonia and septicemia acquired by inhalation to wound infections acquired through inoculation of the bacteria from soil through skin abrasion (Choh *et al.*, 2013). *B. pseudomallei* is now classified as Category B priority agent and represents a worldwide emerging infectious disease problem and a bioterrorism threat due to its severe course of infection, aerosol infectivity, low infectious dose, an intrinsic resistance to commonly used antibiotics, lack of a currently available vaccine and the world wide availability (Choh et al., 2013). A characteristic of this bacterium is the ability to remain latent in the host and cause infections following many years past the initial infection. Regardless of antibiotic therapy rapid progress of acute melioidosis to sepsis, followed by death within 48h of clinical onset has been reported (Choh et al., 2013) .

Burkholderia thailandensis is closely related to *B. pseudomallei*, but in spite of a genes similarity of 99% between the two bacteria, *B. thailandenisis* is apparently non-pathogenic in humans (Smith *et al.*, 1997). This is probably due to the presence, in *B. thailandensis* genome, of a complete arabinose biosynthesis operon, which is an anti-virulence property. This operon is largely deleted in the *B. pseudomallei* genome (Moore *et al.*, 2004, Lazar Adler *et al.*, 2009). Like *B. pseudomallei*, *B. thailandensis* also has an intracellular lifecycle and is often used as a model to study various aspects of *B. pseudomallei* and *B. mallei*.

B. mallei cause glanders typically in solipeds, such as horses, mules, and donkeys. Only occasionally *B. mallei* infect humans (Choh et al., 2013). It has been classified as Category B priority agent, because was among the first bioweapon used during both World Wars I and II, due to its ability to infect *via* inhalation (Wheelis, 1998). The course of infection is reliant on the route of exposure and in an acute infection, general symptoms include fever, malaise, abscess formation, pneumonia, and sepsis. Untreated septicemic infections have a fatality rate of 95% where for antibiotic-treated individuals the 50% fatality has been reported. *B. mallei* is susceptible *in* vitro to various antibiotics, but their efficacies *in vivo* are not well established (Choh et al., 2013).

Finally, several *Burkholderia* species occupy multiple niches, may have both pathogenic and symbiotic interactions with plants, and have become known as opportunistic pathogens in humans. Although they are not considered important pathogens for the normal human population, some are considered serious threats for specific patient groups such as Cystic Fibrosis (CF) patients. These species include *Burkholderia gladioli* and all *Burkholderia cepacia* complex (Bcc) bacteria (Coenye & Vandamme, 2003).

The *Burkholderia cepacia* complex (Bcc)

The *Burkholderia cepacia* complex (Bcc) is a group of closely related bacterial species, with a high (>97,5%) level of 16S rRNA gene sequence similarity and moderate (30-60%) DNA-DNA hybridization value (Coenye *et al.*, 2001c, Vandamme *et al.*, 1997). They posses unusually large genomes (7,5-8,5 Mb), with a GC content of approximately 67mol% and divided in multiple replicons. These large genomes provide them not only unsurpassed metabolic capacities, but also genotypic and phenotypic characteristics that defy our need to classified bacteria in well delineated groups (Vandamme & Dawyndt, 2011). An overview of the 18 actually validly named species within the complex and their isolation sources is reported in Table 1 (Vandamme & Dawyndt, 2011, Peeters *et al.*, 2013).

Despite the progress made in recent years in refining the taxonomy of Bcc bacteria, the differentiation of these species from other related taxa (such as *Ralstonia*, *Cupriavidus*, *Pandorea*, *Achromobacter*, *Brevundimonas*, *Comamonas* and *Delftia* species) is still challenging. Furthermore differentiation of species within the Bcc can be particularly problematic (Vandamme & Dawyndt, 2011).

In the last decades several techniques have been developed and used for classification and identification of Bcc species, such as growth on selective media (Bevivino *et al.*, 1994, Henry *et al.*, 1999), classical biochemical tests and commercial biochemical microtest systems (Henry *et al.*, 2001, Kiska *et al.*, 1996), whole cell protein electrophoresis (Vandamme *et al.*, 1996, Vandamme et al., 1997), whole cell fatty acid analysis (Welch, 1991, Clode *et al.*, 1999, Vandamme et al., 1997), DNA-DNA and DNA-rRNA hybridization (Vandamme et al., 1997), several PCR- based techniques, including 16S rRNA analysis (Vandamme & Dawyndt, 2011), amplified fragment length polymorphism analysis of genomic DNA (Coenye *et al.*, 1999), ribotyping (Brisse *et al.*, 2004, Brisse *et al.*, 2000), restriction fragment length polymorphism analysis of PCR amplified gene fragments (Mahenthiralingam *et al.*, 2000, Segonds *et al.*, 1999, Vermis *et al.*, 2002), tRNA profiling (Storms *et al.*,

2002), Fourier transform infrared spectroscopy (Bosch *et al.*, 2008, Coutinho *et al.*, 2009), *fur* and *hisA* gene sequence analysis (Lynch & Dennis, 2008, Papaleo *et al.*, 2010) and matrix assisted laser desorption ionization time of flight mass spectroscopy (Degand *et al.*, 2008, Vanlaere *et al.*, 2008b).

Most of these techniques were either not very sensitive, not very specific, or neither sensitive nor specific and even when some methods apparently proved useful for Bcc species identification, their sensitivity and specificity needed constant re-evaluation because of the regular emergence of novel members of the complex (Vandamme & Dawyndt, 2011). Recently, *recA* gene analysis (that allow to identify four subpopulations in *Burkholderia cenocepacia*, referred to as *B. cenocepacia* IIIA, IIIB, IIIC, IIID (Mahenthiralingam et al., 2000, Vandamme et al., 2002)) and multilocus sequence-based approaches emerged as powerful, objective and portable taxonomic tools that have been shown to be very useful for both identification and classification purpose (Vandamme & Dawyndt, 2011). In particular a scheme for the amplification and sequence analysis of seven loci that can be used for both multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) of Bcc bacteria was developed by Baldwin *et al.* (Baldwin *et al.*, 2005). This scheme allows to differentiate all the 18 current Bcc species (Baldwin et al., 2005, Peeters et al., 2013).

Finally, the increasing number of whole-genome sequences available is very helpful in identification and classification of these species (Vandamme & Dawyndt, 2011).

Table 1: Overview of *Burkholderia cepacia* complex species and their sources of isolation (CF, Cystic Fibrosis) (adapted from (Vandamme & Dawyndt, 2011)

Name	Type strain	Habitat	Reference
Burkholderia ambifaria	LMG 19182	Humans (CF and non-CF), soil, rhizosphere soil	(Coenye *et al.*, 2001b)
Burkholderia anthina	LMG 20980	Humans (CF), turtle, soil, rhizosphere soil, river water, plant, hospital contaminant	(Vandamme *et al.*, 2002)
Burkholderia arboris	LMG 24066	Humans (CF and non-CF), soil, rhizosphere soil, river water, industrial contaminant	(Vanlaere *et al.*, 2008a)
Burkholderia cenocepacia	LMG 16656	Humans (CF and non-CF), soil, rhizosphere soil,plant, river water, industrial contaminant	(Vandamme *et al.*, 2003, Vandamme *et al.*, 1997)
Burkholderia cepacia	LMG 1222	Humans (CF and non-CF), soil, rhizosphere soil,plant, river water	(Palleroni & Homes, 1981, Vandamme et al., 1997)
Burkholderia contaminans	LMG 23361	Humans (CF and non-CF), sheep, contaminant,plant	(Vanlaere *et al.*, 2009)
Burkholderia diffusa	LMG 24065	Humans (CF and non-CF), soil	(Vanlaere et al., 2008a)
Burkholderia dolosa	LMG 18943	Humans (CF and non-CF), plant, rhizosphere soil	(Coenye *et al.*, 2001a, Vermis *et al.*, 2004)
Burkholderia latens	LMG 24064	Humans (CF)	(Vanlaere et al., 2008a)
Burkholderia lata	LMG 22485	Humans (CF and non-CF), soil, rhizosphere soil,plant, river water, industrial contaminant	(Vanlaere et al., 2009)
Burkholderia metallica	LMG 24068	Humans (CF)	(Vanlaere et al., 2008a)
Burkholderia multivorans	LMG 13010	Humans (CF and non-CF), soil, rhizosphere soil,plant, river water, contaminant	(Vandamme et al., 1997)
Burkholderia pseudomultivorans	LMG 26883	Humans (CF and non-CF), rhizosphere	(Peeters *et al.*, 2013)
Burkholderia pyrrocinia	LMG 14191	Humans (CF and non-CF), soil, rhizosphere soil, river water	(Storms *et al.*, 2004, Vandamme et al., 2002, Vandamme *et al.*, 2000)
Burkholderia seminalis	LMG 24067	Humans (CF and non-CF), soil, rhizosphere soil, plant	(Vanlaere et al., 2008a)
Burkholderia stabilis	LMG 14294	Humans (CF and non-CF), hosptal contaminans, plant	(Vandamme et al., 1997, Vandamme et al., 2000)
Burkholderia ubonensis	LMG 20358	Humans (CF), soil	(Vandamme et al., 2003, Vanlaere et al., 2008a, Yabuuchi *et al.*, 2000)
Burkholderia vietnamiensis	LMG 10929	Humans (CF and non-CF), soil, rhizosphere soil,plant, river water, industrial contaminant	(Gillis *et al.*, 1995, Vandamme et al., 1997)

***Burkholderia cepacia* complex friends or foes?**

The large genomes of Bcc species provide them the capacity to use a wide array of compounds as carbon sources, allowing these bacteria to colonize extremely diverse habitats (Chiarini *et al.*, 2006).

Although the first species belonging to Bcc (*B. cepacia*) has been described as responsible for causing a soft rotting disease in onions (Burkholder, 1950), Bcc species are not important phytopathogens in comparison with other *Burkholderia* species, and the main commercial crops that are affected by Bcc bacteria are onions (Figure 2) (Mahenthiralingam *et al.*, 2005).

In natural environments Bcc species can be found in several soil ecosystems, where, in particular, they inhabit the plant rhizhosphere rather than the soil, and they are generally reported among the dominant bacteria in the rhizosphere of several plants (e.g. rice, maize, pea or cotton) (Vial et al., 2011). Most studies have focused on the maize rhizosphere and revealed that there are differences in the geographical distribution of the species between separate maize rhizosphere locations, with the majority of Bcc isolated belonged to *B. cepacia*, *B. cenocepacia*, *Burkholderia ambifaria* and *Burkholderia pyrrocinia* species (Vial et al., 2011). Bcc bacteria can be also frequently found in stream waters and sediments, while oceans or seas cannot be considered as natural reservoirs (Vial et al., 2011).

Bcc species are considered highly beneficial in the environment: many isolates protect commercially useful crops against bacterial and fungal diseases, can promote plants growth, fix nitrogen and degrade several man-made toxic agents, in particular chlorinated aromatic compounds (Figure 2) (Vial et al., 2011, Mahenthiralingam et al., 2005, Chiarini et al., 2006). The ecologically useful features of these bacteria have generated considerable interest in their possible biotechnological application in agriculture and industry. Although potentially highly beneficial, such widespread commercial use of these bacteria has also raised concerns that the risks of infection for vulnerable individuals might be increased

(see next paragraph), and stringent rules was issued to limit the biotechnological use of Bcc (Chiarini et al., 2006, Mahenthiralingam et al., 2005).

In addition to their presence in different natural environments as free living organisms, Bcc species may also have an intracellular lifestyle and they can be found in association with both plant and animal cells (Vial et al., 2011). Some Bcc strains have been isolated from inside plant cells or tissues, but until now, internal localization of these strains in plants seems to reflect an opportunistic lifestyle rather than an evolutional adaptation, but only few studies about the endophytic lifestyle of Bcc species are available (Vial et al., 2011). Intracellular lifestyle of Bcc species have been more intensively studied in animal cells (Vial et al., 2011). For example amoeba have been suggested to act as a natural reservoir for this strains. They are very frequent in the environment and they can be found also in human

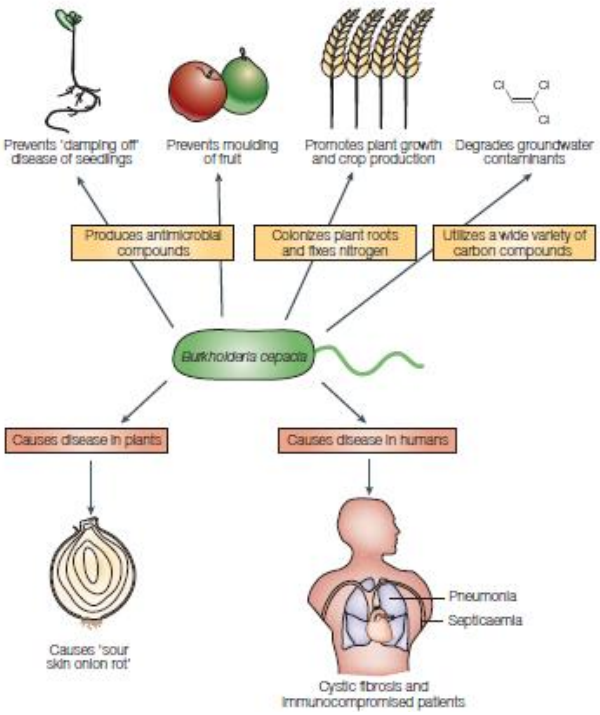


Figure 2: Beneficial and detrimental effects of the *Burkholderia cepacia* complex (Mahenthiralingam et al., 2005)

nasal mucosa. Consequently, they can act as a Trojan horse allowing bacteria to access to respiratory tract (Marolda *et al.*, 1999). Intracellular survival of Bcc strains after phagocytosis was demonstrated in human respiratory epithelial cell and in several human or murine phagocytic cells (Vial et al., 2011). All these elements indicate that a number of Bcc strains are well adapted to survive and even replicate, intracellularly in several eukaryotic cells, especially those implicated in innate immunity and these properties confer them obvious advantages to persist in mammalian lungs (Saldias & Valvano, 2009). Survival and persistence of Bcc bacteria within host cells and tissues are believed to be linked to the life-threatening infections that they can cause in individuals affected by two types of genetic disorders, namely Cystic Fibrosis (CF) and Chronic Granulomatous Disease (CGD) (Saldias & Valvano, 2009, Mahenthiralingam *et al.*, 2008).

***Burkholderia cepacia* complex and Chronic Granulomatous Disease**

Chronic Granulomatous Disease (CGD) is a rare inherited primary immunodeficiency characterized by the absence or malfunction of the NADPH oxidase in phagocytic cells. As a consequence, there is an impaired ability to generate superoxide anions and the subsequent reactive oxygen intermediates and CGD patients suffer from two clinical manifestations: recurrent, life-threatening bacterial and fungal infections and excessive inflammatory reactions leading to granulomatous lesions (Ben-Ari *et al.*, 2012). Patients are particularly susceptible to catalase-positive microorganisms, including *Staphyloccocus aureus*, *Nocardia* spp. and Gram-negative bacteria, such as *Serratia marcescens*, Bcc spp. and *Salmonella* spp. Unusually, catalase-negative microorganisms were also reported (Ben-Ari *et al.*, 2012).

In particular, Bcc species seem to be especially virulent. For example, *B. cenocepacia* induce necrosis of neutrophils in CDG patients but not in healthy donors, adding to the physiologic apoptotic effect. These elements lead to an abnormal inflammatory state that could explain the gravity of infections caused by

Bcc. Also *Burkholderia multivorans* displays increased association and invasion toward phagocytic cells isolated from CDG patients (Vial et al., 2011). In general, there is a great variation between species or even strains virulence among patients, which results in unpredictable prognostics (Vial et al., 2011).

Burkholderia cepacia **complex and Cystic Fibrosis**

Cystic Fibrosis (CF), also known as mucoviscidosis, is the most fatal genetic disease of Caucasians (Vial et al., 2011); it affects about 1 in 2500-2600 newborns, while it is estimated that one person in every 26 is a carrier of the disease. CF, which is transmitted as an autosomal recessive disorder, is caused by the abnormal functioning of CFTR (Cystic Fibrosis Transemembrane Conductance Regulator) protein that is involved in regulation of the passage of chloride ions across the apical membrane of epithelial cells. The CFTR coding gene was found at the q31.2 locus of chromosome 7 (Riordan *et al.*, 1989). More than 1500 different mutations have been identified, that can lead to a partially or totally non-functioning protein, or even to its complete absence. The most common mutation, ΔF508, is a deletion of three nucleotides that results in a loss of the amino acid phenylalanine (F) at the 508th position on the protein. This mutation accounts for 70% of CF cases worldwide (Riordan et al., 1989, Kerem *et al.*, 1989, Rommens *et al.*, 1989).

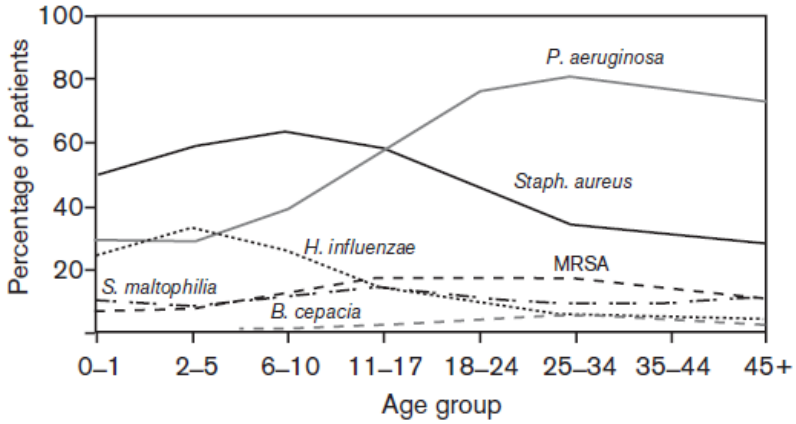
The malfunction, or the absence of CFTR protein, causes abnormal exocrine secretions, which result thick and viscous. These abnormal secretions lead to a progressive deterioration of involved organs. The disease can occur in very different forms between diverse patients, and there is no relationship with the type of mutations present: the same mutations may be found in patients with very different symptoms. In the most severe form different organs and systems are affected, in particular the gastrointestinal and respiratory systems.

The airways of CF patients are clogged by thick and viscous mucus, that is colonized by pathogenic micro-organisms in infancy, and in the vast majority of cases chronic infections are established. Most patients experience recurrent acute respiratory

episodes and eventually die of respiratory failure resulting from infection (Lyczak *et al.*, 2002). The main cause of morbidity and mortality in patients with CF is chronic lung infection with *Pseudomonas aeruginosa*. Other respiratory pathogens play a role at different stages of the lung disease of CF patients, such as *Staphylococcus aureus* and *Haemophilus influenzae* in infants and children and Bcc, *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* and nontuberculous mycobacteria (NTM) in adults (Figure 3a). A role of infection with anaerobe bacteria such as *Prevotella intermedia* in exacerbations of the disease has also been shown in some patients (Ciofu *et al.*, 2013). The range of species reported, the so called "CF microbiome", has widened over the past few decades as life expectancy has increased and detection methods have improved, but in most cases, their role in pathogenesis remains to be confirmed. Pathogenic viruses (e.g. respiratory syncytial virus (RSV), adenoviruses, influenza) and fungi (e.g. Aspergillus and Candida species) are also common (Harrison, 2007). Moreover, co-infections involving different species of bacteria, or bacteria, fungi and viruses, are common and probably the norm, and co-infecting species interact, both syngergistically and antagonistically, and finally, pathogen populations within the lung evolve in response to selection pressures exerted by the within-host environment and by other members of the community (Figure 3b) (Harrison, 2007).

Regarding Bcc species, the prevalence (2009 and 2010) of chronic infection is reported to vary between 0 and 12% of the CF population attending various CF centres (Ciofu et al., 2013). Although it is not high compared to other CF pathogens, mortality in Bcc-infected CF patients is high. Indeed, a common complication of infection is a rapid and uncontrollable clinical deterioration, which included necrotizing pneumonia and septicaemia that result in early death. This rapid decline in patient status is known as "cepacia syndrome" as the bacteremia that is associated with this syndrome rarely occurs during infection with other CF pathogens (Mahenthiralingam et al., 2005, Vial et al., 2011). However, variability

3 a)



3 b)

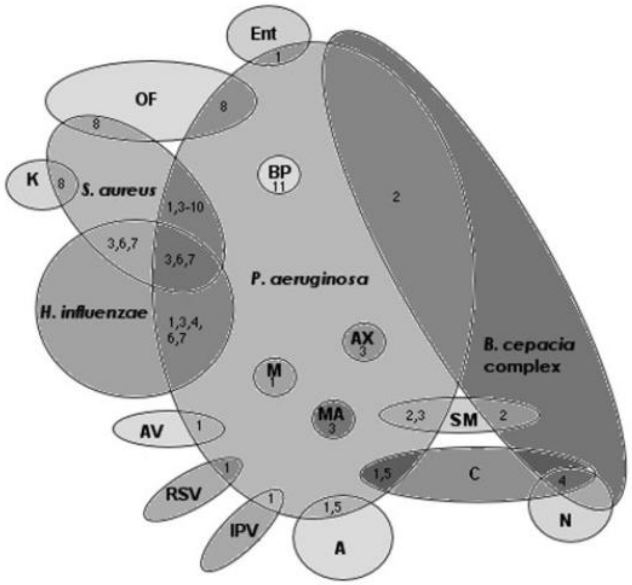


Figure 3: a) Prevalence of bacterial respiratory infections by age group in CF subject. MRSA, methicillin resistant *S. aureus* (Harrison, 2007). b) Venn diagram showing reported co-infection of the CF airways. A, *Aspergillus* spp., AV, adenovirus, AX, *A. xylosoxidans*; BP, bacteriophage; C, *Candida* spp.; Ent, enterobacteria; IPV, influenza and/or parainfluenza virus; K, *Klebsiella* spp.; M, mycoplasma; MA, *Mycobacterium abscessus*; N, *Neisseria* spp.; OF, oropharyngeal flora; RSV, respiratory syncytial virus; SM, *S. maltophilia*. (Harrison, 2007)

in clinical outcome has since been observed in patients infected with the same clonal strain. Overall, infection with Bcc in CF patients correlates with poorer prognosis, longer hospital stays and an increased risk of death (Mahenthiralingam et al., 2005). Lung transplantation in Bcc-infected patients is complicated compared with other CF pathogens, and the mortality within the first years post-transplant is very high. In some transplantation centres, chronic Bcc infection is a contraindication to lung transplantation, because of the worse outcome compared with patients infected with other pathogens (Ciofu et al., 2013).

Prevalence of each Bcc species in CF lungs has been investigated. The distribution is disparate between species, and depends on the geographic localization. The most widespread is *B. cenocepacia*, followed by *B. multivorans*, although the tendency is becoming inverted over recent years (Vial et al., 2011). Bcc species are not commensal bacteria, and the contamination of CF patients comes from environment (20% of strains isolated from clinical samples are clonal with environmental isolates), from organisms, like amoeba, that can be reservoir of Bcc strains, from the contamination of numerous medical solutions, and from transmission among patients (Vial et al., 2011). Bcc transmission is thought to occur by aerosol droplet and direct physical contact with infected individuals or contaminated surfaces, and tight control strategies are imposed on patients to avoid infection (Mahenthiralingam et al., 2005).

***Burkholderia cepacia* complex virulence factors**

As seen in the previous paragraph, *B. cenocepacia* and *B. multivorans* are the two Bcc species that predominate in CF patients. In particular *B. cenocepacia* is considered one of the most serious pathogens because it is frequently associated with reduced survival and highest risk of developing fatal cepacia syndrome and it also contains the majority of epidemic strains described so far, ET-12, Midwest and PHDC lineages (Mahenthiralingam et al., 2005, Drevinek & Mahenthiralingam, 2010). In particular, *B. cenocepacia* ET-12 is responsible for arguably the largest CF

epidemic, which occurred across Canada and United Kindom in the late 1980s and throughout much of the 1990s (Mahenthiralingam et al., 2005). Most of the studies on mechanisms of Bcc virulence have been conducted on this lineage, and in particular on *B.cenocepacia* J2315 (lineage IIIA), whose completely sequenced genome is available (Holden *et al.*, 2009). Figure 4 reported most of the virulence factor identified in Bcc [from (Loutet & Valvano, 2010)].

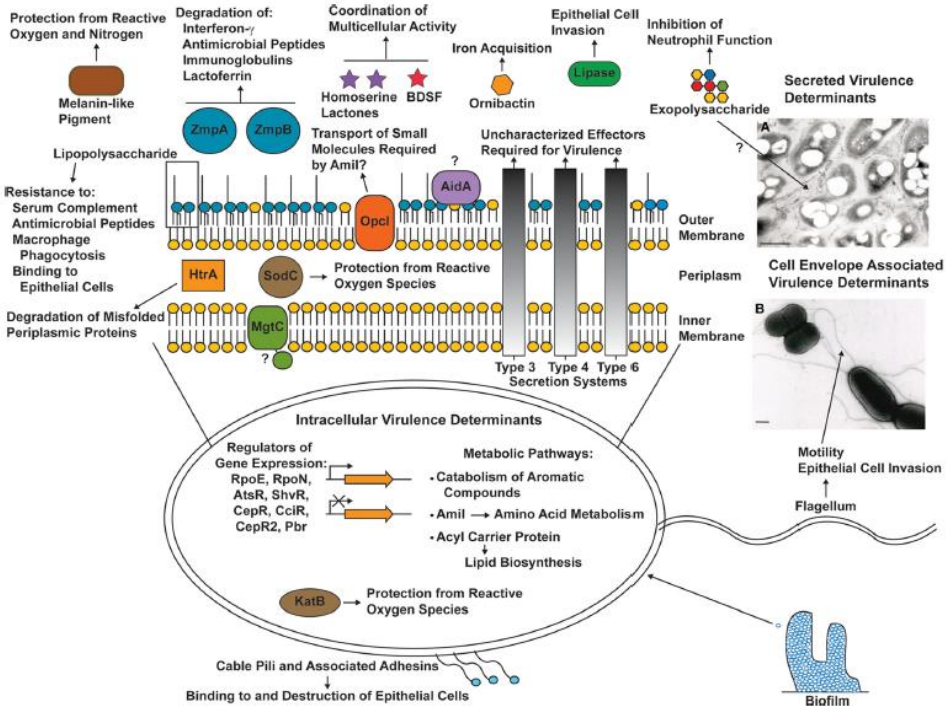


Figure 4: Representation of the localization and known functions of *B. cenocepacia* virulance determinants (Loutet & Valvano, 2010)

Genomic islands and mobile elements

The genome of *B. cenocepacia* J2315 contain fourteen genomic islands (9.3% of the entire size), most of which are still uncharacterized (Drevinek & Mahenthiralingam, 2010, Loutet & Valvano, 2010). None of these islands is conserved in the IIIB lineages available genomes. The most studied is genomic island II, originally

designated as *B. cenocepacia* island (*cci*). The cci encodes the *B. cepacia* epidemic strain marker (BCESM), a marker of epidemic strains. Several coding sequences are included on these genomic island, including putative virulence enhancing factor, like an amidase (AmiI), an outer membrane porin (OpcI) and a *quorum sensing* system *cciRI*. The island also contains a copy of IS*Bcen14*, one of the 22 different IS elements identified in J2315 genome (Loutet & Valvano, 2010, Drevinek & Mahenthiralingam, 2010).

Recently, a genomic island absent from strain J2315 was found in strain K56-B, another ET-12 lineage strain (Loutet & Valvano, 2010).

Alternative sigma factor and related proteins

Alternative sigma factor are regulatory proteins that activate transcription of particular gene subsets by binding at sites within promoter regions and interacting with the RNA polymerase complex, to allow the initiation of transcription. Two alternative sigma factors are identified in *B. cenocepacia* RpoE and RpoN, both required for the ability of engulfed strains to delay phagolysosomal fusion in murine macrophages. RpoE is also required for the ability to growth under conditions of high osmolarity and high temperature, while RpoN is also necessary for motility and biofiom formation (Loutet & Valvano, 2010).

Quorum sensing

Quorum sensing (QS) allows regulation of gene expression on the basis of the density of the bacterial population. Bacteria secrete compounds, usually N-acyl-homoserine lactones (AHL) products by a specific synthase, that accumulate outside the cell, until sufficient cell densities are reached and the concentration of diffusible compounds reach a threshold. Then a regulator, responsive to AHLs, begin to alter gene expression (Loutet & Valvano, 2010).

The *cepRI* system, which mediates the production of a N-octanoylhomoserine lactone, is the first QS system identified in *B. cenocepacia* and it is conserved in all Bcc species. It regulates numerous functions, including siderophore synthesis, protease production, a type III secretion system, motility, biofilm formation and

virulence in different model of infection. It is probably involved also in cross-species communication with *P. aeruginosa* (Loutet & Valvano, 2010, Drevinek & Mahenthiralingam, 2010, Mahenthiralingam et al., 2005).

B. cenocepacia also possesses genes for a second homoserine lactone producing QS system included in the cci, designated *cciRI*, and an orphan regulator, designated *cepR2*, that is not associated with any adjacently encoded AHL synthase. There is also a QS system, that is not based on N- homoserine lactones, but on *cis*-2-dodeconic acid, named BDSF system (Loutet & Valvano, 2010, Drevinek & Mahenthiralingam, 2010).

Analysis of CepR, CciIR, CepR2 and RpfF (BDSF synthase) QS regulons revealed that these QS systems both independently regulate and co-regulate many target genes, often in an opposing manner, suggesting a complex network of gene regulation in response to bacterial cell density (Loutet & Valvano, 2010, Subramoni & Sokol, 2012). The role of QS and several QS-regulated genes in virulence has been determined using vertebrate, invertebrate and plant infection models. Virulence phenotypes are strain and model dependent, suggesting that different QS-regulated genes are important depending on the strain and type of infection (Subramoni & Sokol, 2012).

Biofilms

Biofilms are complex, multicellular bacterial communities that can protect bacteria from antibiotics and the host immune systems. Bcc bacteria are thought to live in CF lungs in biofilms, often in mixed biofilms with *P. aeruginosa*, with which they may even communicate *via* QS systems (Loutet & Valvano, 2010). *B. cenocepacia* can form biofilms also *in vitro*. The genes required for biofilm formation have been identified, and can be divided into three main classes: genes that encode surface proteins, genes that are involved in the biogenesis and maintenance of outer membrane integrity and genes that encode regulatory factor (Mahenthiralingam et al., 2005). Biofilm formation can be affected by multiple gene regulation systems, including QS, and is also affected by motility and iron availability (Loutet & Valvano,

2010). The reports concerning the increased antibiotic resistance in *B. cenocepacia* growing in biofilm compared to that of planktonic cells are conflicting (Loutet & Valvano, 2010).

Biofilm formation is a complex process involving numerous virulence determinant and its disruption is an attractive target for the development of new antibiotics.

Lipopolysaccharide (LPS) and exopolysaccharide (EPS)

Lipopolysaccharide, LPS, is composed of lipid A, core oligosaccharide and O-antigen and is a virulence factor in many Gram-negative bacteria. The LPS of Bcc bacteria is distinct because the core oligosaccharide does not contain as much phosphate or 3-deoxy-D-manno-oct-2-ulosonic acid as that of other Gram-negative bacteria. Furthermore, there are 4-amino-4-deoxyarabinose moieties (Ara4N) attached to the phosphate residues in the lipid A backbone; these have been implicated in resistance to the antibiotics effects of cationic antimicrobial peptides and polymixin (Mahenthiralingam et al., 2005).

All the genes for LPS production are located on chromosome 1 with three main clusters for lipid A, core and O-antigen (Drevinek & Mahenthiralingam, 2010). The latter cluster is part of a genomic island with a low GC content, indicating that is might have been acquired through horizontal gene transfer, as has been previously suggested for O-antigen gene clusters from other bacteria (Mahenthiralingam et al., 2005). The O-antigen is not expressed in some *B. cenocepacia* strains, such as J2315, where there is an interruption of the *wbcE* gene by IS*Bcen20* (Drevinek & Mahenthiralingam, 2010).

Bcc LPS is four to five times more endotoxic of that of *P. aeruginosa* and induces increased neutrophil burst activity and induction of interleukin-8 (IL-8) from epithelial cells (Mahenthiralingam et al., 2005).

Extracellular polysaccharides or exopolysaccharides (EPSs) are high-molecular weight sugar-based polymers that are synthesized and secreted by many microorganisms. Many *Burkholderia* strains have the ability to produce EPS and at least seven different exopolysaccharides have been identified and their structure

determined. Some strains produce a single exopolysaccharide while others produce mixtures. The most common EPS produced by *Burkholderia* is cepacian and has been identified in different species (Ferreira *et al.*, 2011).

The presence of these EPS gives to bacterial colonies mucoid appearance (Drevinek & Mahenthiralingam, 2010). EPS protects bacterial cells from external stresses and contributes to the their virulence through inhibiting both neutrophil chemotaxis and generation of H_2O_2 and O_2^-. The presence of EPS also results in slower clearance of bacteria from murine lungs (Loutet & Valvano, 2010).

B. cenocepacia J2315 appears unable to produce any EPS even though several gene clusters implicated in EPS biosynthesis were identified within its genomes. Cepacian, for instance, is not expressed as a result of a frameshift mutation in *bceB* encoding a putative glycosyltransferase. The role of EPS in Bcc virulence is similar to that of the O-antigen: when expressed, it appears to enhance the virulence of the producer isolate, but the absence of EPS does not rule out that a Bcc strain may still cause a severe infection (Drevinek & Mahenthiralingam, 2010).

Cable pili and 22-kDA adhesin

Bacterial fimbriae (pili) that are expressed in the cell surface can be involved in pathogenesis, mainly through adherence to host cells. Between the five type of pili identified in Bcc strains, only cable (Cbl) pili were found to be associated with epidemic strains. Cable pili are large (2-4 nm), peritrichously expressed appendages, which seem to tether large aggregates of cells. *B. cenocepacia* strains express Cable pili together with an associated 22 kDa adhesin, AdhA. They have been shown to play an essential role in the invasion of respiratory epithelium, using Cytokeratin 13 (CK13) as a host-cell receptor, whose expression is increased in CF airway epithelial cells (Mahenthiralingam et al., 2005).

Flagella

Flagellum is an important virulence factor that besides allowing motility, also serves as an adhesin and enables pathogens to invade host cells. Genes for synthesis and assembly of flagella in *B. cenocepacia* J2315 are distributed within five clusters on

chromosome 1, with two additional genes found on chromosome 2 and 3 (Drevinek & Mahenthiralingam, 2010). Disruption of flagellum results in a non-motile strain that is avirulent in the mouse agar bead model of infection and increased transcriptional activity of flagellar genes was detected in *B. cenocepacia* when the organism was incubated in CF sputum (Drevinek & Mahenthiralingam, 2010, Loutet & Valvano, 2010). Retained motility may account for the pathogen's ability to invade host cells and to cause lifethreatening septicaemia (Drevinek & Mahenthiralingam, 2010). Moreover, bacterial flagellin causes an increased inflammatory response, through an increase of IL-8 level, which leads to increased damage to the lung (Mahenthiralingam et al., 2005).

Iron uptake

The amount of freely available iron in human lungs is very low to a prospective pathogen, and accordingly members of the Bcc appear to possess efficient mechanisms for iron capture. These bacteria specify up to four different types of siderophore (ornibactin, pyochelin, cepabactin and cepaciachelin) that employ the full repertoire of iron-binding groups present in most naturally occurring siderophores (Thomas, 2007). The predominant siderophore produced by most strains of *B. cenocepacia* appears to be ornibactin, while some strains also synthesize small amounts of pyochelin (Drevinek & Mahenthiralingam, 2010). Members of the Bcc are also capable of use some exogenous siderophores that they are not able to synthesize. In addition to siderophore-mediated mechanisms of iron uptake, the Bcc possess mechanisms for acquiring iron from heme and from ferritin. The Bcc therefore appear to be well-equipped for life in an iron-poor environment (Thomas, 2007).

Secretion systems and secreted proteins

Many pathogenic bacteria employ specialized systems for the secretion of effector molecules that contribute to cause disease by disrupting host cellular processes. A role in *B. cenocepacia* virulence has been demonstrated for a type III secretion

system (T3SS), for one of the two predicted type IV secretion systems (T4SS) and recently for a type VI (T6SS) secretion systems (Loutet & Valvano, 2010).

B. cenocepacia produces two distinct extracellular zinc metalloproteases, named ZmpA and ZmpB, which play a role in virulence and also secreted lipases that have been implicated in its ability to cause disease (Loutet & Valvano, 2010).

Colony variants

B. cenocepacia can produce different colony morphologies, at least one of which, the shiny colony morphology variant (shv), results in bacteria that are less virulent with decreases in biofilm formation, motility, extracellular matrices and siderophore production. Some Shv isolates are due to a spontaneous mutation in a LysR-type regulator (ShvR), while others do not have this spontaneous mutation, suggesting that there are multiple pathway that can lead to this phenotype (Loutet & Valvano, 2010).

Resistance to ossidative stress

Phagocytic cells produce reactive oxygen species to help in eliminating bacteria. To resist the harmful effects of oxidative stress, *B. cenocepacia* may utilize several antioxidant enzymes, including superoxide dismutases (cytoplasmic SodB and periplasmic SodC), catalases, catalase-peroxidases (KatA and KatB) and alkyl-hydro-peroxidase. Also a melanin-like pigment expressed by strains of *B. cenocepacia* IIIA lineage seems to be involved in protection both from reactive oxygen and reactive nitrogen species (Loutet & Valvano, 2010, Drevinek & Mahenthiralingam, 2010).

MgtG

MgtC is a virulence protein that is required by distantly related bacterial pathogens, but its role remains elusive (Loutet & Valvano, 2010).

Phenylacetic acid and catabolic pathway

This pathway is the point at which the catabolism of many aromatic compounds coverges, and it is conceivable that this pathway may be important for nutrient acquisition or the metabolism of infecting bacteria in the host environment (Loutet & Valvano, 2010).

***Burkholderia cepacia* antibiotics resistance**

Another characteristic of the Bcc that some consider a virulence factor is its high resistance to antibiotics: they are intrinsically resistant to many antibiotics and can develop *in vivo* resistance to essentially all classes of antimicrobial drugs (Mahenthiralingam et al., 2005, Drevinek & Mahenthiralingam, 2010) (Figure 5).

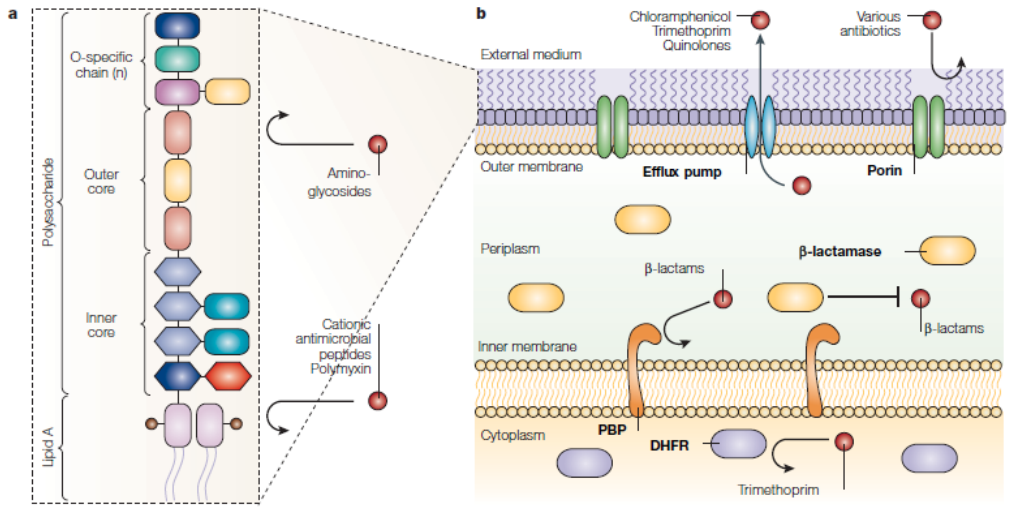


Figure 5: Structure of *Burkholderia cepacia* complex lipopolysaccharide and antibiotics resistance mechanisms (Mahenthiralingam et al., 2005)

This high antibiotics resistance is the result of different mechanisms. Very important is the specific structure of the LPS, indeed the Ara4N moieties attached to the phosphate residues in the lipid A backbone are implicated in resistance to the antibiotics effects of cationic antimicrobial peptides and polymixin (Mahenthiralingam et al., 2005). Moreover the outer membrane is arranged in a way that conceals or protects cation-binding sites on LPS, which are capable of binding polycations such as aminoglycosides or polymxyin (Cox & Wilkinson, 1991, Moore & Hancock, 1986) (Figure 5).

LPS is also involved in resistance to β-lactams, that is due also due to two additional synergistic mechanisms: the presence of β-lactamases and a low membrane permeability. β-lactamases are enzymes that break the β-lactam ring, characteristic of this class of antibiotics, deactivating the molecule's antibacterial properties. In *B. cepacia*, two different β-lactamases have been identified: a penicillinase (PenA) responsible for approximately 80% of the total β-lactamase activity of the strain and a second enzyme with primarily cephalosporinase activities. The expression of *penA* gene is inducible and is associated with the ability of these species of use penicillin as a sole carbon sources (Beckman & Lessie, 1979, Prince *et al.*, 1988, Trepanier *et al.*, 1997). A second mechanism of β-lactams resistance is due to outer membrane porins: a decrease in diffusion caused both by the small size of the channels and by their low conductance and also a diminished porins content was demonstrated in resistant strains (Parr *et al.*, 1987, Aronoff, 1988) (Figure 5).

Regarding trimethoprim, whose cellular target is the enzyme dihydrofolate reductase (DHFR), resistance is due to the presence of a modified enzyme, that is not recognized by the antibiotic (Burns *et al.*, 1989b) (Figure 5).

Moreover, Bcc bacteria growth in biofilm can may be more sensitive to the action of some antibiotics, for example to tobramycin (Messiaen *et al.*, 2013).

In addition to these mechanisms of resistance specific for certain classes of antibiotics, Bcc strains, like all other Gram-negative bacteria tend to be more resistant to lipophilic and amphiphilic inhibitors than Gram-positive bacteria (Nikaido, 1996). Earlier, the only known molecular mechanism that could explain this "intrinsic resistance" was the outer membrane permeation barrier. However, the outer membrane alone is not a sufficient explanation because most drug molecules equilibrate in less than a minute. Now it is recognized that the intrinsic drug resistance of Gram-negative bacteria is a result of the cooperation between the outer membrane barrier and the expression of efflux systems (Li & Nikaido, 2004) (Figure 5). Some of these transporter, such as the tetracycline efflux proteins are dedicated systems mediating the extrusion of a given drug or class of drugs. In

contrast to these specific drug transporters, the so-called multidrug transporters can handle a wide variety of structurally unrelated compounds and the presence of even just one of these kind of transporter can confer resistance to different class of antibiotics (Putman *et al.*, 2000).

Multidrug efflux systems can be classified into five families: one of them, the ABC (ATP-Binding Cassette) is a primary active transporters family, the others are secondary active transporters and are predominant in bacteria. Three families are H^+/drug antiports [the MFS (Major Facilitator Superfamily), the SMR (Small Multidrug Resistance) and the RND (Resistance-Nodulation-Cell Division) families], while the MATE (Multidrug and Toxic Compound Extrusion) family is a Na^+/drug antiporter (Van Bambeke *et al.*, 2000, Murakami & Yamaguchi, 2003). The intrinsic drug resistance of Gram-negative bacteria is mainly attributable to RND-type drug exporters (Murakami & Yamaguchi, 2003).

The Resistance-Nodulation-Cell Division (RND) superfamily

General features

The RND (Resistance-Nodulation-Cell-Division) superfamily includes proteins that are found ubiquitously in Bacteria, Archaea and Eukaryotes (Saier *et al.*, 1994, Tseng *et al.*, 1999, Saier & Paulsen, 2001).

All characterized members of this superfamily probably catalyze substrate efflux via an H^+ antiport mechanism (Saier & Paulsen, 2001). Most of these transport systems consists of a polypeptide chain of a 700-1300 amino acid residues long. These proteins possess a single transmembrane spanner (TMS) at their N-terminus followed by a large extracytoplasmic domain, six additional TMSs, a second large extracytoplasmic domain, and five final C-terminal TMSs. Most RND permeases consist of a single polypeptide chain (Saier & Paulsen, 2001). The first halves of RND family proteins are homologous to the second halves, and the proteins therefore probably arose as a result of an intragenic tandem duplication event that

occurred in the primordial system prior to divergence of the family members. Some archaeal and eukaryotic RND homologues are half size and has no internal duplication (Saier & Paulsen, 2001).

Functionally characterized members of this superfamily fall into eight different families: four of them are overall restricted to Gram-negative bacteria; the other four family have a diverse phylogenetic distribution. The four Gram-negative families have a different substrate specificity, with one responsible for the export of multiple drugs [Hydrophobe/Amphiphile Efflux-1 (HAE-1)], one catalyzing the export of heavy metals [Heavy Metal Efflux (HME)], and one likely catalyzing the export of lipooligosaccharides concerned with plant nodulation related to symbiotic nitrogen fixation [putative Nodulation Factor Exporter (NFE)] (Saier & Paulsen, 2001). The fourth Gram-negative family (the Brominated, Aryl Polyene Pigment Exporter, APPE) has been subsequently identified compared to the other families (Goel *et al.*, 2002). It is very distantly related to the other established members of the superfamily and its representatives were shown to be a pigment exporter in *Xanthomonas oryzae* (Goel et al., 2002).

The RND members of the first three Gram negative families act as complex that can bind various structurally unrelated substrate from the periplasm and/or the cytoplasm and extrude them out directly into the external media using proton-motive force. This complex is composed of a RND protein, located in the cytoplasmic membrane, a periplasmic-located membrane adaptor protein, belonging to the membrane fusion protein family (MFP), and an outer-membrane channel protein (OMP) (Saier & Paulsen, 2001) (Figure 6). Typically, the encoding genes are organized in an operon and the MFP and RND are usually cotranscribed (Poole *et al.*, 1993), whereas in some systems and/or species, the OMP is not linked to the other genes (Ma *et al.*, 1993, Aires *et al.*, 1999) (Figure 6).

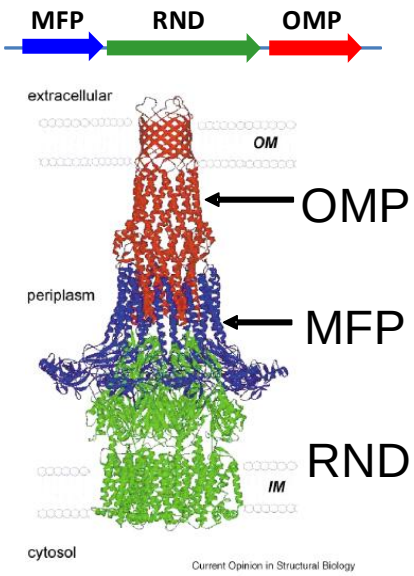


Figure 6: Model of the assembled tripartite RND pump and of the RND operons [adapted from (Eswaran *et al.*, 2004)]

HAE-1 family

The HAE-1 family is the best known of all the eight families, because its representatives are major determinants of Multidrug Resistance (MDR) in Gram-negative bacteria (Poole, 2007, Nikaido & Takatsuka, 2009, Nikaido, 2011). Indeed, in addition to the ability of these transporters to recognize and efficiently expel from the cells a broad range of structurally unrelated compounds (Zgurskaya & Nikaido, 2000), they can also work synergistically with single-components pumps located in the inner membrane (Lee *et al.*, 2000, Tal & Schuldiner, 2009) and furthermore they confer a resistance phenotype that can contribute to the acquisition of additional mechanisms of resistance such as enzymatic inactivation or modification of the drug target(s) (Piddock, 2006a, Poole, 2007, Davin-Regli *et al.*, 2008). In addition, several reports mention an increase in the hospital dissemination of bacterial strains expressing a drug efflux mechanism and a large

number of enterobacterial clinical isolates collected during the antibiotic therapy of infected/colonized patients exhibit a significant overproduction of HAE-1 pumps type (Nikaido & Pages, 2012).

In contrast with other bacterial genes, responsible for antibiotic resistance, acquired by horizontal gene transfer (HGT) (Martinez *et al.*, 2009), genes coding for multidrug efflux pumps are mainly harboured by the chromosome(s) of living organisms. In addition, these genes are highly conserved and their expression is tightly regulated (Martinez et al., 2009). Taken together, these characteristics suggest that the main function of these systems is likely not conferring resistance to antibiotics (used in therapy) and that they might play other roles relevant to the behaviour of bacteria in their natural ecosystems, as also pointed out by Saier and co-workers (Saier *et al.*, 1998). According to this idea, it has been recently proposed, that MDR proteins might have possessed (and, in some cases, might still possess) a role in preventing the build up of excessive osmotic pressure within the cells, thus functioning as safety valves for normal metabolised substrates (Danchin, 2009).

Among the other potential roles, it has been demonstrated that efflux pumps are important for detoxification processes of intracellular metabolites, bacterial virulence in both animal and plant hosts, cell homeostasis, intercellular signal trafficking, bacterial stress responses, cell to cell comunication (Martinez et al., 2009, Li & Nikaido, 2009).

HAE-1 RND proteins are present in all Gram-negative bacteria (Ruggerone *et al.*, 2013) and in recent years numerous studies have shown the presence and the role of these proteins in antibiotic resistance in different species for example *A. xylosoxidans, Acinetobacter baumannii*, *Bacteroides fragilis*, *Burkholderia* spp., *Campylobacter* spp., *E. coli* and other Enterobacteriaceae, *K. pneumoniae, Neisseria* spp., *Pseudomonas* spp., *S. maltophilia*, *Vibrio* spp. (Matsuo *et al.*, 2013, Yoon *et al.*, 2013, Bador *et al.*, 2013, Taylor *et al.*, 2012, Ogawa *et al.*, 2012, Wexler, 2012, Fernandez *et al.*, 2012, Kawakita *et al.*, 2012, Wieczorek *et al.*, 2008, Bina *et*

al., 2008, Perrin *et al.*, 2010, Li & Nikaido, 2004, Li & Nikaido, 2009). In particular HAE-1 proteins have been extensively studied in *E. coli* and *P. aeruginosa* and different transporter systems are well characterized in these two organisms. Among these, to this day, AcrB from *E. coli* and MexB from *P. aeruginosa* [and the associated MFP (AcrA, MexA respectively) and OMP proteins (TolC, OprM respectively)] are the best known RND drug transporter in bacteria, and have served as the prototype for biochemical and structural studies of such pumps (Murakami *et al.*, 2002, Sennhauser *et al.*, 2009, Akama *et al.*, 2004b, Higgins *et al.*, 2004, Mikolosko *et al.*, 2006, Koronakis *et al.*, 2000, Akama *et al.*, 2004a).

AcrB is by far the best- studied RND protein and its most striking characteristics are the extremely wide substrate specificity and the high constitutive expression levels under stress conditions (Nikaido, 2011). The natural function of AcrAB-TolC is probably the removal of bile salts detergents and their derivatives, steroid hormones and host-defence molecules present in high concentrations within the natural habitat of *E. coli* (Piddock, 2006b, Thanassi *et al.*, 1997, Elkins & Mullis, 2006). In addition, this system extrudes several other molecules: neutral, zwitterionic, cationic and anionic compounds, including basic dyes (acriflavine and ethidium), simple solvents (hexane, heptane, octane, nonane, cyclohexane), and moreover most of the known antibiotics (macrolides, fluoroquinolones, β-lactams, tetracyclines, chloramphenicol, rifampin, novobiocin, fusidic acid). All these molecules have in common a certain degree of hydrophobicity/lipophilicity and the only class of antibiotics that is not recognized by AcrB are the aminoglycosides such as kanamycin and streptomycin, which are more hydrophylic (Ruggerone et al., 2013).

In 2002, the first three dimensional X-ray structure of AcrB became available (Murakami et al., 2002) (Figure 7) and represented a symmetric AcrB homotrimer free of substrate. The shape of the protein resembles that of a jellyfish. Viewed orthogonally to the membrane plane, each protomer elongates for ~120 Å, comprising a TM region of ~50 Å composed of 12 α-helices (TM1 to TM12), and a

periplasmic headpiece of about 70 Å. The latter is divided into a pore (porter) region, formed by four β-strand – α-helix – β-strand subdomains (designated PC1, PC2, PN1, PN2), and in an upper region, formed by two mixed β-sheet subdomains (DN and DC) (Murakami et al., 2002) (Figure 7).

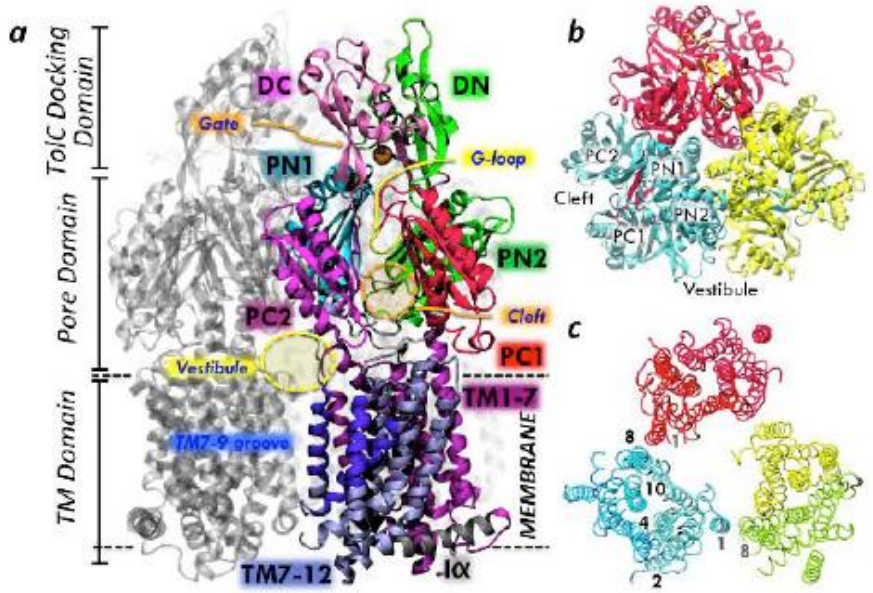


Figure 7: Structure of AcrB proteins. a) Side view of the structure of the AcrB asymmetric homotrimer. The A monomer is shown in solid ribbons, with different colors highlighting the main domains and subdomains of the pump. The remaining monomers are shown in transparent ribbons. b) Top view of the periplasmic domain of AcrB, showing the three monomers A (cyan), B (yellow) and C (red). The TM domain has been removed for clarity. c) Top view of the TM domain of AcrB (adapted from Ref. [131]). The color code is the same as in B, but the TM domains are divided in two regions to highlight the symmetry between the TM1-6 (darker colors) and TM7-12 moieties (lighter colors). Key helices contributing to the proton-relay network and to the transmission of allosteric conformational changes to/from the periplasmic domain are indicated by black labels in the A monomer, while gray labels indicate helices 1 of A and C monomers and 8 of monomer B [from (Ruggerone et al., 2013)].

The three TM domains (one for each protomer) form a large cavity of about 30 Å in diameter, likely to be filled with phospholipids *in vivo*. Notably a hairpin, called G-loop, is present at the depth of the periplasmic cleft formed by subdomains PC1 and PC2 in each protomer, which has been shown to be important for substrate adaptation. The relative arrangement of the AcrB monomers and the putative presence of phospholipids within the central hole delineated by the TM domains of each protomer result in the formation of 3 inter-monomer vestibules, each extending ~15 Å above the membrane plane and connecting the central cavity of the protein with the periplasm. It was suggested that substrates immersed within the outer leaflet of the membrane can enter this central cavity, which was also considered as a possible recognition site for the uptake of substrates (Ruggerone et al., 2013). A third domain is present above the pore domain, featuring an internal funnel with a diameter of ~30 Å at the most distal side but closed at the bottom by the tight interaction of the three pore helices of the pore domain. The upper diameter matches that of the proximal entrance of the TolC protein, and thus that domain was tentatively called TolC docking domain (Ruggerone et al., 2013) (Figure 7).

In subsequent years, several other structures of AcrB featuring a symmetric arrangement of the three monomers were published, both free or in complex with various substrates. These structure supported the hypothesis of substrate entry from the inter-monomer vestibules towards the central cavity or alternatively from the cytoplasm (Ruggerone et al., 2013).

With the publication in three reports in 2006 and 2007 of three asymmetric structures of AcrB, a clearer picture arose concerning the substrate binding and the mechanism of drug transport (Murakami *et al.*, 2006, Seeger *et al.*, 2006, Sennhauser *et al.*, 2007). In the asymmetric structures, each monomer of the trimer adopts a different conformation featuring channels inside the periplasmic domain either open towards the periplasmic space or towards the funnel in the TolC docking domain. The three functional states are called A or Loose (L) or

Access, B or Tight (T) or Binding monomer and C or Open (O) or Extrusion (E) (Murakami *et al.*, 2006, Seeger *et al.*, 2006, Sennhauser *et al.*, 2007)(Figure 8).

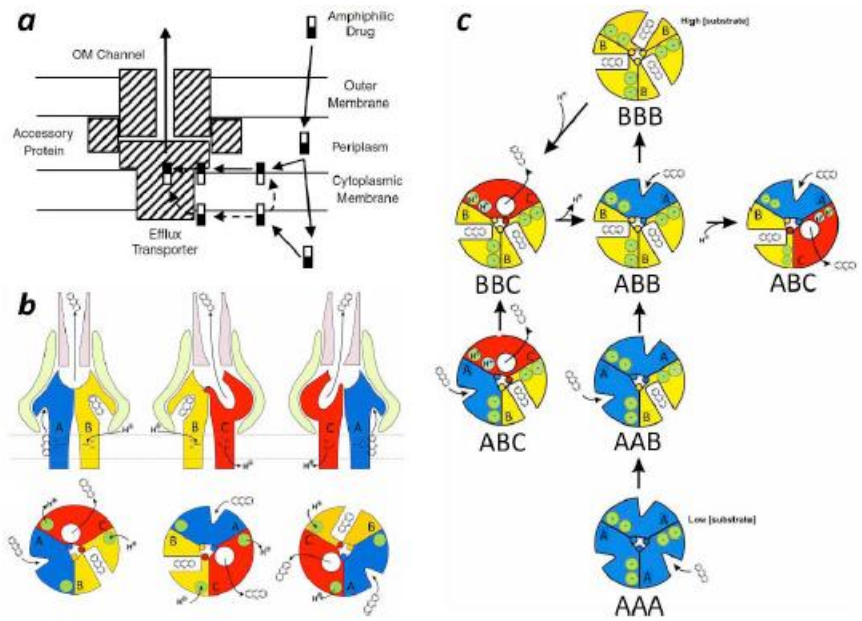


Figure 8: Functional rotation mechanism of substrate extrusion by AcrB. a) Early schematic view of the tripartite AcrAB-TolC complex. b) Schematic representation of the AcrB alternating site functional rotation transport mechanism. The conformational states A, B and C are colored blue, yellow and red, respectively. Upper panel: Side-view schematic representation of two of the three monomers of the AcrB trimer. AcrA and TolC are indicated in light green and light purple colors, respectively. Lower panel: The lateral grooves in the A (blue) and B (yellow) monomers indicate the substrate binding sites. The different geometric forms reflect low (triangle), high (rectangle), or no (circle) binding affinity for the transported substrates. In the first state of the cycle, a monomer binds a substrate (acridine) at the access site (AP in the A monomer), subsequently transports the substrate from AP to the DP (upon conversion to B monomer) and finally releases the substrate in the funnel toward TolC (C monomer). AcrA is postulated to participate in the transduction of the conformational changes from AcrB to TolC, which results in the opening of the TolC channel and the facilitation of drug extrusion to the outside of the cell. c) Schematic representation of the AcrB alternating site functional rotation transport mechanism extended by postulated intermediate steps. The lateral grooves in A and B monomers indicate the substrate binding sites. The different geometric forms reflect low (triangle), high (rectangle), or no (circle) binding affinity for the transported substrates. State BBB is postulated to occur at high substrate concentration, while AAA and AAB are postulated to occur in the absence or at low substrate concentrations [From (Ruggerone et al., 2013)].

Two different binding sites were identified: one called DP is formed by the PN2 subdomain of monomer B shift towards the PN1/PC1 subdomains of monomer A, apparently creating a phenylalanine-rich deep (or distal) binding pocket, and a second more proximal or access pocket (AP) in the A monomer located deep into the PC1/PC2 cleft, and separated from the DP by the G-loop. It has been hypothesized that AcrB recognizes high molecular-mass (HMM) substrates *via* the AP binding site, while low molecular-mass (LMM) substrates are not recognized by the AP but bind directly to the DP (Ruggerone et al., 2013) .

On the basis of the X-ray structures, a "functional rotation" mechanism was postulated to explain drug export by AcrB, involving a concerted cycling of the monomers through any of the asymmetric states A, B, C, and back to A. During a complete cycle ABC → BCA →CAB → ABC occlusions and constrictions inside the pore domain propagate from external gates towards the central funnel, driving the unidirectional transport of substrate (Seeger et al., 2006). In the A conformation, substrates are recruited from the periplasmic space (PC1/PC2 cleft) and/or the membrane (vestibule or central cavity) and bind a wide region that includes the AP. Along the A to B transition substrates move from the AP toward the DP, the second site being more hydrophobic than the former, consistently with the physico-chemical properties of substrates of the pump, which share a certain hydrophobicity. The G-loop was suggested to act as a gate between these two pockets, and indeed its flexibility was shown to be crucial for the functioning of the pump. Upon transition from the B to the C conformation, substrates are squeezed out from the binding pocket and they exit AcrB via its central funnel toward the TolC tunnel (Murakami *et al.*, 2006, Seeger *et al.*, 2006, Sennhauser *et al.*, 2007) (Figure 8).

The electrochemical proton gradient across the IM is most likely the driving force for the aforementioned conformational changes leading to uptake and extrusion of substrates in the periplasmic domain of AcrB. The flux of protons from the periplasm to the cytoplasm most likely involves rearrangements in the TM helices.

Protonation and deprotonation events, involving D407 and D408 primarily but also K940 and R971, induce conformational changes propagating towards the periplasmic domain. It is believed that the B to C transition is the energy-demanding step of the cycle, with 2 protons being necessary to complete one cycle from A to B to C and back to A per monomer and per substrate transported (Ruggerone et al., 2013) (Figure 8).

HME family

The HME family includes proteins involved in heavy-metal efflux. Different from the HAE-1 family, members of the HME family are highly substrate specific, with the ability to differentiate between monovalent and divalent ions (Su *et al.*, 2009), and Nies further subdivided the HME-RND protein into sub-groups, according to the substrate they transport: HME1 (Zn^{2+}, Co^{2+}, Cd^{2+}), HME2 (Co^{2+}, Ni^{2+}), HME3a (divalent cations), HME3b (monovalent cations), HME4 (Cu^{+} or Ag^{+}) and HME5 (Ni^{2+}) (Nies, 2003). The best characterized HME efflux systems are mainly those from *Cupriavidus* (previously called *Ralstonia* and *Alcaligenes*): CzcCBA (Cd^{2+}, Zn^{2+}, Co^{2+} resistance) from *Ralstonia metallidurans* CH34 (Nies, 1995, Grosse *et al.*, 1999, Legatzki *et al.*, 2003), CnrCBA (Ni^{2+} ,Co^{2+}) from *Ralstonia eutropha* (Tibazarwa *et al.*, 2000, Grass *et al.*, 2000), NccCBA (Ni^{2+}, Co^{2+}, Cd^{2+}) from *Alcaligenes xylosoxidans* 31A (Schmidt & Schlegel, 1994). However, other systems such as *P. aeruginosa* Czr (Cd^{2+}, Zn^{2+}) (Hassan *et al.*, 1999), *Helicobacter pylori* Czn (Cd^{2+}, Zn^{2+}, Ni^{2+}) (Stahler *et al.*, 2006), *Caulobacter crescentus* NA1000 CzrCBA (Cd^{2+}, Zn^{2+}) and NczCBA (Ni^{2+} , Co^{2+}) (Valencia *et al.*, 2013) were also studied.

Recently the crystal structure of the three components of one complex belonging to this family, CusA (RND) , CusB (MFP) and CusC (OMP) from *E. coli* (involved in Cu^{+} and Ag^{+} efflux) were obtained (Long *et al.*, 2010, Su et al., 2009, Su *et al.*, 2011, Kulathila *et al.*, 2011). The structure of CusA suggests that this RND pump exists as a homotrimer and each of the three subunit consists of the 12 transmembrane α-helices and of the large periplasmic domain formed by two periplasmic loops

characteristic of RND proteins. Similarly to the AcrB structure, the periplasmic domain can be divided into a pore domain (comprising sub-domains PN1, PN2, PC1 and PC2) and a CusC docking domain (containing sub-domains DN and DC) (Long et al., 2010). The structure of CusB demonstrates that this adaptor protein is folded into a four-domain elongated structure, ~120 Å long and ~40 Å wide (Su et al., 2009) and the co-crystal structure of the CusBA adaptor–transporter reveals that the trimeric CusA pump associates with six CusB molecules to form the CusB6–CusA3 complex (Su et al., 2011). The crystal structure of the CusC channel has also been resolved, suggesting that the architecture of this protein resembles those of TolC and OprM (Kulathila et al., 2011).

Kim *et al.* (Kim *et al.*, 2011) have proposed two models for the extrusion of heavy metals by HME proteins from the periplasm to the extracellular medium, i.e.the 'funnel' and the 'swich' mechanisms. The funnel model involves the shuttling of periplasmic substrate from the membrane fusion protein to the RND transporter and further on through the outer membrane factor to the extracellular space. Conversely, the switch model requires substrate binding to the membrane fusion protein, inducing a conformational change and creating an open-access state of the tripartite protein complex. They favor this second mechanism.

Recently, also the structure and a model for substrate efflux of the Zn^{2+} transport ZneA from *Cupriavidus metallidurans* CH34 is reported (Pak *et al.*, 2013).

NFE family

The representative protein of NFE family is NolG, a putative nodulation factor exporter in rhizobia, that probably function together with the product of the neighboring gene *nolF* (MFP family) (Saier et al., 1994, Tseng et al., 1999, Barnett *et al.*, 2001). Recently homologs of this protein have been identified in the genome sequence of many other bacteria (Pinto *et al.*, 2009).

APPE family

The fourth Gram-negative family (the Brominated, Aryl Polyene Pigment Exporter, APPE) has been identified subsequently compared to the other RND families (Goel et al., 2002). It is very distantly related to the other established members of the superfamily and its representatives were shown to be a pigment exporter in *Xanthomonas oryzae* (Goel et al., 2002). This protein has close homologues in various species of *Xanthomonas* as well as *Xylella*, *Ralstonia* and *E. coli* (from TCDB database, http://www.tcdb.org/).

SecDF family

The SecD-F proteins can be found in both Bacteria and Archaea (Tseng et al., 1999, Eichler, 2003, Hand *et al.*, 2006). It has been shown in *E. coli* that the coding gene of these two proteins are associated in a operon that contain also the *yajC* gene (Pogliano & Beckwith, 1994), and that they form a membrane protein complex that associates in a transient fashion with the Sec translocon (the major facilitator in the translocation and insertion of proteins across or into the inner membrane of prokaryotes) and stimulates pre-protein translocation (du Plessis *et al.*, 2011). Although SecD and SecF are not essential, their inactivation in *E. coli* results in a severe pleiotropic protein secretion defect as well as a severe growth inhibition and a cold sensitive phenotype (du Plessis et al., 2011, Tseng et al., 1999). Recently, the crystal structure of SecD-F was obtained in *Thermus thermophilus* and the overall structure confirms the presence of the twelve transmembrane domains and the the two periplasmic loops typical of RND proteins (Tsukazaki *et al.*, 2011). Additionally, the crystal structure of the isolated periplasmic domain corresponding to SecD (P1) was resolved, revealing two conformation that seem to occur upon a rotation by 120° (Echizen *et al.*, 2011). These two structures suggest that this complex functions as a membrane-integrated chaperone, powered by proton motive force, that interact with emerging secretory protein by P1 domain, promoting the forward movement and increasing translocation efficiency (Tsukazaki et al., 2011, Echizen et al., 2011). The role of YajC is still unclear (Fang &

Wei, 2011). SecD-F was also found to interact with the membrane protein insertase YidC (Nouwen & Driessen, 2002, Kol *et al.*, 2008, Li *et al.*, 2013).

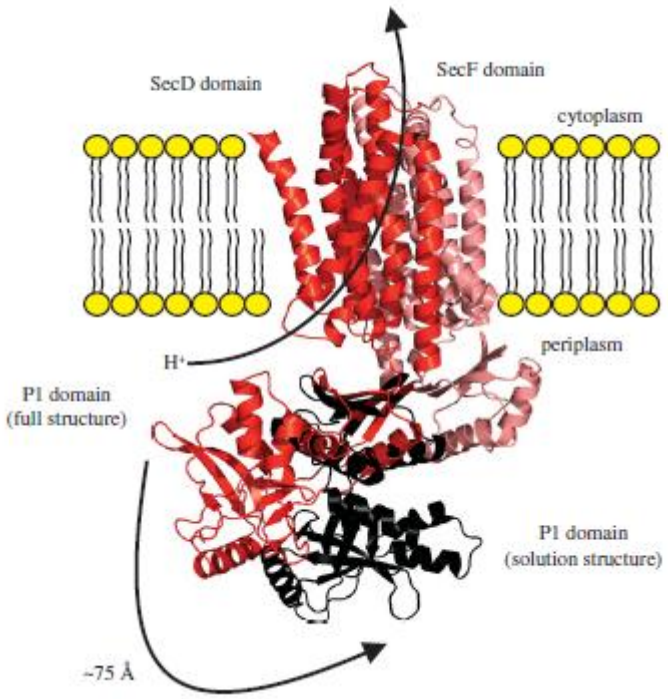


Figure 9: Structure and proposed mechanism of the SecDF complex. *Thermus thermophilus* SecDF (3AQP) is shown with the SecF domain in pink and the SecD domain in red. The structure obtained from the isolated cytoplasmic portion of SecD (3AQO; black) is docked on the transmembrane part of SecD. The proposed turn of the head domain directed by the PMF as indicated by an arrow is shown. The distance indicated is measured from the tip of the head domain in its two conformations. [From (Lycklama & Driessen, 2012)]

It has been demonstrated in *S. aureus* that the *secDF* deletion has a combination of different effects on transcription, regulation and translocation that lead to impaired cell division, reduced antibiotics resistance and altered expression of virulence determinants suggesting SecDF to be of major relevance in *S. aureus* (Quiblier *et al.*, 2011). Moreover, a quantitative secretome analysis performed on the *secDF* deletion mutant revealed that numerous Sec signal containing proteins involved in virulence are decreased in the supernatant of mutant compared to

wild-type strain. However, two Sec-dependent hydrolases were increased in comparison to the wild type, suggesting additional indirect, regulatory effects to occur upon deletion of secDF. Adhesion, invasion, and cytotoxicity of the secDF mutant were reduced in human umbilical vein endothelial cells and virulence was significantly reduced using a *Galleria mellonella* insect model (Quiblier *et al.*, 2013). A role of SecDF in secretion of virulence factors has been demonstrated also in *Listeria monocytogenes* (Burg-Golani *et al.*, 2013).

HAE_2 family

The Hydrophobe/Amphiphile Efflux-2 (HAE-2) family comprises members exclusively from Gram-positive bacteria. Almost all members of the family are from high G+C Gram-positive bacteria or from *Bacillus subtilis*, an organism with about 43.5% G+C content (Tseng et al., 1999). Among the representatives of this family that have been studied there are the ActII3 protein of *Streptomyces coelicolor* that has been implicated in drug resistance and TmtpC of *Mycobacterium smegmatis* that may be a glycopeptidolipid exporter that function with the MmpS4 protein to form a scaffold for coupled biosynthesis and transport (Saier & Paulsen, 2001, Deshayes *et al.*, 2010).

The *Mycobacterium tuberculosis* genome contains 13 putative RND-type transporters, designated MmpL (mycobacterial membrane proteins, large). However, the inactivation of 11 out of 13 of these genes did not alter the drug susceptibility (Domenech *et al.*, 2005). Some of them are instead involved in virulence in mice.

Several Mmpl proteins have been experimentally characterized or in *M. tubercolosis* or in *M. smegmatis*:

- Mmpl3 of *M. tuberculosis* exports trehalose monomycolate, involved in mycolic acid donation to the cell wall core (Tahlan *et al.*, 2013) and it is also involved with Mmpl11 in iron up-take (Owens *et al.*, 2013a, Owens *et al.*, 2013b). It is the target of some antitubercular drugs like the pyrrole

derivative BM212 (La Rosa V *et al.*, 2012), of SQ109, a 1,2-diamine related to ethambutol (Tahlan et al., 2013), of adamantyl ureas (Scherman *et al.*, 2012), of a benzimidazole (Stanley *et al.*, 2012), of a number of tetrahydropyrazolo analogues (Remuinan *et al.*, 2013).

- In *M. tuberculosis* MmpL4 and Mmpl 5, that functions with MmpS4 and MmpS5 respectively, are siderophore exporters, that overlap in function (Wells *et al.*, 2013). Mmpl5-MmpS5 are also involved in azole resistance (Milano *et al.*, 2009).
- MmpL7 of *M. tuberculosis* was determined to be essential for virulence (Camacho *et al.*, 2001), presumably because it transports phthiocerol dimycoceroserate (PDIM) (Cox *et al.*, 1999) and a related but distinct phenolic glycolipid(Reed *et al.*, 2004). When expressed in *M. smegmatis*, MmpL7 confers a high-level resistance to isoniazid due to efflux and this resistance level decreases in the presence of the EPIs (Efflux pumps inhibitors) (Pasca *et al.*, 2005).
- In *M. tuberculosis* MmpL8 is required for sulfolipid-1 biosynthesis (exports a sulfatide precursor) and for virulence (Converse *et al.*, 2003, Li & Nikaido, 2009, Domenech et al., 2005).
- Mmpl11 in *M. tuberculosis* is involved, with Mmpl3, in iron up-take mechanisms (Owens *et al.*, 2013a, Owens *et al.*, 2013b).

  In *M. smegmatis* the lack of MmpL11 results in a reduced membrane permeability that leads to resistance to host antimicrobial peptides (Purdy *et al.*, 2009). Moreover MmpL11 protein transports mycolic acid-containing lipids to the mycobacterial cell wall and contributes to biofilm formation in *M. smegmatis*. The role in mycobacterial cell wall biogenesis of Mmpl11 could be conserved in other mycobacteria (Pacheco *et al.*, 2013).

HAE_3 family

The Hydrophobe/Amphiphile Efflux-3 (HAE-3) family comprises members from archaea and spirochete. All the proteins included in this family were revealed by genome sequencing, and the functions of these proteins have not been investigated (Tseng et al., 1999, Saier & Paulsen, 2001).

EST family

Some or all the eukaryotic proteins may function in cholesterol/steroid hormone transport, reception, regulation, and catalysis. Non-transporter members of this unusual family possess the sterol recognition domain and do not exhibit the typical RND family internal duplication (Saier & Paulsen, 2001). Examples of proteins belonging to this family are the Patched (Ptc) protein that is the likely receptor for the cholesterol-modified morphogen Hedgehog (Hh), the Dispatched (Disp) protein that acts in Hh signaling to facilitate the release of the Hh protein from Hh-secreting cells and the membrane bound Niemann-Pick C1 and the water-soluble Niemann-Pick C2 proteins (NPC1 and NPC2), that are cholesterol-binding lysosomal proteins required for export of lipoprotein-derived cholesterol from lysosomes (Tseng et al., 1999, Hausmann *et al.*, 2009, Kuwabara & Labouesse, 2002).

In addition to these eight families, there is one group of RND proteins that have been termed “hopanoid biosynthesis-associated RND transporters” or “HpnN” (Hausmann et al., 2009). Hopanoid are pentacyclic triterpenoids that may be surrogates for eukaryotic sterols in bacteria (Schmerk *et al.*, 2011), although relatively little about their biological functions is known (Doughty *et al.*, 2011). The HpnN coding genes are associated with hopanoid biosynthesis genes in many bacterial genome (Doughty et al., 2011). It is not clear if these proteins are really involved in hopanoid transport: this seems true in *Rhodopseudomonas palustris* TIE-1 (Doughty et al., 2011), but not in *B. cenocepacia* (hopanoid are likely capable of reaching their proper location also in absence of this protein) (Schmerk et al., 2011). Also the location of this subfamily within RND superfamily is not so clear:

they seems to be related to APPE and HAE-3 family (Doughty et al., 2011), but some authors argued that the eukaryotic RND-transporters arose from this particular group of proteins (Hausmann et al., 2009). It is very interesting that these proteins are involved in antibiotics resistance in some *Burkholderia* strains (Schmerk et al., 2011, Malott *et al.*, 2012).

Regulation of expression RND proteins

Both the presence of numerous RND efflux systems and the fact that some of them may have overlapping functions, require a well-regulated expression of these efflux systems, which can be subject to multiple levels of regulation. Indeed, the existence of a variety of local and global transcriptional regulators and other modulators highlights the complexity and diversity of the regulatory mechanisms of drug efflux pumps (Li & Nikaido, 2009, Li & Nikaido, 2004).

In most cases, local transcriptional regulators encoded by genes linked to the efflux genes were identified, and many of them are repressors belonging to TetR family. In some cases also two-component regulatory systems are associated with the genes coding for RND transporters. *P. aeruginosa* contains various RND systems, which have been characterized and whose complex regulation and co-regulation has been studied (Li & Nikaido, 2009), and the regulation network of these systems is reported in Figure 10. Multiple mechanisms are also involved in regulation of RND transporters in other bacteria (Li & Nikaido, 2009).

Regarding the global regulators of RND expressions, the control system of the *E.coli* AcrAB-TolC is the best studied example and involved at least four global transcriptional activators, MarA, SoxS, Rob and SdiA (Li & Nikaido, 2004)(Figure 11). The regulation *via* the *mar* systems (multiple antibiotics resistance) operates by the regulation of MarA levels. This regulator controls differential expression of over 60

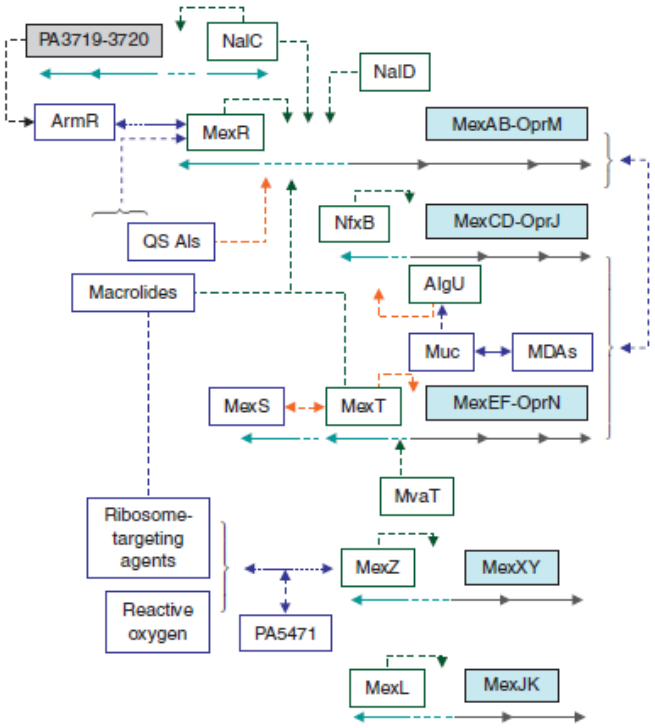


Figure 10: Regulation of the resistance-nodulation-division (RND) superfamily Mex transporters of *P. aeruginosa*. The efflux systems are shown in the light-blue blocks with the respective transcriptional units presented in the solid-grey lines. All regulators are shown in the green boxes, and their functions as repressors or activators are indicated, respectively, in the green- or orange-dotted arrows. The inverse relationship between MexAB-OprM expression and MexCD-OprJ/MexEF-OprN expression is marked by a double-arrowed line. Interaction of the regulators (MexR or MexT) with the modulators (ArmR or MexT) is denoted by the double-arrowed dotted lines. MDA= membrane-damaging agents; QS AIs = quorum-sensing autoinducers [From (Li & Nikaido, 2009)].

chromosomal genes, including the *acrAB* operon, whose transcription is activated, incrising drug efflux (Li & Nikaido, 2004) (Figure 11). Also SoxS, the effector of the global superoxide response regulon SoxSR, increases the transcription of *acrAB*, allowing *E. coli*, to increase is antibacterial resistance in presence of superoxide radicals (Li & Nikaido, 2004) (Figure 11). The Rob regulator is involved in regulation of *acrAB* and also in the activation of the transcription of *mar-sox* regulons, and this activation occours through the binding of some inducers (Li & Nikaido, 2004). MarA, SoxS or Rob are also involved in increasing TolC expression, indeed a

putative *mar/sox/rob* box has been identified upstream of the *tolC* gene. Moreover, the overexpression of MarA and SoxR decreases the synthesis of the porin OmpF thus preventing the entry of drugs into the cells (Li & Nikaido, 2004) (Figure 11). Finally, SdiA positively regulates the AcrAB expression (Li & Nikaido, 2004). Homologues of these *E. coli* global regulators has been identified also in other bacteria, where they are involved in global regulation of RND proteins expression (Li & Nikaido, 2004). In many cases the expression of RND proteins can be induced by the substrates of the pumps (Li & Nikaido, 2009).

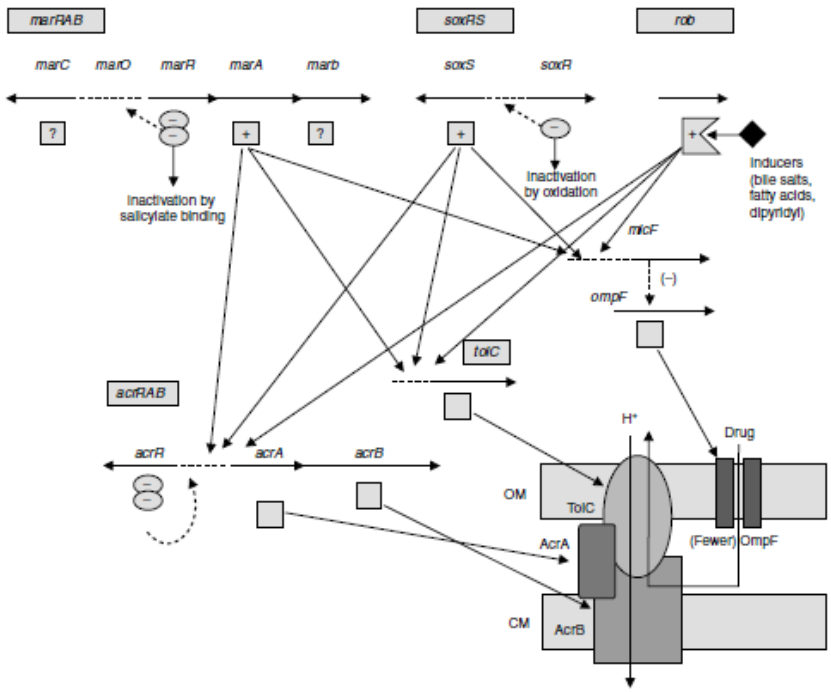


Figure 11: Regulation of AcrAB-TolC expression. The acrAB operon is negatively regulated by the AcrR repressor (bottom left). In addition, acrAB and tolC are positively regulated by the three global activators, depicted on top, MarA, SoxS and Rob. The activators are shown by squares and a polygon with plus signs (+) in them; the repressors are shown by ellipses with minus (–) signs in them. The levels of MarA and SoxS are regulated by repressors MarR and SoxR, and the activity of Rob is apparently regulated by small ligands such as bile salts. These three activators also up-regulate micF, whose transcript inhibits the translation of ompF porin mRNA. Thus, all three activators increase the expression of efflux complex AcrAB-TolC, at the same time decreasing the influx of drugs through decreased expression of OmpF [from (Li & Nikaido, 2004)].

Strategy to overcome RND-mediated drugs resistance

The low number of new antibiotics discovered in the last years and the continuous spread of resistant bacteria has prompted research efforts towards the search for strategies to overcome resistance mediated by multidrug efflux pumps (Nikaido & Pages, 2012, Ruggerone et al., 2013). Two main strategies can be used. The first consists in bypassing efflux pumps by improving the molecular design of existing antibiotics or by designing new ones in such a way that they are not recognized by transporters. Despite some new molecules less amenable for efflux have been produced, even for these compounds resistance has been described very shortly after their deployment (Ruggerone et al., 2013).

The second strategy is the inactivation of efflux pumps that can increase the intracellular concentration of antibiotics, decrease the intrinsic bacteria resistance to antibiotics and reverse the acquired resistance due to overexpression of efflux pumps, reduce the emergence of highly resistant strains and finally prevent the export of virulance factors (Ruggerone et al., 2013).

There are several possible ways to inactivate RND efflux pumps:

- Inhibitors of proton motive force. They can completely abolish the efflux activity of different pumps, but these inhibitors have a general mode of action that affect the entire energetics of the cells, including eukaryotic cells (Ruggerone et al., 2013).
- Biological inhibitors. This class of inhibitors affects efflux pump activity by blocking either the proteins themselves or by inactivating the genes encoding the pumps. This approach has been shown to work for AcrAB of *E. coli* and can be extended to other RND proteins (Ruggerone et al., 2013).
- Pharmacological inhibitors, also called Efflux Pumps Inhibitors (EPIs). These are chemical compounds used or to interfere with the assembly or function of efflux pumps, or in combination therapy to increase the antibiotic concentration inside the cell, by competitive/non-competitive

inhibition of thr pump (Ruggerone et al., 2013). Example of EPIs are peptidomimetic EPIs, for example PAβN, piperazines, pyridopyrimidines (Ruggerone et al., 2013, Nikaido & Pages, 2012).

RND proteins in the *Burkholderia* genus

As seen in the previous paragraphs RND proteins have been extensively studied in many organisms, mainly in *E. coli* and *P. aeruginosa*. Much less information are available on RND proteins in the *Burkholderia* genus. In 2010 an *in silico* analysis of RND proteins belonging to HAE-1 and HME families in 21 completely sequenced *Burkholderia* genomes revealed the presence and distribution of these proteins in the *Burkholderia* genus (Perrin et al., 2010). Some of these proteins have been experimentally characterized in recent years mostly in *B. pseudomallei* and in *B. cenocepacia* species.

The genome sequence of *B. pseudomallei* K96243 reveals the presence of at least 10 operons that may encode for RND efflux pump components (Holden *et al.*, 2004), and although differently annotated these pumps are conserved in other *B. pseudomallei* strains (Kumar *et al.*, 2008, Perrin et al., 2010) (Figure 12). Only three of these systems have been characterized in detail, AmrAB–OprA, BpeAB–OprB and BpeEF–OprC (Schweizer, 2012) (Figure 12).

AmrAB–OprA was the first efflux pump characterized in *B. pseudomallei* and it is responsible for the intrinsic aminoglycoside and macrolide resistance observed in most clinical and environmental strains (Moore *et al.*, 1999). This system also extrudes acriflavine, fluoroquinolones and tetracyclines but the resistance levels are low and probably clinically insignificant (Mima & Schweizer, 2010). Some clinical isolates susceptible to aminoglycoside and macrolide have been identified and such variants may be more frequent than originally thought, since clinical diagnosis of melioidosis in many instances still relies on use of Ashdown's agar, whose main selective ingredient is gentamicin (Trunck *et al.*, 2009). This susceptibility is due to lack of, or greatly reduced, AmrAB-OprA expression, either

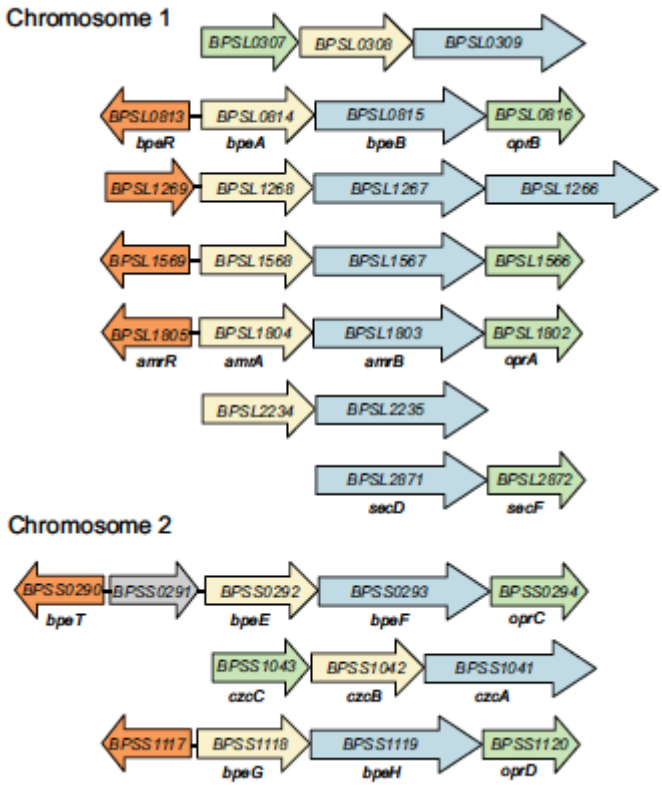


Figure 12: Resistance-nodulation-cell-division (RND) efflux systems encoded by the *B. pseudomallei* K96243 genome. Membrane fusion protein, RND transporter and outer membrane channel protein-encoding genes are indicated by yellow, blue and green arrows, respectively. Orange arrows indicate known or probable regulatory genes. Note that the bpEF-oprC operon is preceded by BPSS0291 (grey arrow) encoding a putative lipase. Actual gene names are given for characterized or highly probable gene products
[from (Kumar et al., 2008)]

due to deletion or as to as yet unknown mechanisms (Trunck et al., 2009). Recently, several *B. pseudomallei* strains isolated from Sarawak, Malaysian Borneo were found to be susceptible to aminoglycosides and macrolides and whole genome sequencing of these strains identified a novel non-synonymous mutation within *amrB* gene (Podin *et al.*, 2013). AmrAB-OprA is also responsible for the reduced efficacy of novel therapeutic agents, such as the ketolide, cethromycin, which was shown to select for mutants overexpressing AmrAB–OprA in response to *in vitro* cethromycin exposure,resulting in high-level resistance (MIC ≥128 µg/ml) (Mima *et al.*, 2011).

AmrAB-OprA is responsible for highlevel aminoglycoside resistance also in *B.* thailandensis, while the closely related *B. mallei* strain ATCC 23344 lacks this system, which likely contributes to its aminoglycoside susceptibility (Mima & Schweizer, 2010).

BpeAB-OprB of Singapore strain KHW was reported to mediate efflux of aminoglycosides (gentamicin and streptomycin) and macrolides (erythromycin) and of the dye acriflavine (Chan *et al.*, 2004), as well as to play an important role in virulence (optimal production of biofilms, siderophores, and phospholipase C) and quorum sensing (excretion of acyl homoserine lactone (AHL)) (Chan & Chua, 2005, Chan *et al.*, 2007). However, subsequent molecular genetic studies with strain 1026b, a Thai clinical isolate, revealed that BpeAB–OprB did not bestow significant levels of aminoglycoside resistance in this strain (Mima & Schweizer, 2010). This pump extrudes macrolides, fluoroquinolones, tetracyclines and, to a lesser extent, chloramphenicol. Despite contributing to intrinsic resistance to these antibiotics, resistance levels are low (with the exception of some macrolides) and, therefore, probably clinically insignificant (Mima & Schweizer, 2010). Moreover, strain 1026b BpeAB-OprB mutants were not impaired in extrusion of AHLs, swimming motility, or siderophore production, and showed only an impaired biofilm formation (Mima & Schweizer, 2010).

The BpeABOprB homologus of *B. thailandensis* is overexpressed in association with also the OMP of the BpeEF-OprC homologus in chloramphenicol resistant strains (Biot *et al.*, 2011).

BpeEF–OprC has been characterized for the first time ina a *P. aeruginosa* surrogate, where it is involved in chloramphenicol and trimethoprim efflux (Kumar *et al.*, 2006). This finding was subsequently corroborated using *B. pseudomallei* mutants defective in the cognate BpeT activator protein, which constitutively express BpeEF–OprC and exhibit resistance to chloramphenicol, fluoroquinolones, tetracyclines and trimethoprim (Schweizer, 2012). An analysis of a comprehensive collection of clinical and environmental isolates from Thailand demonstrated that

trimethoprim resistance is widespread and attributable to BpeEF–OprC expression. All trimethoprim-resistant isolates remain susceptible to sulfamethoxazole, thus preserving the clinical utility of cotrimoxazole (Podnecky *et al.*, 2013). The clinical significance ofBpeEF–OprC is further corroborated by the identification of a pair of sequential isolates, 354b and 354e, from a Thai melioidosis patient, with the secondary isolate, 354e, exhibiting significantly increased resistance to chloramphenicol, ofloxacin and co-trimoxazole. Genomic analyses indicated that a large 800-kb inversion had deleted the last 24 codons of bpeT, which encodes the BpeEFOprC transcriptional regulator BpeT (Schweizer, 2012).

The expression of RND proteins in commonly used *B. pseudomallei* strains and in clinical isolates from Northen Australia have been evaluated and seven of them was found to be widespread in both clinical and other isolates (Kumar et al., 2008). In particular over AmrAB-OprA, BpeAB-OprB and BpeEF-OprC, four other yet uncharacterized pumps (*bpeH, BPSL0309, BPSL1267, BPSL1567* genes) were found to be expressed. In 45 of the 50 isolates, mRNA was detected for at least one of the seven RND pumps, and of these 45 isolates, 41 expressed multiple pumps with 9 strains expressing all seven pumps tested. One of the commonly used strains over-expressed AmrAB-OprA, BpeAB-OprB, BpeEF-OprC and BpeGH-OprD. BpeAB-OprB was expressed in most of clinical isolates, and it is often co-expressed with AmrAB-OprA. The high prevalence of expression of the BpeEF-OprC and BpeGH-OprD as well as the *BPSL1267* and *BPSL1567*- encoded pump components in clinical isolates may be indicative of a significan role(s) of these efflux pumps (Kumar et al., 2008). The expression of *bpeH* (and of *BMAA1045* of B. mallei) were found to be increased also in *B. pseudomallei* strains highly resistant to fluoroquinolones (Viktorov *et al.*, 2008).

Regarding Bcc species, RND proteins have been studied mainly in *B. cenocepacia* J2315 strain. In the genome of this strain 16 genes encoding putative RND proteins have been identified and named RND-1 to RND-16, and most of them are

associated in a operon with genes coding for MFP and OMP proteins (Holden et al., 2009, Guglierame *et al.*, 2006, Buroni *et al.*, 2009, Perrin et al., 2010) (Figure 13).

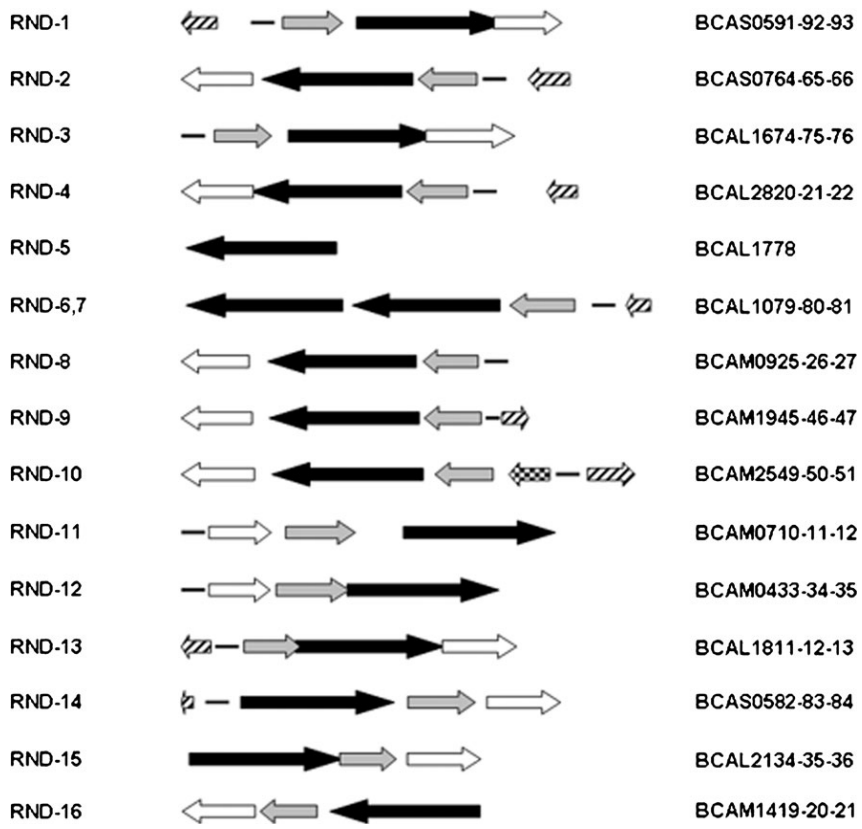


Figure 13: Organization of the genes encoding RND efflux systems in B. cenocepacia J2315. The striped arrows represent the regulatory genes, the membrane fusion proteins are depicted in grey, the RND encoding ones in black and the genes encoding outer membrane proteins are in white. The checked arrow shows llpE gene (lipase-like protein), and the promoter regions are represented as a black dash (Bazzini *et al.*, 2011).

The first experimentally characterized of these 16 RND was the *ceo* operon (RND-10, BCAM2549-50-51) that confers resistance to chloramphenicol, trimethoprim and ciprofloxacin, and is induced by salicylate (Burns *et al.*, 1989a, Burns *et al.*, 1996, Nair *et al.*, 2004, Nair *et al.*, 2005).

In 2006 Guglierame et al. (Guglierame et al., 2006), found that RND-3 (BCAL1675), RND-9 (BCAM1946), RND-11 (BCAM0713) and RND-13 (BCAL1812) are expressed at detectable levels in *B. cenocepacia* J2315 grown on LB medium and characterized RND-2 (BCAS0765) in *E. coli*, where it conferred resistance to fluoroquinolones, tetraphenylphosphonium, streptomycin and ethidium bromide. The same group also characterized RND-1 (BCAS0592), RND-3 (BCAL1674) and RND-4 (BCAL2821), through the deletion of the three operons encoding these transporters (Buroni et al., 2009). Strain D1 (ΔBCAS0591-BCAS0593) did not shown any increased susceptibility as compared to the parental strains, while strain D3 (ΔBCAL1674-BCAL1676) havea reduced MIC to nalidixic acid. The deletion of RND-4 (BCAL2820-BCAL2822) led to an increased sensitivity to various compounds (aztreonam, chloramphenicol, gentamicin, tobramicin, different fluoroquinolones, such as nalidixic acid, ciprofloxacin, levofloxacin, norfloxacin, and sparfloxacin and also ethidium bromide) (Buroni et al., 2009). RND-3 and RND-4 operons mutants demonstrated also a reduced accumulation of N-acyl homoserine lactones in the growth medium (Buroni et al., 2009). These proteins are involved also in resistance to the disinfectant chlorhedixine, in lifestyle-specific tolerance mechanisms: RND-4 operon was more responsible for chlorhedixine tollerance in planktonic *Burkholderia* cells, while RND-3 and RND-9 (BCAM1945-BCAM1947) operons were linked to resistance in sessile cells (Coenye *et al.*, 2011). Moreover RND-8 operon (BCAM0925-BCAM0927) is over-expressed in sessile cells exposed to chlorhedixine (Coenye et al., 2011).

An altered expression of RND genes or operons was observed in strains of *Burkholderia* grown under different conditions:

- the RND-3 gene and the MFP subunit of RND-9 (BCAM1947) were found to be up-regualted in *B. cenocepacia* J2315 grown in a CF-sputum based infection model, while the OMP component (BCAL1813) of RND-13 is down-regulated (Drevinek *et al.*, 2008);

- the three genes of the RND-6-7 operon and the MFP subunit (BCAL2822) of RND-4 are over-expressed in a strain of *B. cenocepacia* isolated from a CF patient compared to a less antibiotics resistant clonal strain isolated three years before from the same patient (Mira *et al.*, 2011);
- RND-8 operon were found to be up-regulated in a *B. cenocepacia* J2315 strain exposed to chlorpromazine (Sass *et al.*, 2011).

HpnN transporters were identified and characterized in *B. cenocepacia* and in *B. multivorans* where they are involved in antibiotics resistance (Schmerk et al., 2011, Malott *et al.*, 2012).

Finally, an RND operon involved in the transport of the phytotoxin toxofalvin have been identified in *B. glumae* (Kim *et al.*, 2004).

Bibliography

TCDB (Transport Classification Database). In., pp.

Aires, J. R., T. Kohler, H. Nikaido & P. Plesiat, (1999) Involvement of an active efflux system in the natural resistance of Pseudomonas aeruginosa to aminoglycosides. *Antimicrob Agents Chemother* **43**: 2624-2628.

Akama, H., M. Kanemaki, M. Yoshimura, T. Tsukihara, T. Kashiwagi, H. Yoneyama, S. Narita, A. Nakagawa & T. Nakae, (2004a) Crystal structure of the drug discharge outer membrane protein, OprM, of Pseudomonas aeruginosa: dual modes of membrane anchoring and occluded cavity end. *J Biol Chem* **279**: 52816-52819.

Akama, H., T. Matsuura, S. Kashiwagi, H. Yoneyama, S. Narita, T. Tsukihara, A. Nakagawa & T. Nakae, (2004b) Crystal structure of the membrane fusion protein, MexA, of the multidrug transporter in Pseudomonas aeruginosa. *J Biol Chem* **279**: 25939-25942.

Aronoff, S. C., (1988) Outer membrane permeability in *Pseudomonas cepacia*: diminished porin content in a beta-lactam-resistant mutant and in resistant cystic fibrosis isolates. *Antimicrob Agents Chemother* **32**: 1636-1639.

Bador, J., L. Amoureux, E. Blanc & C. Neuwirth, (2013) Innate aminoglycoside resistance of *Achromobacter xylosoxidans* is due to AxyXY-OprZ, an RND-type multidrug efflux pump. *Antimicrob Agents Chemother* **57**: 603-605.

Baldwin, A., E. Mahenthiralingam, K. M. Thickett, D. Honeybourne, M. C. Maiden, J. R. Govan, D. P. Speert, J. J. Lipuma, P. Vandamme & C. G. Dowson, (2005) Multilocus sequence typing scheme that provides both species and strain differentiation for the *Burkholderia cepacia* complex. *J Clin Microbiol* **43**: 4665-4673.

Barnett, M. J., R. F. Fisher, T. Jones, C. Komp, A. P. Abola, F. Barloy-Hubler, L. Bowser, D. Capela, F. Galibert, J. Gouzy, M. Gurjal, A. Hong, L. Huizar, R. W. Hyman, D. Kahn, M. L. Kahn, S. Kalman, D. H. Keating, C. Palm, M. C. Peck, R. Surzycki, D. H. Wells, K. C. Yeh, R. W. Davis, N. A. Federspiel & S. R. Long, (2001) Nucleotide sequence and predicted functions of the entire Sinorhizobium meliloti pSymA megaplasmid. *Proc Natl Acad Sci U S A* **98**: 9883-9888.

Bazzini, S., C. Udine & G. Riccardi, (2011) Molecular approaches to pathogenesis study of *Burkholderia cenocepacia*, an important cystic fibrosis opportunistic bacterium. *Appl Microbiol Biotechnol* **92**: 887-895.

Beckman, W. & T. G. Lessie, (1979) Response of *Pseudomonas cepacia* to beta-Lactam antibiotics: utilization of penicillin G as the carbon source. *J Bacteriol* **140**: 1126-1128.

Ben-Ari, J., O. Wolach, R. Gavrieli & B. Wolach, (2012) Infections associated with chronic granulomatous disease: linking genetics to phenotypic expression. *Expert Rev Anti Infect Ther* **10**: 881-894.

Bevivino, A., S. Tabacchioni, L. Chiarini, M. V. Carusi, M. Del Gallo & P. Visca, (1994) Phenotypic comparison between rhizosphere and clinical isolates of *Burkholderia cepacia*. *Microbiology* **140 (Pt 5)**: 1069-1077.

Bina, X. R., D. Provenzano, N. Nguyen & J. E. Bina, (2008) *Vibrio cholerae* RND family efflux systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. *Infect Immun* **76**: 3595-3605.

Biot, F. V., E. Valade, E. Garnotel, J. Chevalier, C. Villard, F. M. Thibault, D. R. Vidal & J. M. Pages, (2011) Involvement of the efflux pumps in chloramphenicol selected strains of *Burkholderia thailandensis*: proteomic and mechanistic evidence. *PLoS One* **6**: e16892.

Bosch, A., A. Minan, C. Vescina, J. Degrossi, B. Gatti, P. Montanaro, M. Messina, M. Franco, C. Vay, J. Schmitt, D. Naumann & O. Yantorno, (2008) Fourier transform infrared spectroscopy for rapid identification of nonfermenting gram-negative bacteria isolated from sputum samples from cystic fibrosis patients. *J Clin Microbiol* **46**: 2535-2546.

Brisse, S., C. Cordevant, P. Vandamme, P. Bidet, C. Loukil, G. Chabanon, M. Lange & E. Bingen, (2004) Species distribution and ribotype diversity of *Burkholderia cepacia* complex isolates from French patients with cystic fibrosis. *J Clin Microbiol* **42**: 4824-4827.

Brisse, S., C. M. Verduin, D. Milatovic, A. Fluit, J. Verhoef, S. Laevens, P. Vandamme, B. Tummler, H. A. Verbrugh & A. van Belkum, (2000) Distinguishing species of the *Burkholderia cepacia* complex and *Burkholderia gladioli* by automated ribotyping. *J Clin Microbiol* **38**: 1876-1884.

Burg-Golani, T., Y. Pozniak, L. Rabinovich, N. Sigal, R. Nir Paz & A. A. Herskovits, (2013) Membrane Chaperone SecDF Plays a Role in the Secretion of *Listeria monocytogenes* Major Virulence Factors. *J Bacteriol* **195**: 5262-5272.

Burkholder, W. H., (1942) Three bacterial plant pathogens: *Phytomonas caryophylli* sp.n., *Phytomonas alliicola* sp.n. and *Phytomonas manihotis* (Artaud, Berthet and Bondar) Vigas. . *Phytopathology* **32**: 141-149.

Burkholder, W. H., (1950) Sour skin, a bacterial rot of onion bulbs. *Phytopathology* **40**: 115-117.

Burns, J. L., L. A. Hedin & D. M. Lien, (1989a) Chloramphenicol resistance in *Pseudomonas cepacia* because of decreased permeability. *Antimicrob Agents Chemother* **33**: 136-141.

Burns, J. L., D. M. Lien & L. A. Hedin, (1989b) Isolation and characterization of dihydrofolate reductase from trimethoprim-susceptible and trimethoprim-resistant *Pseudomonas cepacia*. *Antimicrob Agents Chemother* **33**: 1247-1251.

Burns, J. L., C. D. Wadsworth, J. J. Barry & C. P. Goodall, (1996) Nucleotide sequence analysis of a gene from *Burkholderia* (*Pseudomonas*) *cepacia* encoding an outer membrane lipoprotein involved in multiple antibiotic resistance. *Antimicrob Agents Chemother* **40**: 307-313.

Buroni, S., M. R. Pasca, R. S. Flannagan, S. Bazzini, A. Milano, I. Bertani, V. Venturi, M. A. Valvano & G. Riccardi, (2009) Assessment of three Resistance-Nodulation-Cell Division drug efflux transporters of *Burkholderia cenocepacia* in intrinsic antibiotic resistance. *BMC Microbiol* **9**: 200.

Camacho, L. R., P. Constant, C. Raynaud, M. A. Laneelle, J. A. Triccas, B. Gicquel, M. Daffe & C. Guilhot, (2001) Analysis of the phthiocerol dimycocerosate locus of *Mycobacterium tuberculosis*. Evidence that this lipid is involved in the cell wall permeability barrier. *J Biol Chem* **276**: 19845-19854.

Chan, Y. Y., H. S. Bian, T. M. Tan, M. E. Mattmann, G. D. Geske, J. Igarashi, T. Hatano, H. Suga, H. E. Blackwell & K. L. Chua, (2007) Control of quorum sensing by a *Burkholderia pseudomallei* multidrug efflux pump. *J Bacteriol* **189**: 4320-4324.

Chan, Y. Y. & K. L. Chua, (2005) The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence. *J Bacteriol* **187**: 4707-4719.

Chan, Y. Y., T. M. Tan, Y. M. Ong & K. L. Chua, (2004) BpeAB-OprB, a multidrug efflux pump in *Burkholderia pseudomallei*. *Antimicrob Agents Chemother* **48**: 1128-1135.

Chiarini, L., A. Bevivino, C. Dalmastri, S. Tabacchioni & P. Visca, (2006) *Burkholderia cepacia* complex species: health hazards and biotechnological potential. *Trends Microbiol* **14**: 277-286.

Choh, L. C., G. H. Ong, K. M. Vellasamy, K. Kalaiselvam, W. T. Kang, A. R. Al-Maleki, V. Mariappan & J. Vadivelu, (2013) *Burkholderia* vaccines: are we moving forward? *Front Cell Infect Microbiol* **3**: 5.

Ciofu, O., C. R. Hansen & N. Hoiby, (2013) Respiratory bacterial infections in cystic fibrosis. *Curr Opin Pulm Med* **19**: 251-258.

Clode, F. E., L. A. Metherell & T. L. Pitt, (1999) Nosocomial Acquisition of *Burkholderia gladioli* in patients with cystic fibrosis. *Am J Respir Crit Care Med* **160**: 374-375.

Coenye, T., J. J. LiPuma, D. Henry, B. Hoste, K. Vandemeulebroecke, M. Gillis, D. P. Speert & P. Vandamme, (2001a) *Burkholderia cepacia* genomovar VI, a new member of the *Burkholderia cepacia* complex isolated from cystic fibrosis patients. *Int J Syst Evol Microbiol* **51**: 271-279.

Coenye, T., E. Mahenthiralingam, D. Henry, J. J. LiPuma, S. Laevens, M. Gillis, D. P. Speert & P. Vandamme, (2001b) *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. *Int J Syst Evol Microbiol* **51**: 1481-1490.

Coenye, T., L. M. Schouls, J. R. Govan, K. Kersters & P. Vandamme, (1999) Identification of *Burkholderia* species and genomovars from cystic fibrosis patients by AFLP fingerprinting. *Int J Syst Bacteriol* **49 Pt 4**: 1657-1666.

Coenye, T., H. Van Acker, E. Peeters, A. Sass, S. Buroni, G. Riccardi & E. Mahenthiralingam, (2011) Molecular mechanisms of chlorhexidine tolerance in *Burkholderia cenocepacia* biofilms. *Antimicrob Agents Chemother* **55**: 1912-1919.

Coenye, T. & P. Vandamme, (2003) Diversity and significance of *Burkholderia* species occupying diverse ecological niches. *Environ Microbiol* **5**: 719-729.

Coenye, T., P. Vandamme, J. R. Govan & J. J. LiPuma, (2001c) Taxonomy and identification of the *Burkholderia cepacia* complex. *J Clin Microbiol* **39**: 3427-3436.

Compant, S., J. Nowak, T. Coenye, C. Clement & E. Ait Barka, (2008) Diversity and occurrence of *Burkholderia* spp. in the natural environment. *FEMS Microbiol Rev* **32**: 607-626.

Converse, S. E., J. D. Mougous, M. D. Leavell, J. A. Leary, C. R. Bertozzi & J. S. Cox, (2003) MmpL8 is required for sulfolipid-1 biosynthesis and *Mycobacterium tuberculosis* virulence. *Proc Natl Acad Sci U S A* **100**: 6121-6126.

Coutinho, C. P., I. Sa-Correia & J. A. Lopes, (2009) Use of Fourier transform infrared spectroscopy and chemometrics to discriminate clinical isolates of bacteria of the *Burkholderia cepacia* complex from different species and ribopatterns. *Anal Bioanal Chem* **394**: 2161-2171.

Cox, A. D. & S. G. Wilkinson, (1991) Ionizing groups in lipopolysaccharides of *Pseudomonas cepacia* in relation to antibiotic resistance. *Mol Microbiol* **5**: 641-646.

Cox, J. S., B. Chen, M. McNeil & W. R. Jacobs, Jr., (1999) Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature* **402**: 79-83.

Danchin, A., (2009) Cells need safety valves. *Bioessays* **31**: 769-773.

Davin-Regli, A., J. M. Bolla, C. E. James, J. P. Lavigne, J. Chevalier, E. Garnotel, A. Molitor & J. M. Pages, (2008) Membrane permeability and regulation of drug "influx and efflux" in enterobacterial pathogens. *Curr Drug Targets* **9**: 750-759.

Degand, N., E. Carbonnelle, B. Dauphin, J. L. Beretti, M. Le Bourgeois, I. Sermet-Gaudelus, C. Segonds, P. Berche, X. Nassif & A. Ferroni, (2008) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of nonfermenting gram-negative bacilli isolated from cystic fibrosis patients. *J Clin Microbiol* **46**: 3361-3367.

Deshayes, C., H. Bach, D. Euphrasie, R. Attarian, M. Coureuil, W. Sougakoff, F. Laval, Y. Av-Gay, M. Daffe, G. Etienne & J. M. Reyrat, (2010) MmpS4 promotes glycopeptidolipids biosynthesis and export in *Mycobacterium smegmatis*. *Mol Microbiol* **78**: 989-1003.

Domenech, P., M. B. Reed & C. E. Barry, 3rd, (2005) Contribution of the Mycobacterium tuberculosis MmpL protein family to virulence and drug resistance. *Infect Immun* **73**: 3492-3501.

Doughty, D. M., M. L. Coleman, R. C. Hunter, A. L. Sessions, R. E. Summons & D. K. Newman, (2011) The RND-family transporter, HpnN, is required for hopanoid localization to the outer membrane of Rhodopseudomonas palustris TIE-1. *Proc Natl Acad Sci U S A* **108**: E1045-1051.

Drevinek, P., M. T. Holden, Z. Ge, A. M. Jones, I. Ketchell, R. T. Gill & E. Mahenthiralingam, (2008) Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when *Burkholderia cenocepacia* grows in cystic fibrosis sputum. *BMC Infect Dis* **8**: 121.

Drevinek, P. & E. Mahenthiralingam, (2010) *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. *Clin Microbiol Infect* **16**: 821-830.

du Plessis, D. J., N. Nouwen & A. J. Driessen, (2011) The Sec translocase. *Biochim Biophys Acta* **1808**: 851-865.

Echizen, Y., T. Tsukazaki, N. Dohmae, R. Ishitani & O. Nureki, (2011) Crystallization and preliminary X-ray diffraction of the first periplasmic domain of SecDF, a translocon-associated membrane protein, from *Thermus thermophilus*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **67**: 1367-1370.

Eichler, J., (2003) Evolution of the prokaryotic protein translocation complex: a comparison of archaeal and bacterial versions of SecDF. *Mol Phylogenet Evol* **27**: 504-509.

Elkins, C. A. & L. B. Mullis, (2006) Mammalian steroid hormones are substrates for the major RND- and MFS-type tripartite multidrug efflux pumps of Escherichia coli. *J Bacteriol* **188**: 1191-1195.

Estrada-de los Santos, P., P. Vinuesa, L. Martinez-Aguilar, A. M. Hirsch & J. Caballero-Mellado, (2013) Phylogenetic analysis of burkholderia species by multilocus sequence analysis. *Curr Microbiol* **67**: 51-60.

Eswaran, J., E. Koronakis, M. K. Higgins, C. Hughes & V. Koronakis, (2004) Three's company: component structures bring a closer view of tripartite drug efflux pumps. *Curr Opin Struct Biol* **14**: 741-747.

Fang, J. & Y. Wei, (2011) Expression, purification and characterization of the *Escherichia coli* integral membrane protein YajC. *Protein Pept Lett* **18**: 601-608.

Fernandez, M., S. Conde, J. de la Torre, C. Molina-Santiago, J. L. Ramos & E. Duque, (2012) Mechanisms of resistance to chloramphenicol in *Pseudomonas putida* KT2440. *Antimicrob Agents Chemother* **56**: 1001-1009.

Ferreira, A. S., I. N. Silva, V. H. Oliveira, R. Cunha & L. M. Moreira, (2011) Insights into the role of extracellular polysaccharides in *Burkholderia* adaptation to different environments. *Front Cell Infect Microbiol* **1**: 16.

Gillis, M., T. Vanvan, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kersters, T. Heulin & M. P. Fernandez, (1995) Polyphasic taxonomy in the genus *Burkholderia* leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. nov. for N_2-fixing isolates from rice in Vietnam. *Int J Syst Bacteriol* **45**: 274-289.

Goel, A. K., L. Rajagopal, N. Nagesh & R. V. Sonti, (2002) Genetic locus encoding functions involved in biosynthesis and outer membrane localization of xanthomonadin in Xanthomonas oryzae pv. oryzae. *J Bacteriol* **184**: 3539-3548.

Grass, G., C. Grosse & D. H. Nies, (2000) Regulation of the cnr cobalt and nickel resistance determinant from *Ralstonia* sp. strain CH34. *J Bacteriol* **182**: 1390-1398.

Grosse, C., G. Grass, A. Anton, S. Franke, A. N. Santos, B. Lawley, N. L. Brown & D. H. Nies, (1999) Transcriptional organization of the czc heavy-metal homeostasis determinant from *Alcaligenes eutrophus*. *J Bacteriol* **181**: 2385-2393.

Guglierame, P., M. R. Pasca, E. De Rossi, S. Buroni, P. Arrigo, G. Manina & G. Riccardi, (2006) Efflux pump genes of the resistance-nodulation-division family in *Burkholderia cenocepacia* genome. *BMC Microbiol* **6**: 66.

Hand, N. J., R. Klein, A. Laskewitz & M. Pohlschroder, (2006) Archaeal and bacterial SecD and SecF homologs exhibit striking structural and functional conservation. *J Bacteriol* **188**: 1251-1259.

Harrison, F., (2007) Microbial ecology of the cystic fibrosis lung. *Microbiology* **153**: 917-923.

Hassan, M. T., D. van der Lelie, D. Springael, U. Romling, N. Ahmed & M. Mergeay, (1999) Identification of a gene cluster, czr, involved in cadmium and zinc resistance in *Pseudomonas aeruginosa*. *Gene* **238**: 417-425.

Hausmann, G., C. von Mering & K. Basler, (2009) The hedgehog signaling pathway: where did it come from? *PLoS Biol* **7**: e1000146.

Henry, D., M. Campbell, C. McGimpsey, A. Clarke, L. Louden, J. L. Burns, M. H. Roe, P. Vandamme & D. Speert, (1999) Comparison of isolation media for recovery of *Burkholderia cepacia* complex from respiratory secretions of patients with cystic fibrosis. *J Clin Microbiol* **37**: 1004-1007.

Henry, D. A., E. Mahenthiralingam, P. Vandamme, T. Coenye & D. P. Speert, (2001) Phenotypic methods for determining genomovar status of the *Burkholderia cepacia* complex. *J Clin Microbiol* **39**: 1073-1078.

Higgins, M. K., E. Bokma, E. Koronakis, C. Hughes & V. Koronakis, (2004) Structure of the periplasmic component of a bacterial drug efflux pump. *Proc Natl Acad Sci U S A* **101**: 9994-9999.

Holden, M. T., H. M. Seth-Smith, L. C. Crossman, M. Sebaihia, S. D. Bentley, A. M. Cerdeno-Tarraga, N. R. Thomson, N. Bason, M. A. Quail, S. Sharp, I. Cherevach, C. Churcher, I. Goodhead, H. Hauser, N. Holroyd, K. Mungall, P. Scott, D. Walker, B. White, H. Rose, P. Iversen, D. Mil-Homens, E. P. Rocha,

A. M. Fialho, A. Baldwin, C. Dowson, B. G. Barrell, J. R. Govan, P. Vandamme, C. A. Hart, E. Mahenthiralingam & J. Parkhill, (2009) The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J Bacteriol* **191**: 261-277.

Holden, M. T., R. W. Titball, S. J. Peacock, A. M. Cerdeno-Tarraga, T. Atkins, L. C. Crossman, T. Pitt, C. Churcher, K. Mungall, S. D. Bentley, M. Sebaihia, N. R. Thomson, N. Bason, I. R. Beacham, K. Brooks, K. A. Brown, N. F. Brown, G. L. Challis, I. Cherevach, T. Chillingworth, A. Cronin, B. Crossett, P. Davis, D. DeShazer, T. Feltwell, A. Fraser, Z. Hance, H. Hauser, S. Holroyd, K. Jagels, K. E. Keith, M. Maddison, S. Moule, C. Price, M. A. Quail, E. Rabbinowitsch, K. Rutherford, M. Sanders, M. Simmonds, S. Songsivilai, K. Stevens, S. Tumapa, M. Vesaratchavest, S. Whitehead, C. Yeats, B. G. Barrell, P. C. Oyston & J. Parkhill, (2004) Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proc Natl Acad Sci U S A* **101**: 14240-14245.

Kawakita, Y., F. Taguchi, Y. Inagaki, K. Toyoda, T. Shiraishi & Y. Ichinose, (2012) Characterization of each aefR and mexT mutant in *Pseudomonas syringae* pv. tabaci 6605. *Mol Genet Genomics* **287**: 473-484.

Kerem, B., J. M. Rommens, J. A. Buchanan, D. Markiewicz, T. K. Cox, A. Chakravarti, M. Buchwald & L. C. Tsui, (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science* **245**: 1073-1080.

Kim, E. H., D. H. Nies, M. M. McEvoy & C. Rensing, (2011) Switch or funnel: how RND-type transport systems control periplasmic metal homeostasis. *J Bacteriol* **193**: 2381-2387.

Kim, J., J. G. Kim, Y. Kang, J. Y. Jang, G. J. Jog, J. Y. Lim, S. Kim, H. Suga, T. Nagamatsu & I. Hwang, (2004) Quorum sensing and the LysR-type transcriptional activator ToxR regulate toxoflavin biosynthesis and transport in *Burkholderia glumae*. *Mol Microbiol* **54**: 921-934.

Kiska, D. L., A. Kerr, M. C. Jones, J. A. Caracciolo, B. Eskridge, M. Jordan, S. Miller, D. Hughes, N. King & P. H. Gilligan, (1996) Accuracy of four commercial systems for identification of *Burkholderia cepacia* and other gram-negative nonfermenting bacilli recovered from patients with cystic fibrosis. *J Clin Microbiol* **34**: 886-891.

Kol, S., N. Nouwen & A. J. Driessen, (2008) Mechanisms of YidC-mediated insertion and assembly of multimeric membrane protein complexes. *J Biol Chem* **283**: 31269-31273.

Koronakis, V., A. Sharff, E. Koronakis, B. Luisi & C. Hughes, (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405**: 914-919.

Kulathila, R., M. Indic & B. van den Berg, (2011) Crystal structure of *Escherichia coli* CusC, the outer membrane component of a heavy metal efflux pump. *PLoS One* **6**: e15610.

Kumar, A., K. L. Chua & H. P. Schweizer, (2006) Method for regulated expression of single-copy efflux pump genes in a surrogate *Pseudomonas aeruginosa* strain: identification of the BpeEF-OprC chloramphenicol and trimethoprim efflux pump of *Burkholderia pseudomallei* 1026b. *Antimicrob Agents Chemother* **50**: 3460-3463.

Kumar, A., M. Mayo, L. A. Trunck, A. C. Cheng, B. J. Currie & H. P. Schweizer, (2008) Expression of resistance-nodulation-cell-division efflux pumps in commonly used *Burkholderia pseudomallei* strains and clinical isolates from northern Australia. *Trans R Soc Trop Med Hyg* **102 Suppl 1**: S145-151.

Kuwabara, P. E. & M. Labouesse, (2002) The sterol-sensing domain: multiple families, a unique role? *Trends Genet* **18**: 193-201.

La Rosa V, Poce G, Canseco JO, Buroni S, Pasca MR, Biava M, Raju RM, Porretta GC, Alfonso S, Battilocchio C, Javid B, Sorrentino F, Ioerger TR, Sacchettini JC, Manetti F, Botta M, De Logu A, Rubin EJ & D. R. E., (2012) MmpL3 is the cellular target of the antitubercular pyrrole derivative BM212. *Antimicrob Agents Chemother.* **56**: 324-331.

Lazar Adler, N. R., B. Govan, M. Cullinane, M. Harper, B. Adler & J. D. Boyce, (2009) The molecular and cellular basis of pathogenesis in melioidosis: how does *Burkholderia pseudomallei* cause disease? *FEMS Microbiol Rev* **33**: 1079-1099.

Lee, A., W. Mao, M. S. Warren, A. Mistry, K. Hoshino, R. Okumura, H. Ishida & O. Lomovskaya, (2000) Interplay between efflux pumps may provide either additive or multiplicative effects on drug resistance. *J Bacteriol* **182**: 3142-3150.

Legatzki, A., S. Franke, S. Lucke, T. Hoffmann, A. Anton, D. Neumann & D. H. Nies, (2003) First step towards a quantitative model describing Czc-mediated heavy metal resistance in *Ralstonia metallidurans*. *Biodegradation* **14**: 153-168.

Li, X. Z. & H. Nikaido, (2004) Efflux-mediated drug resistance in bacteria. *Drugs* **64**: 159-204.

Li, X. Z. & H. Nikaido, (2009) Efflux-mediated drug resistance in bacteria: an update. *Drugs* **69**: 1555-1623.

Li, Z., D. Boyd, M. Reindl & M. B. Goldberg, (2013) Identification of YidC residues that define interactions with the Sec apparatus. *J Bacteriol*.

Long, F., C. C. Su, M. T. Zimmermann, S. E. Boyken, K. R. Rajashankar, R. L. Jernigan & E. W. Yu, (2010) Crystal structures of the CusA efflux pump suggest methionine-mediated metal transport. *Nature* **467**: 484-488.

Loutet, S. A. & M. A. Valvano, (2010) A decade of *Burkholderia cenocepacia* virulence determinant research. *Infect Immun* **78**: 4088-4100.

Lycklama, A. N. J. A. & A. J. Driessen, (2012) The bacterial Sec-translocase: structure and mechanism. *Philos Trans R Soc Lond B Biol Sci* **367**: 1016-1028.

Lyczak, J. B., C. L. Cannon & G. B. Pier, (2002) Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* **15**: 194-222.

Lynch, K. H. & J. J. Dennis, (2008) Development of a species-specific fur gene-based method for identification of the *Burkholderia cepacia* complex. *J Clin Microbiol* **46**: 447-455.

Ma, D., D. N. Cook, M. Alberti, N. G. Pon, H. Nikaido & J. E. Hearst, (1993) Molecular cloning and characterization of acrA and acrE genes of Escherichia coli. *J Bacteriol* **175**: 6299-6313.

Mahenthiralingam, E., A. Baldwin & C. G. Dowson, (2008) *Burkholderia cepacia* complex bacteria: opportunistic pathogens with important natural biology. *J Appl Microbiol* **104**: 1539-1551.

Mahenthiralingam, E., J. Bischof, S. K. Byrne, C. Radomski, J. E. Davies, Y. Av-Gay & P. Vandamme, (2000) DNA-Based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. *J Clin Microbiol* **38**: 3165-3173.

Mahenthiralingam, E., T. A. Urban & J. B. Goldberg, (2005) The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat Rev Microbiol* **3**: 144-156.

Malott, R. J., B. R. Steen-Kinnaird, T. D. Lee & D. P. Speert, (2012) Identification of hopanoid biosynthesis genes involved in polymyxin resistance in Burkholderia multivorans. *Antimicrob Agents Chemother* **56**: 464-471.

Marolda, C. L., B. Hauroder, M. A. John, R. Michel & M. A. Valvano, (1999) Intracellular survival and saprophytic growth of isolates from the *Burkholderia cepacia* complex in free-living amoebae. *Microbiology* **145 (Pt 7)**: 1509-1517.

Martinez, J. L., M. B. Sanchez, L. Martinez-Solano, A. Hernandez, L. Garmendia, A. Fajardo & C. Alvarez-Ortega, (2009) Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol Rev* **33**: 430-449.

Matsuo, T., K. Nakamura, T. Kodama, T. Mikami, H. Hiyoshi, T. Tsuchiya, W. Ogawa & T. Kuroda, (2013) Characterization of all RND-type multidrug efflux transporters in Vibrio parahaemolyticus. *Microbiologyopen* **2**: 725-742.

Messiaen, A. S., H. Nelis & T. Coenye, (2013) Investigating the role of matrix components in protection of *Burkholderia cepacia* complex biofilms against tobramycin. *J Cyst Fibros*.

Mikolosko, J., K. Bobyk, H. I. Zgurskaya & P. Ghosh, (2006) Conformational flexibility in the multidrug efflux system protein AcrA. *Structure* **14**: 577-587.

Milano, A., M. R. Pasca, R. Provvedi, A. P. Lucarelli, G. Manina, A. L. Ribeiro, R. Manganelli & G. Riccardi, (2009) Azole resistance in *Mycobacterium tuberculosis* is mediated by the MmpS5-MmpL5 efflux system. *Tuberculosis (Edinb)* **89**: 84-90.

Mima, T. & H. P. Schweizer, (2010) The BpeAB-OprB efflux pump of *Burkholderia pseudomallei* 1026b does not play a role in quorum sensing, virulence factor production, or extrusion of aminoglycosides but is a broad-spectrum drug efflux system. *Antimicrob Agents Chemother* **54**: 3113-3120.

Mima, T., H. P. Schweizer & Z. Q. Xu, (2011) In vitro activity of cethromycin against *Burkholderia pseudomallei* and investigation of mechanism of resistance. *J Antimicrob Chemother* **66**: 73-78.

Mira, N. P., A. Madeira, A. S. Moreira, C. P. Coutinho & I. Sa-Correia, (2011) Genomic expression analysis reveals strategies of *Burkholderia cenocepacia* to adapt to cystic fibrosis patients' airways and antimicrobial therapy. *PLoS One* **6**: e28831.

Moore, R. A., D. DeShazer, S. Reckseidler, A. Weissman & D. E. Woods, (1999) Efflux-mediated aminoglycoside and macrolide resistance in *Burkholderia pseudomallei*. *Antimicrob Agents Chemother* **43**: 465-470.

Moore, R. A. & R. E. Hancock, (1986) Involvement of outer membrane of *Pseudomonas cepacia* in aminoglycoside and polymyxin resistance. *Antimicrob Agents Chemother* **30**: 923-926.

Moore, R. A., S. Reckseidler-Zenteno, H. Kim, W. Nierman, Y. Yu, A. Tuanyok, J. Warawa, D. DeShazer & D. E. Woods, (2004) Contribution of gene loss to the pathogenic evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*. *Infect Immun* **72**: 4172-4187.

Murakami, S., R. Nakashima, E. Yamashita, T. Matsumoto & A. Yamaguchi, (2006) Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. *Nature* **443**: 173-179.

Murakami, S., R. Nakashima, E. Yamashita & A. Yamaguchi, (2002) Crystal structure of bacterial multidrug efflux transporter AcrB. *Nature* **419**: 587-593.

Murakami, S. & A. Yamaguchi, (2003) Multidrug-exporting secondary transporters. *Curr Opin Struct Biol* **13**: 443-452.

Nair, B. M., K. J. Cheung, Jr., A. Griffith & J. L. Burns, (2004) Salicylate induces an antibiotic efflux pump in *Burkholderia cepacia* complex genomovar III (*B. cenocepacia*). *J Clin Invest* **113**: 464-473.

Nair, B. M., L. A. Joachimiak, S. Chattopadhyay, I. Montano & J. L. Burns, (2005) Conservation of a novel protein associated with an antibiotic efflux operon in *Burkholderia cenocepacia*. *FEMS Microbiol Lett* **245**: 337-344.

Nies, D. H., (1995) The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in Escherichia coli. *J Bacteriol* **177**: 2707-2712.

Nies, D. H., (2003) Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol Rev* **27**: 313-339.

Nikaido, H., (1996) Multidrug efflux pumps of gram-negative bacteria. *J Bacteriol* **178**: 5853-5859.

Nikaido, H., (2011) Structure and mechanism of RND-type multidrug efflux pumps. *Adv Enzymol Relat Areas Mol Biol* **77**: 1-60.

Nikaido, H. & J. M. Pages, (2012) Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. *FEMS Microbiol Rev* **36**: 340-363.

Nikaido, H. & Y. Takatsuka, (2009) Mechanisms of RND multidrug efflux pumps. *Biochim Biophys Acta* **1794**: 769-781.

Nouwen, N. & A. J. Driessen, (2002) SecDFyajC forms a heterotetrameric complex with YidC. *Mol Microbiol* **44**: 1397-1405.

Ogawa, W., M. Onishi, R. Ni, T. Tsuchiya & T. Kuroda, (2012) Functional study of the novel multidrug efflux pump KexD from *Klebsiella pneumoniae*. *Gene* **498**: 177-182.

Owens, C. P., N. Chim & C. W. Goulding, (2013a) Insights on how the *Mycobacterium tuberculosis* heme uptake pathway can be used as a drug target. *Future Med Chem* **5**: 1391-1403.

Owens, C. P., N. Chim, A. B. Graves, C. A. Harmston, A. Iniguez, H. Contreras, M. D. Liptak & C. W. Goulding, (2013b) The *Mycobacterium tuberculosis* secreted protein Rv0203 transfers heme to membrane proteins MmpL3 and MmpL11. *J Biol Chem* **288**: 21714-21728.

Pacheco, S. A., F. F. Hsu, K. M. Powers & G. E. Purdy, (2013) MmpL11 protein transports mycolic acid-containing lipids to the mycobacterial cell wall and contributes to biofilm formation in *Mycobacterium smegmatis*. *J Biol Chem* **288**: 24213-24222.

Pak, J. E., E. N. Ekende, E. G. Kifle, J. D. O'Connell, 3rd, F. De Angelis, M. B. Tessema, K. M. Derfoufi, Y. Robles-Colmenares, R. A. Robbins, E. Goormaghtigh, G. Vandenbussche & R. M. Stroud, (2013) Structures of intermediate transport states of ZneA, a Zn(II)/proton antiporter. *Proc Natl Acad Sci U S A* **110**: 18484-18489.

Palleroni, N. J. & B. Homes, (1981) *Pseudomonas cepacia* sp. nov. nom. rev. . *Int. J. Syst. Bacteriol.* **31**: 479-481.

Papaleo, M. C., E. Perrin, I. Maida, M. Fondi, R. Fani & P. Vandamme, (2010) Identification of species of the *Burkholderia cepacia* complex by sequence analysis of the *hisA* gene. *J Med Microbiol* **59**: 1163-1170.

Parr, T. R., Jr., R. A. Moore, L. V. Moore & R. E. Hancock, (1987) Role of porins in intrinsic antibiotic resistance of *Pseudomonas cepacia*. *Antimicrob Agents Chemother* **31**: 121-123.

Partida-Martinez, L. P., I. Groth, I. Schmitt, W. Richter, M. Roth & C. Hertweck, (2007) *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*. *Int J Syst Evol Microbiol* **57**: 2583-2590.

Pasca, M. R., P. Guglierame, E. De Rossi, F. Zara & G. Riccardi, (2005) mmpL7 gene of *Mycobacterium tuberculosis* is responsible for isoniazid efflux in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* **49**: 4775-4777.

Peeters, C., J. E. Zlosnik, T. Spilker, T. J. Hird, J. J. Lipuma & P. Vandamme, (2013) *Burkholderia pseudomultivorans* sp. nov., a novel *Burkholderia cepacia* complex species from human respiratory samples and the rhizosphere. *Syst Appl Microbiol*.

Perrin, E., M. Fondi, M. C. Papaleo, I. Maida, S. Buroni, M. R. Pasca, G. Riccardi & R. Fani, (2010) Exploring the HME and HAE1 efflux systems in the genus *Burkholderia*. *BMC Evol Biol* **10**: 164.

Piddock, L. J., (2006a) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin Microbiol Rev* **19**: 382-402.

Piddock, L. J., (2006b) Multidrug-resistance efflux pumps - not just for resistance. *Nat Rev Microbiol* **4**: 629-636.

Pinto, F. G., L. M. Chueire, A. T. Vasconcelos, M. F. Nicolas, L. G. Almeida, R. C. Souza, P. Menna, F. G. Barcellos, M. Megias & M. Hungria, (2009) Novel genes related to nodulation, secretion systems, and surface structures revealed by a genome draft of Rhizobium tropici strain PRF 81. *Funct Integr Genomics* **9**: 263-270.

Podin, Y., D. S. Sarovich, E. P. Price, M. Kaestli, M. Mayo, K. Hii, H. Ngian, S. Wong, I. Wong, J. Wong, A. Mohan, M. Ooi, T. Fam, A. Tuanyok, P. Keim, P. M. Giffard & B. J. Currie, (2013) *Burkholderia pseudomallei* from Sarawak, Malaysian Borneo are predominantly susceptible to aminoglycosides and macrolides. *Antimicrob Agents Chemother*.

Podnecky, N. L., V. Wuthiekanun, S. J. Peacock & H. P. Schweizer, (2013) The BpeEF-OprC efflux pump is responsible for widespread trimethoprim resistance in clinical and environmental B*urkholderia pseudomallei* isolates. *Antimicrob Agents Chemother* **57**: 4381-4386.

Pogliano, K. J. & J. Beckwith, (1994) Genetic and molecular characterization of the Escherichia coli secD operon and its products. *J Bacteriol* **176**: 804-814.

Poole, K., (2007) Efflux pumps as antimicrobial resistance mechanisms. *Ann Med* **39**: 162-176.

Poole, K., K. Krebes, C. McNally & S. Neshat, (1993) Multiple antibiotic resistance in Pseudomonas aeruginosa: evidence for involvement of an efflux operon. *J Bacteriol* **175**: 7363-7372.

Prince, A., M. S. Wood, G. S. Cacalano & N. X. Chin, (1988) Isolation and characterization of a penicillinase from *Pseudomonas cepacia* 249. *Antimicrob Agents Chemother* **32**: 838-843.

Purdy, G. E., M. Niederweis & D. G. Russell, (2009) Decreased outer membrane permeability protects mycobacteria from killing by ubiquitin-derived peptides. *Mol Microbiol* **73**: 844-857.

Putman, M., H. W. van Veen & W. N. Konings, (2000) Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* **64**: 672-693.

Quiblier, C., K. Seidl, B. Roschitzki, A. S. Zinkernagel, B. Berger-Bachi & M. M. Senn, (2013) Secretome analysis defines the major role of SecDF in Staphylococcus aureus virulence. *PLoS One* **8**: e63513.

Quiblier, C., A. S. Zinkernagel, R. A. Schuepbach, B. Berger-Bachi & M. M. Senn, (2011) Contribution of SecDF to Staphylococcus aureus resistance and expression of virulence factors. *BMC Microbiol* **11**: 72.

Reed, M. B., P. Domenech, C. Manca, H. Su, A. K. Barczak, B. N. Kreiswirth, G. Kaplan & C. E. Barry, 3rd, (2004) A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature* **431**: 84-87.

Remuinan, M. J., E. Perez-Herran, J. Rullas, C. Alemparte, M. Martinez-Hoyos, D. J. Dow, J. Afari, N. Mehta, J. Esquivias, E. Jimenez, F. Ortega-Muro, M. T. Fraile-Gabaldon, V. L. Spivey, N. J. Loman, M. J. Pallen, C. Constantinidou, D. J. Minick, M. Cacho, M. J. Rebollo-Lopez, C. Gonzalez, V. Sousa, I. Angulo-Barturen, A. Mendoza-Losana, D. Barros, G. S. Besra, L. Ballell & N. Cammack, (2013) Tetrahydropyrazolo[1,5-a]pyrimidine-3-carboxamide and N-benzyl-6',7'-dihydrospiro[piperidine-4,4'-thieno[3,2-c]pyran] analogues with bactericidal efficacy against *Mycobacterium tuberculosis* targeting MmpL3. *PLoS One* **8**: e60933.

Riordan, J. R., J. M. Rommens, B. Kerem, N. Alon, R. Rozmahel, Z. Grzelczak, J. Zielenski, S. Lok, N. Plavsic, J. L. Chou & et al., (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**: 1066-1073.

Rommens, J. M., M. C. Iannuzzi, B. Kerem, M. L. Drumm, G. Melmer, M. Dean, R. Rozmahel, J. L. Cole, D. Kennedy, N. Hidaka & et al., (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* **245**: 1059-1065.

Ruggerone, P., S. Murakami, K. M. Pos & A. V. Vargiu, (2013) RND Efflux Pumps: Structural Information Translated Into Function And Inhibition Mechanisms. *Curr Top Med Chem*.

Saier, M. H., Jr. & I. T. Paulsen, (2001) Phylogeny of multidrug transporters. *Semin Cell Dev Biol* **12**: 205-213.

Saier, M. H., Jr., I. T. Paulsen, M. K. Sliwinski, S. S. Pao, R. A. Skurray & H. Nikaido, (1998) Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. *FASEB J* **12**: 265-274.

Saier, M. H., Jr., R. Tam, A. Reizer & J. Reizer, (1994) Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. *Mol Microbiol* **11**: 841-847.

Saldias, M. S. & M. A. Valvano, (2009) Interactions of *Burkholderia cenocepacia* and other *Burkholderia cepacia* complex bacteria with epithelial and phagocytic cells. *Microbiology* **155**: 2809-2817.

Sass, A., A. Marchbank, E. Tullis, J. J. Lipuma & E. Mahenthiralingam, (2011) Spontaneous and evolutionary changes in the antibiotic resistance of *Burkholderia cenocepacia* observed by global gene expression analysis. *BMC Genomics* **12**: 373.

Scherman, M. S., E. J. North, V. Jones, T. N. Hess, A. E. Grzegorzewicz, T. Kasagami, I. H. Kim, O. Merzlikin, A. J. Lenaerts, R. E. Lee, M. Jackson, C. Morisseau & M. R. McNeil, (2012) Screening a library of 1600 adamantyl ureas for anti-*Mycobacterium tuberculosis* activity in vitro and for better physical chemical properties for bioavailability. *Bioorg Med Chem* **20**: 3255-3262.

Schmerk, C. L., M. A. Bernards & M. A. Valvano, (2011) Hopanoid production is required for low-pH tolerance, antimicrobial resistance, and motility in Burkholderia cenocepacia. *J Bacteriol* **193**: 6712-6723.

Schmidt, T. & H. G. Schlegel, (1994) Combined nickel-cobalt-cadmium resistance encoded by the ncc locus of *Alcaligenes xylosoxidans* 31A. *J Bacteriol* **176**: 7045-7054.

Schweizer, H. P., (2012) Mechanisms of antibiotic resistance in *Burkholderia pseudomallei*: implications for treatment of melioidosis. *Future Microbiol* **7**: 1389-1399.

Seeger, M. A., A. Schiefner, T. Eicher, F. Verrey, K. Diederichs & K. M. Pos, (2006) Structural asymmetry of AcrB trimer suggests a peristaltic pump mechanism. *Science* **313**: 1295-1298.

Segonds, C., T. Heulin, N. Marty & G. Chabanon, (1999) Differentiation of *Burkholderia* species by PCR-restriction fragment length polymorphism analysis of the 16S rRNA gene and application to cystic fibrosis isolates. *J Clin Microbiol* **37**: 2201-2208.

Sennhauser, G., P. Amstutz, C. Briand, O. Storchenegger & M. G. Grutter, (2007) Drug export pathway of multidrug exporter AcrB revealed by DARPin inhibitors. *PLoS Biol* **5**: e7.

Sennhauser, G., M. A. Bukowska, C. Briand & M. G. Grutter, (2009) Crystal structure of the multidrug exporter MexB from Pseudomonas aeruginosa. *J Mol Biol* **389**: 134-145.

Smith, M. D., B. J. Angus, V. Wuthiekanun & N. J. White, (1997) Arabinose assimilation defines a nonvirulent biotype of *Burkholderia pseudomallei*. *Infect Immun* **65**: 4319-4321.

Stahler, F. N., S. Odenbreit, R. Haas, J. Wilrich, A. H. Van Vliet, J. G. Kusters, M. Kist & S. Bereswill, (2006) The novel *Helicobacter pylori* CznABC metal efflux pump is required for cadmium, zinc, and nickel resistance, urease modulation, and gastric colonization. *Infect Immun* **74**: 3845-3852.

Stanley, S. A., S. S. Grant, T. Kawate, N. Iwase, M. Shimizu, C. Wivagg, M. Silvis, E. Kazyanskaya, J. Aquadro, A. Golas, M. Fitzgerald, H. Dai, L. Zhang & D. T. Hung, (2012) Identification of novel inhibitors of *M. tuberculosis* growth using whole cell based high-throughput screening. *ACS Chem Biol* **7**: 1377-1384.

Storms, V., N. Van Den Vreken, T. Coenye, E. Mahenthiralingam, J. J. LiPuma, M. Gillis & P. Vandamme, (2004) Polyphasic characterisation of *Burkholderia cepacia*-like isolates leading to the emended description of *Burkholderia pyrrocinia*. *Syst Appl Microbiol* **27**: 517-526.

Storms, V., N. van den Vreken, M. Gillis & P. Vandamme, (2002) Evaluation of tRNA intergenic length polymorphism (tDNA-pCR) for the differentiation of the members of the *Burkholderia cepacia* complex. *Syst Appl Microbiol* **25**: 376-385.

Su, C. C., F. Long, M. T. Zimmermann, K. R. Rajashankar, R. L. Jernigan & E. W. Yu, (2011) Crystal structure of the CusBA heavy-metal efflux complex of Escherichia coli. *Nature* **470**: 558-562.

Su, C. C., F. Yang, F. Long, D. Reyon, M. D. Routh, D. W. Kuo, A. K. Mokhtari, J. D. Van Ornam, K. L. Rabe, J. A. Hoy, Y. J. Lee, K. R. Rajashankar & E. W. Yu, (2009) Crystal structure of the membrane fusion protein CusB from Escherichia coli. *J Mol Biol* **393**: 342-355.

Suarez-Moreno, Z. R., J. Caballero-Mellado, B. G. Coutinho, L. Mendonca-Previato, E. K. James & V. Venturi, (2012) Common features of environmental and potentially beneficial plant-associated *Burkholderia*. *Microb Ecol* **63**: 249-266.

Subramoni, S. & P. A. Sokol, (2012) Quorum sensing systems influence *Burkholderia cenocepacia* virulence. *Future Microbiol* **7**: 1373-1387.

Tahlan, K., R. Wilson, D. B. Kastrinsky, K. Arora, V. Nair, E. Fischer, S. W. Barnes, J. R. Walker, D. Alland, C. E. Barry, 3rd & H. I. Boshoff, (2013) SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **56**: 1797-1809.

Tal, N. & S. Schuldiner, (2009) A coordinated network of transporters with overlapping specificities provides a robust survival strategy. *Proc Natl Acad Sci U S A* **106**: 9051-9056.

Taylor, D. L., X. R. Bina & J. E. Bina, (2012) *Vibrio cholerae* VexH encodes a multiple drug efflux pump that contributes to the production of cholera toxin and the toxin co-regulated pilus. *PLoS One* **7**: e38208.

Thanassi, D. G., L. W. Cheng & H. Nikaido, (1997) Active efflux of bile salts by Escherichia coli. *J Bacteriol* **179**: 2512-2518.

Thomas, M. S., (2007) Iron acquisition mechanisms of the *Burkholderia cepacia* complex. *Biometals* **20**: 431-452.

Tibazarwa, C., S. Wuertz, M. Mergeay, L. Wyns & D. van Der Lelie, (2000) Regulation of the cnr cobalt and nickel resistance determinant of *Ralstonia eutropha* (*Alcaligenes eutrophus*) CH34. *J Bacteriol* **182**: 1399-1409.

Trepanier, S., A. Prince & A. Huletsky, (1997) Characterization of the *penA* and *penR* genes of *Burkholderia cepacia* 249 which encode the chromosomal class A penicillinase and its LysR-type transcriptional regulator. *Antimicrob Agents Chemother* **41**: 2399-2405.

Trunck, L. A., K. L. Propst, V. Wuthiekanun, A. Tuanyok, S. M. Beckstrom-Sternberg, J. S. Beckstrom-Sternberg, S. J. Peacock, P. Keim, S. W. Dow & H. P. Schweizer, (2009) Molecular basis of rare aminoglycoside susceptibility and pathogenesis of *Burkholderia pseudomallei* clinical isolates from Thailand. *PLoS Negl Trop Dis* **3**: e519.

Tseng, T. T., K. S. Gratwick, J. Kollman, D. Park, D. H. Nies, A. Goffeau & M. H. Saier, Jr., (1999) The RND permease superfamily: an ancient, ubiquitous and

diverse family that includes human disease and development proteins. *J Mol Microbiol Biotechnol* **1**: 107-125.

Tsukazaki, T., H. Mori, Y. Echizen, R. Ishitani, S. Fukai, T. Tanaka, A. Perederina, D. G. Vassylyev, T. Kohno, A. D. Maturana, K. Ito & O. Nureki, (2011) Structure and function of a membrane component SecDF that enhances protein export. *Nature* **474**: 235-238.

Valencia, E. Y., V. S. Braz, C. Guzzo & M. V. Marques, (2013) Two RND proteins involved in heavy metal efflux in *Caulobacter crescentus* belong to separate clusters within proteobacteria. *BMC Microbiol* **13**: 79.

Van Bambeke, F., E. Balzi & P. M. Tulkens, (2000) Antibiotic efflux pumps. *Biochem Pharmacol* **60**: 457-470.

Vandamme, P. & P. Dawyndt, (2011) Classification and identification of the *Burkholderia cepacia* complex: Past, present and future. *Syst Appl Microbiol* **34**: 87-95.

Vandamme, P., D. Henry, T. Coenye, S. Nzula, M. Vancanneyt, J. J. LiPuma, D. P. Speert, J. R. Govan & E. Mahenthiralingam, (2002) *Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools. *FEMS Immunol Med Microbiol* **33**: 143-149.

Vandamme, P., B. Holmes, T. Coenye, J. Goris, E. Mahenthiralingam, J. J. LiPuma & J. R. Govan, (2003) *Burkholderia cenocepacia* sp. nov.--a new twist to an old story. *Res Microbiol* **154**: 91-96.

Vandamme, P., B. Holmes, M. Vancanneyt, T. Coenye, B. Hoste, R. Coopman, H. Revets, S. Lauwers, M. Gillis, K. Kersters & J. R. Govan, (1997) Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of *Burkholderia multivorans* sp. nov. *Int J Syst Bacteriol* **47**: 1188-1200.

Vandamme, P., E. Mahenthiralingam, B. Holmes, T. Coenye, B. Hoste, P. De Vos, D. Henry & D. P. Speert, (2000) Identification and population structure of *Burkholderia stabilis* sp. nov. (formerly *Burkholderia cepacia* genomovar IV). *J Clin Microbiol* **38**: 1042-1047.

Vandamme, P., B. Pot, M. Gillis, P. de Vos, K. Kersters & J. Swings, (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**: 407-438.

Vanlaere, E., A. Baldwin, D. Gevers, D. Henry, E. De Brandt, J. J. LiPuma, E. Mahenthiralingam, D. P. Speert, C. Dowson & P. Vandamme, (2009) Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. *Int J Syst Evol Microbiol* **59**: 102-111.

Vanlaere, E., J. J. Lipuma, A. Baldwin, D. Henry, E. De Brandt, E. Mahenthiralingam, D. Speert, C. Dowson & P. Vandamme, (2008a) *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia*

seminalis sp. nov. and *Burkholderia metallica* sp. nov., novel species within the *Burkholderia cepacia* complex. *Int J Syst Evol Microbiol* **58**: 1580-1590.

Vanlaere, E., K. Sergeant, P. Dawyndt, W. Kallow, M. Erhard, H. Sutton, D. Dare, B. Devreese, B. Samyn & P. Vandamme, (2008b) Matrix-assisted laser desorption ionisation-time-of of-flight mass spectrometry of intact cells allows rapid identification of *Burkholderia cepacia* complex. *J Microbiol Methods* **75**: 279-286.

Vermis, K., T. Coenye, J. J. LiPuma, E. Mahenthiralingam, H. J. Nelis & P. Vandamme, (2004) Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov. *Int J Syst Evol Microbiol* **54**: 689-691.

Vermis, K., C. Vandekerckhove, H. J. Nelis & P. A. Vandamme, (2002) Evaluation of restriction fragment length polymorphism analysis of 16S rDNA as a tool for genomovar characterisation within the *Burkholderia cepacia* complex. *FEMS Microbiol Lett* **214**: 1-5.

Vial, L., A. Chapalain, M. C. Groleau & E. Deziel, (2011) The various lifestyles of the *Burkholderia cepacia* complex species: a tribute to adaptation. *Environ Microbiol* **13**: 1-12.

Viktorov, D. V., I. B. Zakharova, M. V. Podshivalova, E. V. Kalinkina, O. A. Merinova, N. P. Ageeva, V. A. Antonov, L. K. Merinova & V. V. Alekseev, (2008) High-level resistance to fluoroquinolones and cephalosporins in *Burkholderia pseudomallei* and closely related species. *Trans R Soc Trop Med Hyg* **102 Suppl 1**: S103-110.

Welch, D. F., (1991) Applications of cellular fatty acid analysis. *Clin Microbiol Rev* **4**: 422-438.

Wells, R. M., C. M. Jones, Z. Xi, A. Speer, O. Danilchanka, K. S. Doornbos, P. Sun, F. Wu, C. Tian & M. Niederweis, (2013) Discovery of a siderophore export system essential for virulence of *Mycobacterium tuberculosis*. *PLoS Pathog* **9**: e1003120.

Wexler, H. M., (2012) Pump it up: occurrence and regulation of multi-drug efflux pumps in *Bacteroides fragilis*. *Anaerobe* **18**: 200-208.

Wheelis, M., (1998) First shots fired in biological warfare. *Nature* **395**: 213.

Wieczorek, P., P. Sacha, T. Hauschild, M. Zorawski, M. Krawczyk & E. Tryniszewska, (2008) Multidrug resistant *Acinetobacter baumannii*--the role of AdeABC (RND family) efflux pump in resistance to antibiotics. *Folia Histochem Cytobiol* **46**: 257-267.

Yabuuchi, E., Y. Kawamura, T. Ezaki, M. Ikedo, S. Dejsirilert, N. Fujiwara, T. Naka & K. Kobayashi, (2000) *Burkholderia uboniae* sp. nov., L-arabinose-assimilating but different from *Burkholderia thailandensis* and *Burkholderia vietnamiensis*. *Microbiol Immunol* **44**: 307-317.

Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki & M. Arakawa, (1992) Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus,

with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol* **36**: 1251-1275.

Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta & Y. Nishiuchi, (1995) Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. Nov.: Proposal of *Ralstonia pickettii* (Ralston, Palleroni and Doudoroff 1973) comb. Nov., *Ralstonia solanacearum* (Smith 1896) comb. Nov. and *Ralstonia eutropha* (Davis 1969) comb. Nov. *Microbiol Immunol* **39**: 897-904.

Yoon, E. J., P. Courvalin & C. Grillot-Courvalin, (2013) RND-type efflux pumps in multidrug-resistant clinical isolates of *Acinetobacter baumannii*: major role for AdeABC overexpression and AdeRS mutations. *Antimicrob Agents Chemother* **57**: 2989-2995.

Zgurskaya, H. I. & H. Nikaido, (2000) Multidrug resistance mechanisms: drug efflux across two membranes. *Mol Microbiol* **37**: 219-225.