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REAL-TIME ASSESSMENT OF THE ACTIVITY OF ANTIBIOTIC EFFLUX PUMPS IN *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA*

Doctoral dissertation
Physical sciences, Biochemistry (04 P)

Vilnius, 2010

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ANTIBIOTIKUS IŠMETANČIŲ POMPŲ VEIKLOS *ESCHERICHIA COLI* IR *PSEUDOMONAS AERUGINOSA* LĄSTELĖSE TIESIOGINIS VERTINIMAS

Daktaro disertacija
Fiziniai mokslai, biochemija (04 P)

Vilnius, 2010

Disertacija rengta 2005-2010 metais Vilniaus universiteto Gamtos mokslų fakultete, Biochemijos ir biofizikos katedroje ir Helsinkio universiteto Biologijos mokslų katedroje.

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Abbreviation list

$\Delta\psi$ – membrane potential

cipR – resistance to ciprofloxacin

EDTA – ethylenediaminetetraacetic acid

GD – gramicidin D

meroR – resistance to meropenem

OM – outer membrane

PAβN – phenylalanyl-arginyl-β-naphtylamide

PM – plasma membrane

PMB – polymyxin B

rep-PCR – repetitive-sequence-based PCR

RND – resistance-nodulation-cell division

TC – tetracycline

tobraR – resistance to tobramycin

TPP^+ – tetraphenylphosphonium ions

INTRODUCTION

The discovery of antibiotics was one of the greatest achievements in the science. However, after penicillin was industrialized and entered clinical use in the early 1940's humans gradually encountered bacterial resistance to antimicrobials (Fernandes, 2006). Microorganisms have developed resistance to every approved antibiotic (Smith & Romesberg, 2007). Consequently, multiple antibiotic resistance (MAR) causes difficulties in infection treatments. Today antibiotic pressure is not limited only to the hospitals. Irresponsible usage of antibiotics in communities and food industry highly increases occurrences of resistant microorganisms. For partial prevention of the accumulation of antibacterial resistance the use of most antibiotics as growth promoters had been banned in European Union from 1 January 2006 (COD/2002/0073:22/09/2003 - Final legislative act).

Bacteria have developed various ways of defense to high concentrations of toxic compounds such as different drugs and antiseptics. Target alteration, antibiotic-degrading enzymes, low cell envelope permeability and active efflux are the main resistance mechanisms found in bacteria. However, low specificity efflux pumps give the highest input to multiple drug resistance (MDR) phenotypes (Wright, 2007). The main evolution driving forces mutations and horizontal gene transfer rapidly increase the emergence of bacterial drug resistance. Moreover, together with acquired resistance, virulence factors can be transferred among bacteria. In such cases even common bacteria are becoming invasive pathogens (Smith & Romesberg, 2007).

Pseudomonas aeruginosa is a highly adaptable Gram-negative bacterium occupying broad spectrum of ecological niches. Moreover, it is an opportunistic pathogen causing serious infections in patients with compromised immune system (Wilson & Dowling, 1998). *P. aeruginosa* infections are difficult to cure for their antimicrobial resistance conferred by

MDR efflux pumps belonging to the resistance-nodulation-cell division (RND) superfamily (Kumar & Schweizer, 2005). Noteworthy, resistance to all anti-pseudomonal drugs had been observed in several clinical isolates of *P. aeruginosa* (Bonomo & Szabo, 2006).

It is of high importance to completely understand and evaluate mechanisms of MDR pumps so that clinicians could introduce efficient interventions for overcoming infections caused by "superbugs". Targeting and inhibition of active efflux is one of the most researched MDR overcoming strategies (Pages & Amaral, 2009). There are several methods developed to characterize MDR efflux pumps in bacteria (Mesaros *et al.*, 2007; Vedel, 2005; Zhu *et al.*, 2007). Assays for the monitoring efflux pump activities are based on the accumulation of fluorescent probes, such as ethidium bromide (Babayan & Nikaido, 2004), pyronin Y (Pannek *et al.*, 2006) and acriflavine (Li *et al.*, 2004). Also radiolabeled antibiotics (Aires & Nikaido, 2005; Li *et al.*, 2004), tetraphenylphosphonium (TPP^+) ions (Rotem & Schuldiner, 2004) or other lipophilic compounds are used for the assays. Such methods include procedures (pelleting or filtration of the cells, incubation in closed vials), leading to conditions affecting the energy state of bacteria and, therefore, the functioning of energy-dependent pumps. However, direct efflux pump functions real-time assays are missing. Such assays would improve the evaluation of MDR efflux pump activities and the search for potential inhibitors.

The aim of this study was to develop method for on-line assessment of the activities of antibiotic efflux pumps in Gram-negative bacteria.

The following **tasks** have been formulated:

- to assay the activity of tetracycline efflux pump TetA(B) in *Escherichia coli*;
- to determine optimal conditions for the real time evaluation of RND-type efflux pumps in *Pseudomonas aeruginosa*;
- to apply the developed protocols for evaluation of RND-type efflux pump activities in clinical isolates of *P. aeruginosa*.

Scientific novelty

An assay for real-time monitoring of the activity of MDR efflux pumps was designed, employing tetraphenylphosphonium (TPP^+) ions and TPP^+-selective electrodes. Neither radiolabeled nor aggressive (e.g. ethidium bromide) compounds are used in this assay. Importantly, during the recording cell suspension is aerated and thermostated, reagents are added to the reaction mixture and samples can be taken for additional analysis without interrupting the experiment or changing the culture conditions.

It was demonstrated that the temperature and the composition of medium, the presence of nutrients and the level of aeration affect the efficiency of the TPP^+ extrusion in *P. aeruginosa*. The control of these parameters ensures robust and reproducible results obtained by the assay. These results also indicate that the change of medium composition can be the main reason for the culture growth phase-related changes of MDR pump activities. Also was shown that tetracycline-specific efflux pump TetA(B) in *Escherichia coli* has a high selectivity to tetracycline-unrelated compound TPP^+.

Current findings give better understanding of how bacterial cell physiology affects activities of MDR efflux pumps and point out the importance for standardization of experimental conditions to obtain quantitative and comparable results.

1. BACKGROUND – antimicrobial resistance

Alexander Fleming, the discoverer of penicillin, pointed out, during his Nobel Prize lecture in 1945 (http://nobelprize.org/nobel_prizes/medicine/laureates/1945/), his concerns about the ability of microorganisms to become tolerant to significant concentrations of antibiotics. After several decades, Fleming's concern turned out to be the reality. Bacterial tolerance to high concentrations of various antibiotics caused difficulties in treating bacterial infections. The old, the young and the immunocompromised were susceptible to serious infections, while in the 21st century new virulent and drug-resistant bacteria may cause severe illnesses also to healthy individuals (Fernandes, 2006).

Microorganisms employ several strategies for resistance to antibiotics: i) low permeability of the cell membrane; ii) target alterations; iii) active efflux; iv) enzymatic modification of the drug inside the cell (Delcour, 2009; Kumar & Schweizer, 2005). Moreover, biofilms (the natural phenomenon in bacterial communities) are commonly associated with many health problems and reduces the susceptibility of pathogens to therapeutics (Kvist *et al.*, 2008). All these strategies allow pathogenic microorganism to survive under harsh pressure of antibiotics. Genes responsible for such defense are encoded in chromosomes and mobile genetic elements such as plasmids, transposons or prophages (Livermore, 2004). Furthermore, along with acquired resistance, microorganisms develop higher virulence levels (Hirakata *et al.*, 2002; Levy & Marshall, 2004). In those cases former commensal bacteria become pathogens (like meticillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and drug resistant *Escherichia coli*). Other examples of resistant microbes are opportunistic pathogens, such as *Stenotrophomonas maltophilia, Acinetobacter baumannii, Burkholderia cepacia* and *Pseudomonas aeruginosa*, which infect immunocompromised patients and are frequently intrinsically resistant to multiple antibiotics (Wright, 2007).

The spread of resistant bacteria is of great concern to clinicians (Bonomo & Szabo, 2006). Multidrug resistant pathogens originated in hospitals where high antibiotic consumption took place. Today so called "superbugs" are no longer limited to hospitals. Resistant strains can be traced from the community to the hospital and vice versa, indicating that drug resistance is no longer localized (Fernandes, 2006; Levy & Marshall, 2004). The occurrence and spread of resistance genes among microorganisms, even between species, is due to mobile genetic elements (Wright, 2007). The origin of drug resistance is often due to unnecessary usage of antibiotics (Livermore, 2004; Nuutila & Lilius, 2007).

1.1. Active efflux

Extrusion of antibiotics out of the cell was the most recently identified mechanism of resistance in bacteria (Poole, 2005a). Multiple drug resistance (MDR) in many cases is attributable to active efflux pumps and reduced cell envelope permeability (Kumar & Schweizer, 2005; Lomovskaya & Watkins, 2001). The latter property is connected to Gram-negative bacteria as their cell envelope consists of two membranes. The outer membrane (OM), due to its negatively charged hydrophilic lipopolisacharide layer, makes the passage of hydrophobic compounds much more restricted compared to Gram-positive bacteria (Nikaido, 1996; Zhang *et al.*, 2008). During the active efflux processes drugs, disinfectants and other toxic compounds are expelled from cytoplasm and periplasm (in Gram-negative bacteria) to the external media. Historically, the first MDR efflux protein, the P-glycoprotein, was described in mammalian cancer cells in the middle of 1970's (Juliano & Ling, 1976). Decreased uptake of tetracycline was discovered in late 1960's – early 1970's (Reynard *et al.*, 1971) and was recognized as antibiotic resistance, caused by efflux systems (Ball *et al.*, 1977; Ball *et al.*, 1980). Since then many findings about efflux-mediated resistance to antibiotics, solvents, biocides and detergents have been reported (Kumar & Schweizer, 2005; Mahamoud *et al.*, 2007). Nowadays it is

considered that antibiotic efflux systems contribute significantly to the acquired MDR as they recognize a broad variety of substrates and are expressed in important pathogens (Lomovskaya & Bostian, 2006; Van Bambeke *et al.*, 2000). MDR efflux mechanisms are broadly conserved and invariably encoded by bacterial chromosomes. On the contrary, specific efflux pumps are encoded in plasmids and other mobile genetic elements. Often mutations in their regulator genes result in overexpression of such defense systems (Poole, 2005a). Moreover, there is evidence suggesting that the increased expression of MDR pumps may be the first step in the development of resistance to clinically relevant antibiotics (Piddock, 2006a). In addition, it has been observed that bacteria can express more than one type of MDR efflux pumps simultaneously. The different types of efflux pumps are capable of extruding the same antibiotics leading to significantly higher resistance (Lee *et al.*, 2000; Llanes *et al.*, 2004). It is noteworthy that an activity of a lost or inactive efflux pump (due to mutations) can be compensated by the induction and activity of the others (Li *et al.*, 2000). However, increased expression of one type of efflux pumps may lead to lower expression of others (Gotoh *et al.*, 1998).

Efflux systems can be divided into single and multiple-domain ones. The first ones are composed only of one type of protein(s) and located always in the plasma membrane (PM). For example MDR efflux pump LmrA in *Lactococcus lactis* belongs to this category (van Veen *et al.*, 1996). The multiple component efflux pumps are formed of several proteins (efflux pump, membrane fusion protein and OM channel), like AcrAB-TolC pump in *E. coli* (Blair & Piddock, 2009). This type of pumps is found exclusively in Gram-negative bacteria. The substrates can be transported directly to external milieu through the OM from the cytoplasm, the plasma membrane leaflets or the periplasm (Lomovskaya *et al.*, 2007).

Based on the structure and energy type used by the different MDR efflux pumps they fall into five families (Fig. 1.1): i) major facilitator superfamily (MFS), ii) ATP-binding cassette (ABC) superfamily, iii) small

multidrug resistance family (SMR), iv) multidrug and toxic compound extrusion family (MATE) and v) resistance nodulation cell division superfamily (RND) (Kumar & Schweizer, 2005; Misra & Bavro, 2009). Efflux pumps belonging to the ABC superfamily are the only ones using primary energy from ATP hydrolysis. Members of other four families, commonly referred as secondary transporters, utilize the energy of transmembrane electrochemical gradient of protons (SMR, MFS, RND, MATE) or sodium ions (MATE) which is commonly referred as proton motive force (PMF) (Misra & Bavro, 2009; Putman *et al.*, 2000). All these efflux systems make a substantial contribution to intrinsic and acquired bacterial antimicrobial resistance (Krulwich *et al.*, 2005).

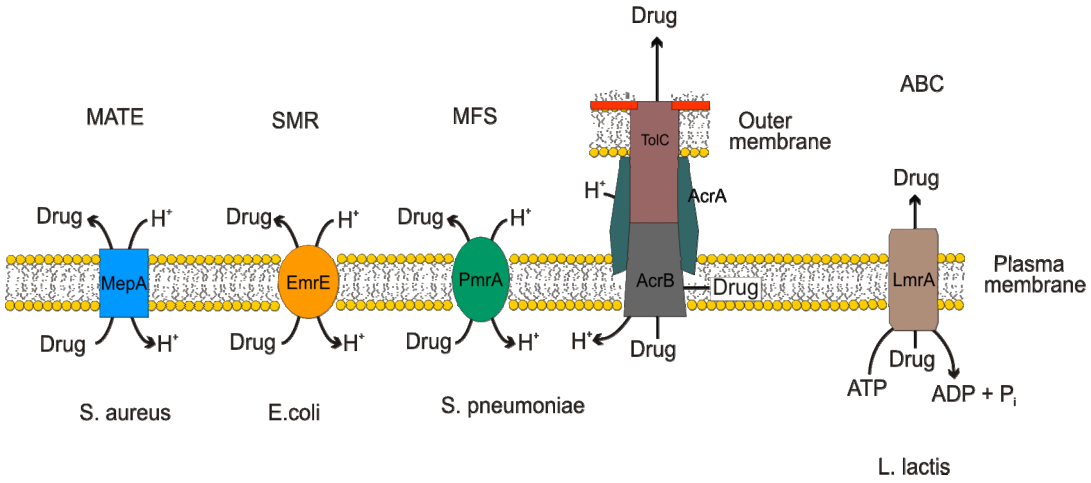

Fig. 1.1. A schematic illustration of five families of bacterial MDR efflux pumps.

In addition to the role in MDR efflux pumps are taking part in various physiological functions of pro- and eukaryotic cells, these transport systems participate in cell stress responses, environmental adaptation, biofilm formation, membrane reparation, osmosis regulation, signal transduction (e.g. export of signaling molecules), virulence and many others (Kvist *et al.*, 2008; Poole, 2008; Wright, 2007).

1.1.1. The major facilitator superfamily

The major facilitator superfamily is the largest known family of transport proteins (permeases) (Fluman & Bibi, 2009). This very old and diverse superfamily is found in all living organisms and contains more than a thousand sequenced members (Saier & Paulsen, 2001). The MFS is subdivided into seventeen families of secondary transporters that employ proton motive force as energy for their function (Piddock, 2006a). The members of those families utilize uniport, sinport and antiport as mechanisms for the transport of various compounds (metabolites, sugars, amino acids, peptides, drugs and inorganic anions). The plurality of MFS transporters can be illustrated by an *E. coli* example. There are 37 putative MDR transport systems in annotated *E. coli* genomes, where 19 of them belong to the MFS. Genes encoding these proteins can be found in the chromosome, plasmids and mobile genetic elements (Fluman & Bibi, 2009). For example, the genes of Tet proteins (to be discussed bellow) which are responsible for the resistance to tetracyclines, are located in transposon *Tn10* (Nishino & Yamaguchi, 2001). Certain efflux pumps are transcriptionally upregulated by the presence of the drug in the cell. Moreover, even endogenous low level expression of such transporters may increase the resistance to antimicrobial compounds (Fluman & Bibi, 2009).

The MFS proteins (400-600 amino acid residues long) are located in plasma membrane. They incorporate into membrane by spanning it by 12 or 14 trans-membrane-spanners (TMS). The efflux pumps harboring 14 TMS are assigned only to three families compared to other 16 families of proteins forming 12 TMS. It is proposed that the 12 TMS have evolved by duplication from primordial six TMSs (Pao *et al.*, 1998). While 14 TMS evolved from a primordial 12 TMS drug transporter by gene insertion into the middle of the 12 spanner-encoding gene. Such insertion resulted in the formation of the 7^{th} and 8^{th} tansmembrane spanners. From the more general point of view, evolutionary research results suggest that the MFS was derived from a single primordial system (Saier *et al.*, 1998). There are two strongly conserved protein motives

(A and B) among the members of MFS proteins. Motif A (GxxxD(R/K)xGR(K/R)) is present in the cytoplasmic loop between TMS 2 and 3, and it is a common feature of most members of MFS proteins. Motif B (GpilGPvlGG) is found at the end of TMS 5, and contrary to motif A is present only in MFS drug transporters. It is believed that motif B plays an important role in initial drug recognition and determining the direction of the transport. While motif A is responsible for conformational changes of the transporter (Bolhuis *et al.*, 1997).

Six MFS families are known to participate in drug extrusion. Three of them belong to drug/H^+ antiporter (DHA) families: DHA1, DHA2 and DHA3. DHA1 and DHA2 families are ubiquitous and the most analyzed. DHA2 family pumps have more restricted specificity compared to DHA1 (Saier & Paulsen, 2001). Members of DHA3 family are restricted only to prokaryotes, but are found in both Gram-positive and Gram-negative bacteria. The transporters of all three families have a broad specificity for substrates including high variety of drugs. However, the substrate specificity of the MFS efflux pumps is referred to be relatively narrow (Mahamoud *et al.*, 2007). Usually, these transporters function as single component proteins, like NorA in *S. aureus* (Yoshida *et al.*, 1990). Nevertheless, in Gram-negative bacteria such efflux systems are functioning as multi-complex systems, e.g. EmrAB-TolC system (confers resistance to uncouplers) in *E. coli* (Kumar & Schweizer, 2005; Lomovskaya & Lewis, 1992). Generally, these exporters extrude the drugs from the cell in exchange to one or two protons. However, the energy transduction during transport as well as many other aspects of MDR's are still poorly understood (Bapna *et al.*, 2007; Fluman & Bibi, 2009).

1.1.2. The ATP-binding cassette superfamily

The ABC superfamily, is the second superfamily of transport proteins found in all classified living organisms (van Veen & Konings, 1998). According to evolutionary data, this family originated more than 3 billion years

ago (Saier *et al.*, 1998). Transport systems with active uptake and efflux fall into this family. The members participating in active efflux are best exemplified by mammalian MDR efflux pump P-glycoprotein (Bolhuis *et al.*, 1997) and bacterial MDR transporter LmrA (Velamakanni *et al.*, 2009). The ABC transporters have a wide range of substrates – from ions and small biological compounds such as sugars, vitamins, amino acids and xenobiotics up to peptides, proteins and polysaccharides (Lubelski *et al.*, 2007). The ABC efflux pumps are the only MDR transporters which use primary energy from ATP hydrolysis to extrude antibiotics, toxic compounds and metabolites out of the cell. Because of the wide range of substrates utilized by this superfamily, ABC transporters play many roles in the physiology of microbial cells, e.g. immunity, virulence, pathogenesis, differentiation, extrusion of cellular waste products, osmotic stress and defense to antibiotics (Higgins, 2001; Jones & George, 2004). In spite of many functions, most of the ABC efflux systems are substrate specific (Lage, 2003; Lomovskaya & Watkins, 2001). The bacterial ABC transporters functioning as MDR efflux pumps are rare. Mostly they are found in Gram-positive bacteria. In the beginning of this millennium there were no experimental evidence of ABC transporters conferring MDR in Gram-negative microorganisms (Lage, 2003). However, today there are already examples of active ABC transport systems in Gram-negative bacteria (Al-Hamad *et al.*, 2009).

In contrast to MFS, the members of ABC superfamily are highly variable in protein size (Lomovskaya & Watkins, 2001). One of the best-characterized class of bacterial ABC transporters, involved in extracellular secretion of RTX (repeats in toxin) toxins, vary in size from 600 to 750 amino acid residues (Fath & Kolter, 1993). ABC transporters are membrane proteins consisting of transmembrane and nucleotide-binding domains (TMDs and NBDs respectively). Typically, the TMD is composed of 6 α-helices which are designated as membrane spanners. TMDs form the transmembrane channel and it is believed that they are responsible for the substrate specificity of the transporter. Protein sequence homology among TMDs in various ABC

transporters is rather low, but only few transporters deviate from the canonical rule of 6+6 helices in TMD dimers. On the contrary, NBDs share a high level of homology in different members of ABC superfamily. The NBD functions as a dimer and is located at the cytoplasmic side of the plasma membrane (Lubelski *et al.*, 2007). It plays the role of an engine which transforms ATP-derived energy to change protein conformations (Jones & George, 2004). NBDs have a conservative core of 215 amino acid residues which is an important property in defining and limiting the family (Higgins, 2001). Minimal requirements for functional transporter are the presence of 2 TMDs and 2 NBDs. However, the functional transporter can be formed as either a homo- or heterodimer, e.g. *L. lactis* transporters LmrA and LmrCD, respectively (Lubelski *et al.*, 2004). Moreover, some of the ABC transporters share closely related functionality as well. van Veen and colleagues (1998) have shown that mammalian ABC transporter P-glycoprotein can be fully complemented by bacterial transporter LmrA. Surprisingly, expression of LmrA in a heterologous host resulted in incorporation of bacterial transporter into the membrane of a human fibroblast cell (van Veen & Konings, 1998).

The genes encoding ABC efflux systems in bacteria can be located in the chromosome or plasmids (Piddock, 2006b; Poole, 2004). Most of bacterial ABC transporter genes are organized into an operons that encode different ABC and membrane domains as separate subunits requiring assembly to form biologically active transporters (Lage, 2003). Separate genes for all transporter subunits is a characteristic for the uptake ABC proteins, while efflux transporters are encoded by fewer genes as a result of the fusion of two TMDs, two NBDs or TMD with the NBD. In eukaryotes, ABC transporters can be found as individual proteins (Lubelski *et al.*, 2007).

The mechanisms of ABC transporters in both prokaryotes and eukaryotes share mechanistic similarities. The transport cycle of ABC systems can be divided into 4 steps (Fig. 1.2). The transport starts when a substrate interacts with TMDs at the inner surface of the membrane bilayer. This process initiates conformational changes in NBDs resulting higher affinity to ATP.

During the second step, ATP induces the formation of a closed NBD dimer resulting in large conformational changes in TMDs where the ligand is translocated and released to the outer surface of the cytoplasmic membrane. During the third transport step, ATP is hydrolyzed resulting in dissociation of the tightly closed NBDs dimer. Finally, ADP and P_i are released from NBDs and the transporter returns to the initial state with high affinity to the ligand. However, the detailed mechanisms of ABC transporters are still not clear and needs to be elucidated (Higgins, 2001; Linton, 2007; van Veen *et al.*, 2001).

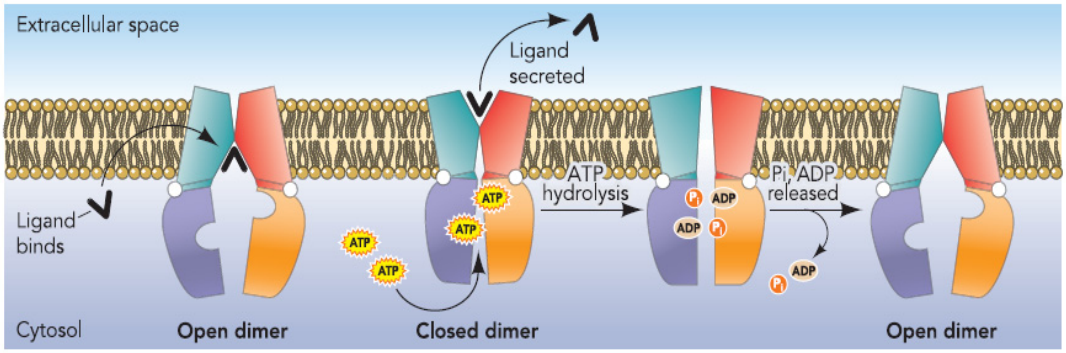

Fig. 1.2. Schematic representation of ABC transporter activity. Adopted from (Linton, 2007).

1.1.3. The small multidrug resistance family

The small multidrug resistance family (Paulsen *et al.*, 1996) was reviewed for the first time in 1996 by Paulsen and colleagues. In 1992, Grinius and colleagues (Grinius *et al.*, 1992) described one of the first proteins from this family and named it staphylococcal multidrug (Smr) protein. However, *E. coli* efflux protein EmrE is today the best understood MDR efflux system of the SMR family. It is used as a model system and as a starting point for structure predictions of other SMR family members (Bay & Turner, 2009; Kikukawa *et al.*, 2006). The SMR family is widespread but restricted only to bacteria and archea (Bay *et al.*, 2008). There are seven SMR homologues in the genome of *Bacillus subtilis* and four in the genome of *E. coli* (Jack *et al.*,

2000). At least one SMR efflux pump is found in about half of all sequenced bacteria and archea (Ninio & Schuldiner, 2003; Poulsen *et al.*, 2009). Genes encoding SMR proteins can be found in chromosomes, MDR encoding plasmids, transposons and integrons (Paulsen *et al.*, 1996).

SMR efflux systems confer clinically significant resistance to multiple lipophilic cationic drugs, dyes, disinfectants and antibiotics (β-lactams, aminoglycosides and cephalosporins) (Bay *et al.*, 2008; Grinius & Goldberg, 1994; Poulsen *et al.*, 2009). SMR efflux pumps utilize proton motive force for the extrusion of toxic compounds. The substrates are transported to the external milieu in exchange to one or more protons (Bay *et al.*, 2008; Grinius & Goldberg, 1994). The minimal structural unit required for the functional SMR efflux pump is a dimer. This is the main structural property of all characterized SMR pumps (Butler *et al.*, 2004; Jack *et al.*, 2000; Ninio & Schuldiner, 2003). However, there are presumptions of functional trimers and tetramers as well (Elbaz *et al.*, 2004). Though the SMR family efflux pumps confer resistance to toxic compounds, still little is known about natural substrates of these transport systems (Bay & Turner, 2009).

The SMR family proteins are the smallest MDR efflux pumps when compared to other pump families. Their monomeric sizes vary from 100 to 140 amino acid residues (Lomovskaya & Watkins, 2001). Although it is assumed that some larger proteins may also be distantly related to this family. For example, *E. coli* tellurite-resistance protein TehA, a 330 amino acid protein, shares similarities with SMR protein sequences and has similar functionalities, e.g. resistance to various organic cations (Paulsen *et al.*, 1996; Turner *et al.*, 1997).

SMR efflux pumps are integral membrane proteins and form four putative α-helical transmembrane spanners (TMS) (Kikukawa *et al.*, 2006). SMR family proteins are considered to be the progenitors of other MDR pump families. This assumption was made due to their low substrate specificity, size, relatively low number of TMS and similarity to protein sequences of larger families (Bay & Turner, 2009).

The analysis of available SMR protein sequences revealed the structural and functional diversity of the proteins of this family. In the first classification SMR family was divided into two subclasses (Paulsen *et al.*, 1996). While after recent phylogenetic analyses, where 685 amino acid sequences were analyzed (Bay & Turner, 2009), the third subclass was introduced (Bay *et al.*, 2008). Overall, there are 3 subclasses of the SMR family proteins today, with the following nomenclature: i) small multidrug protein (SMP); ii) supresor of *groEL* mutations (SUG) (GroEL is a part of the chaperonine system GroEL/GroES which takes part in the protein folding in bacteria); iii) paired small multidrug (PSMR) subclass (Bay *et al.*, 2008; Bay & Turner, 2009). Each subclass is thought to have a similar architecture. The SMP class proteins are mainly characterized by their ability to confer multiple drug resistance in Gram-positive and Gram-negative bacteria and Archea (Bay & Turner, 2009; Paulsen *et al.*, 1996). However, there is only one identified efflux pump in Archea (Bay *et al.*, 2008; Ninio & Schuldiner, 2003). On the contrary to the SMP SUG efflux pumps show a very narrow substrate range. In addition to their export function, these proteins may also play a role in importing small molecules (Bay & Turner, 2009). It is assumed that these transporters may be important in the uptake of regulatory compounds for chaperones and partially function as chaperones by themselves (Chung & Saier, 2002). However, little is known about the nature of relations between SUG proteins and GroEL (Bay *et al.*, 2008). The paired small multidrug class is the latest characterized class of SMR family proteins. This class of proteins has the most specific sequence distribution compared to the members of the other two classes. Such a phenomenon proposes that PSMR class proteins are maintained under selective pressure, different from the SMP and SUG classes (Bay & Turner, 2009). PSMR class efflux systems consist of two proteins where one has the length of the SMR proteins and the other one is longer (for example *E. coli* proteins YdgE 109 aa long and YdgF 121 aa long) (Bay *et al.*, 2008). The expression of both PSMR proteins is obligatory for the function of the efflux pump (Masaoka *et al.*, 2000). However, the dimeric architecture was proven in most of the

SMR family proteins, where SMP and SUG class efflux system dimers are formed from protein molecules in opposite orientation (Rapp *et al.*, 2006).

Not all members of the SMR family confer antimicrobial resistance. Some identified transporters of this family are proposed to transport unknown substrates and metabolites. However, new members of this family are waiting to be discovered and mechanisms elucidated (Bay *et al.*, 2008; Srinivasan *et al.*, 2009).

1.1.4. The multidrug and toxic compound extrusion superfamily

The MATE superfamily is the most recently described efflux pump family (Omote *et al.*, 2006). Previously the members of the MATE superfamily belonged to the MF superfamily. However, in the early 1999 Brown and colleagues separated this superfamily into two distinct ones. In spite of the similar membrane topology there are no sequence homology between them (Brown *et al.*, 1999; Kumar & Schweizer, 2005). In 1998, Morita and colleagues identified the first members of this family in *Vibrio parahaemolyticus* and *E. coli.* The efflux proteins were named NorM and YdhE, respectively (Morita *et al.*, 1998). The members of MATE superfamily are widely spread among all domains of life. Genes for these transport systems has been found even in humans. These gene products, named hMATE1 and hMATE2, export organic cations and toxic compounds and share sequence similarities with bacterial MATE efflux system NorM (Kuroda & Tsuchiya, 2009; Otsuka *et al.*, 2005).

The size of MATE transporters varies form 400 up to 1000 amino acid residues (Lomovskaya & Watkins, 2001). Nevertheless, most of them are composed of 400-550 amino acid residues and contain 12 transmembrane helices (Omote *et al.*, 2006; Putman *et al.*, 2000). Phylogenetic analysis of MATE transporters revealed that this superfamily could be divided into three subfamilies comprising 14 subgroups. Part of the bacterial MATE transporters, including NorM, belong to the family 1, while family 2 comprises eukaryotic

efflux pumps. The third family consists of bacterial and archeal MATE transporters (Moriyama *et al.*, 2008; Omote *et al.*, 2006). It is noteworthy, that all MATE proteins encoding genes may have evolved from one duplicated primordial gene. However, no apparent consensus sequence is found among MATE efflux pumps, yet they share ~40% sequence similarities (Omote *et al.*, 2006).

MATE efflux pumps are secondary transporters that utilize H^+ or Na^+ ion motive force in their transport processes. Bacterial efflux pumps commonly employ Na^+ ion motive force, however, this is not an obligatory property (He *et al.*, 2004; Omote *et al.*, 2006). The substrate specificity for MATE transporters is rather low, compared to the RND superfamily (discussed bellow). However, these efflux pumps play a significant role in multidrug resistance. The transporters belonging to MATE superfamily extrude fluoroquinolones, cationic dyes (such as ethidium bromide and acriflavine), aminoglycosides, metabolic waste products and toxic compounds (Moriyama *et al.*, 2008). Still, no common substrate for the characterized MATE efflux pumps has been found yet (Kuroda & Tsuchiya, 2009).

In spite of super-polyspecificity of MATE transporters, the physiological role of them and full substrate extrusion mechanisms are still not clear. Furthermore, there is very little information on the contribution of MATE transporters to bacterial virulence (Kuroda & Tsuchiya, 2009; Moriyama *et al.*, 2008; Nishino *et al.*, 2006).

1.1.5. The resistance nodulation cell division superfamily

The RND superfamily is the best studied and analyzed among all MDR efflux system families. The efflux systems belonging to this family have the highest impact in intrinsic and acquired MDR resistance in Gram-negative bacteria (Misra & Bavro, 2009). All known members of the RND superfamily are known to be MDR efflux pumps (Kumar & Schweizer, 2005). RND efflux systems are found ubiquitously in bacteria, archea and eukaryotes (Blair &

Piddock, 2009). Moreover, they are assumed to be as abundant as the MFS transporters (Mahamoud *et al.*, 2007). Most of bacterial RND pumps are known to reside in Gram-negative bacteria (Bolhuis *et al.*, 1997) giving the advantage for pathogenic microbes to overcome antibiotic treatments (Poole, 2004). The best studied RND efflux systems are AcrAB-TolC (*E. coli*) and MexAB-OprM (*P. aeruginosa*) (Akama *et al.*, 2004b; Mokhonov *et al.*, 2005; Mokhonov *et al.*, 2004; Symmons *et al.*, 2009). These protein complexes are the main model-systems used for analysis of transport mechanisms and predicting of structures and putative mechanisms of other related RND efflux systems.

The genes for the RND efflux systems are commonly located in bacterial chromosome (Zgurskaya & Nikaido, 2000). However, there are some cases where RND pump genes are in plasmids (Hansen *et al.*, 2004). The genes of all RND-type efflux system components often are organized into one gene cluster (Kumar & Schweizer, 2005). There are, however, some exceptions when a distant gene encodes one of the components of a tripartite efflux system. For example, *P. aeruginosa* efflux system MexXY-OprM utilizes outer membrane protein OprM, which is a gene product of the gene cluster for the MexAB-OprM efflux system (Aires *et al.*, 1999). Commonly the chromosomal genes are expressed constitutively (where they contribute to instrinsic resistance) or following a mutation (where they contribute to acquired resistance) (Poole, 2007).

RND proteins belong to the secondary transporters and utilize proton motive force for their catalytic processes. During the transport process one drug molecule is extruded to the external milieu in exchanged with one H^+ (Piddock, 2006b). The substrates may be obtained from both the cytoplasm and from the periplasmic space of Gram-negative bacteria. In addition to these to pathways, RND efflux pumps can capture the substrate from the plasma membrane (Aires & Nikaido, 2005). However, all these transport mechanisms are different from each other (Piddock, 2006b).

The size of RND proteins varies from 700 up to 1300 amino acid residues (Lomovskaya & Watkins, 2001; Saier & Paulsen, 2001). All RND pumps fall into seven phylogenetic families, where three primary families are largely restricted to Gram-negative bacteria. It is proposed that all members of this family evolved from one primordial protein (Saier & Paulsen, 2001). RND efflux pumps function as tripartite systems composed of: i) the transport (efflux) protein (RND integral membrane protein forming 12 TMS, e.g. AcrB); ii) the membrane fusion protein (MFP, e.g. AcrA); iii) the outer membrane protein (OMP, e.g. TolC), either termed as an outer membrane factor or an outer membrane channel (Misra & Bavro, 2009; Piddock, 2006a). For more detailed schematic representation of the efflux pump AcrAB-TolC see Fig. 1.3.

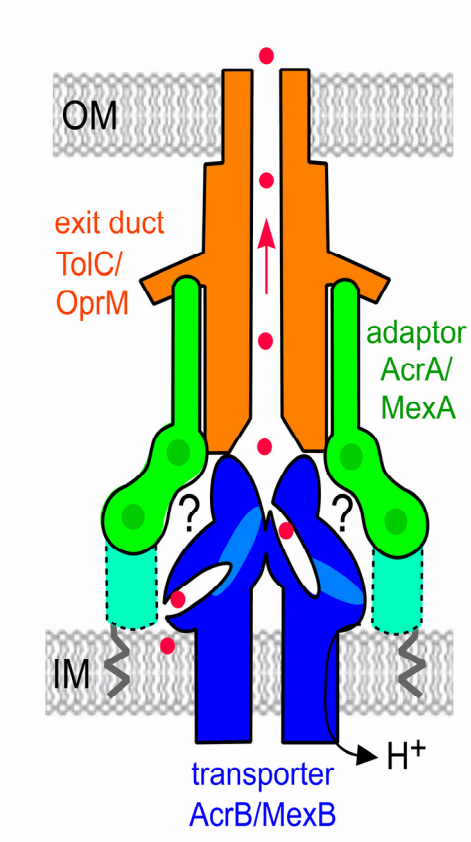

Fig. 1.3. Schematic presentation of the tripartite multidrug efflux pump. These pumps comprise an OMP (exit duct) (shown in orange), a transporter (in blue) and a MFS (adaptor) protein (in green) linked to the inner membrane by a fatty acid (zigzag line). The adaptor binds the exit duct domain via a hairpin (Lobedanz *et al.*, 2007) and the transporter via unknown interactions (indicated by ?). The linear multidomain adaptor structure is flexible, but incomplete, missing the MP domain indicated by the dotted outlines. Red dots indicate antibacterial drugs bound to the putative pockets in the transporter, passing through TolC, and out of the cell. Adopted from (PNAS, (Symmons *et al.*, 2009))

Together these proteins form a tunnel witch transports toxic compounds directly from the cytoplasm or periplasm to the exterior. Such a passage ensures that during the extrusion process antibiotics do not interact with periplasmic space. The transport of toxic compounds through the cell envelope

and the outer membrane permeability works in synergy (Zgurskaya & Nikaido, 2000), as the transported compound has to traverse the barrier of OM again to reenter the cell (Nikaido & Takatsuka, 2009). It was calculated, that these tripartite efflux pumps may have a 100 fold-reduced activity, compared to substrate-specific pumps and still ensure an adequate resistance level to the bacterial cell (Lomovskaya & Watkins, 2001). Furthermore, in spite of the high efficiency of the substrate extrusion, these complex efflux systems can extrude extremely high number of antibiotics (aminoglycosides, β-lactams, tetracyclines, macrolides, quinolones and others), cationic dyes (acriflavine, ethidium bromide), disinfectants, bile salts and other toxic compounds (Li *et al.*, 1994a; Li *et al.*, 1994b; Li *et al.*, 2003a; Mao *et al.*, 2001; Masuda *et al.*, 2000a; Nishino *et al.*, 2006).

Besides the defense functions, RND pumps serve other physiological functions as well. Cell metabolites, toxic products of metabolism, virulence factors, toxins and signaling molecules are transported by the RND efflux systems (Blair & Piddock, 2009; Evans *et al.*, 1998; Jeannot *et al.*, 2008; Nishino *et al.*, 2006). Moreover, expression of other RND pumps in the cell are induced in the cases when the principal physiological efflux pumps are deficient or inactive (Li *et al.*, 2000).

1.2. Antibiotic efflux pumps in *E. coli*

E. coli is a natural symbiotic microorganism in the gastrointestinal trackts of humans and warm-blooded animals. However, some strains can cause food-borne diseases which can be fatal, especially to the young (under 15 years) and the old. Enterohaemorrhagic *E. coli* (EHEC) is one of the most important and most studied strain in this category (www.who.int). Antibiotic resistance has a huge impact on the treatment of *E. coli* infections as resistance is spreading not only in hospitals, but also in the communities (Rice, 2009). *E. coli* was chosen as a model microorganism in the late 1930's. It was a choice of bacterial physiologists as it had minimal virulence and could be grown

readily in predefined media (Schaechter & Neidhardt, 1987). In addition, *E. coli* genome was sequenced already in 1997. This gave a huge advantage for researches to analyze this microorganism (Blattner *et al.*, 1997). In spite of the complications in treating infections, antibiotic resistance genes have been used as genetic markers. Frequently β-lactamases and tetracycline efflux systems are employed for this purpose (May *et al.*, 2009).

The sequenced genome of *E. coli* revealed that there are all the five efflux protein families present in this bacterium (Blattner *et al.*, 1997). Nishino and Yamaguchi showed that 37 open reading frames (ORFs) code for putative drug resistance efflux proteins. However, only 20 of them have been confirmed to be active (11 MF, 2 SMR, 6 RND, and 1 ABC) (Nishino & Yamaguchi, 2001).

In spite of intrinsic resistance, additional genes conferring antibiotic resistance may be obtained from the environment by horizontal gene transfer. Resistance genes are often carried by mobile genetic elements like MDR plasmids (also called as R-plasmids), transposons and integrons (May *et al.*, 2009). However, particular attention has been given to *E. coli* tetracycline resistance (Palmer *et al.*) and RND efflux pumps (Nishino *et al.*, 2009) (see bellow).

1.2.1. Tetracycline efflux pumps

TetA is one of the most common efflux pump conferring specific resistance to tetracyclines in bacteria (Hillen & Berens, 1994). TetA protein was discovered in 1974 (Levy & McMurry, 1974). Since the discovery, different types of TetA proteins have been characterized. TetA may be encoded by the chromosome or by mobile genetic elements such as plasmids, integrons and transposons. TetA proteins have been grouped alphabetically. TetA(A-E), (G) proteins are found among Gram-negative bacteria. While Gram-positive bacteria carry Tet(K), (L), (P) and (Z) efflux proteins (Ammor *et al.*, 2008; Hillen & Berens, 1994; McMurry *et al.*, 1980; Randall *et al.*, 2004; Tauch *et*

al., 2000). *tet* genes are commonly found on mobile elements (e.g. transposon *Tn10* encodes for TetA(B)). However, in some cases tetracycline efflux pump genes can be found also in the chromosome (like TetA(L) in the chromosome of *Bacillus subtilis*) (Cheng *et al.*, 1996). The genes of all TetA proteins are inducible by tetracycline and strictly regulated by the *tetR* genes, as the overproduction of TetA proteins may affect the osmoliarity and other physiological functions of the cell (Stavropoulos & Strathdee, 2000). Besides, the *tet* dependent tetracycline resistance level is dose dependent (Allard & Bertrand, 1993).

TetA proteins are integral cytoplasmic membrane polypeptides commonly forming 12 TMS (Hillen & Berens, 1994; Levy, 1992), with the exception of TetA(L) spanning the membrane 14 times (Cheng *et al.*, 1996). The size of TetA proteins varies from 35 to 50 kDa. TetA proteins utilize proton motive force and belong to the MFS efflux pumps (Speer *et al.*, 1992; Yamaguchi *et al.*, 1991). It is noteworthy that based on their amino acid similarity ranging from 78 to 45 % and common energy source it has been proposed that all TetA proteins had the same progenitor (Hillen & Berens, 1994; Speer *et al.*, 1992) which may have evolved from the cell transport proteins by gene duplication (Levy, 1992).

Tet proteins of Gram-negative bacteria confer resistance to tetracyclines by causing active efflux from cytoplasm into the periplasmic space (Thanassi *et al.*, 1995). However, it was shown (de Cristobal *et al.*, 2006) that TetA(B) confers high resistance to tetracyclines only when the main *E. coli* efflux pump AcrAB-TolC is operational. TetA(B) is the best studied pump in the whole Tet protein group and is commonly found in *E. coli* (Yamaguchi *et al.*, 1993). TetA(B) has rather low specificity to substrates when compared to other Tet proteins (Guay *et al.*, 1994; Yamaguchi *et al.*, 1993). Moreover, it was shown that TetA(B) can transport unrelated substrates to tetracycline.

1.2.2. RND-type efflux pumps of *E. coli*

There are seven RND efflux protein ORFs in the chromosome of *E. coli* (*acrB*, *acrD*, *acrF*, *yhiV*, *cusA* (formerly *ybdE*) (Nishino & Yamaguchi, 2001), *mdtB* and *mdtC* (formerly *yegN* and *yegO*, respectively) (Nagakubo *et al.*, 2002)) assumed to confer drug resistance. Six of them encode RND proteins for five drug efflux systems (Kumar & Schweizer, 2005; Li & Nikaido, 2004). MdtABC is a multiheteromeric efflux system while *cusA* encodes for a protein participating in copper and silver resistance (Franke *et al.*, 2003). Most of those pumps are weakly expressed under normal laboratory growth conditions in wild type cells, except the AcrAB-TolC drug efflux system (Li & Nikaido, 2004; Nishino *et al.*, 2009).

1.2.2.1. AcrAB-TolC efflux system

In 1965, the role of *acr* genes in bacterial resistance to cationic dyes (acriflavine, methylene blue, toluidine blue, crystal violet, methyl green, and pyronine B) was discovered (Nakamura, 1965). However, the first molecular characterization of those genes and their products started in 1993 (Ma *et al.*, 1993). AcrAB-TolC is probably one of the best studied RND efflux systems today. There are high resolution structures available for all three components of this efflux system (Murakami *et al.*, 2006; Symmons *et al.*, 2009).

AcrB is the integral cytoplasmic membrane RND component, which is responsible for the drug recognition and energy utilization during the drug extrusion. AcrB spans the cytoplasmic membrane 12 times (Yu *et al.*, 2003). The energy used during the efflux process is the proton motive force (Ma *et al.*, 1993). AcrA is a membrane fusion protein taking part in stabilizing the complex (Dinh *et al.*, 1994). AcrA is not specific only to AcrAB-TolC system but takes part in forming other efflux system with AcrD, where AcrD does not employ its own membrane fusion protein. In addition, the AcrB and AcrD are

homologous proteins (Elkins & Nikaido, 2002). TolC is an outer membrane protein, which enables direct connection with the external milieu. Structural analysis of AcrAB-TolC system revealed that AcrB docks directly to the TolC. However, this phenomenon of direct interaction has not been biochemically detected (Murakami & Yamaguchi, 2003). Moreover, it was shown (Tikhonova & Zgurskaya, 2004) that the process of the efflux system assembly does not require any energy, however, it was proposed that energy is used to disassemble the AcrAB-TolC system and recycle its components.

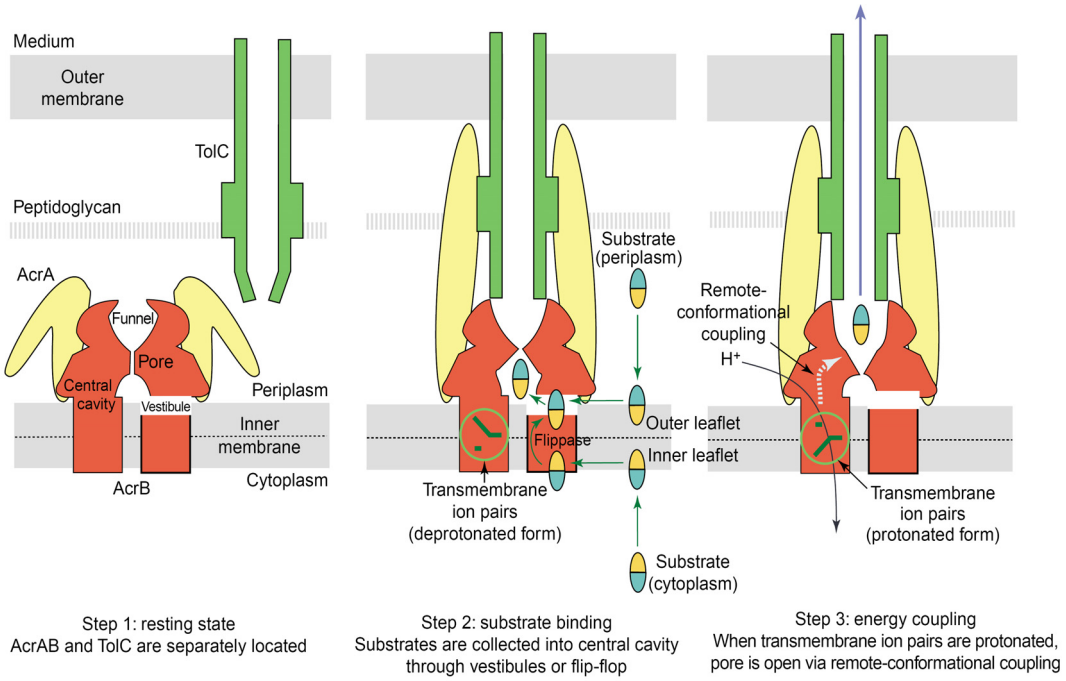

Fig. 1.4. Proposed mechanism for AcrAB-TolC mediated drug extrusion. Addopted with permission from (Murakami & Yamaguchi, 2003).

AcrAB-TolC system is composed of homotrimers of all components: $AcrA_3$:$AcrB_3$:$TolC_3$ (Symmons *et al.*, 2009). The molecular masses for each component monomer are 43, 110 (Tikhonova & Zgurskaya, 2004) and 51 kDa (Yamanaka *et al.*, 2001), respectively. All three components are essential to form the active efflux pump. It was shown that introducing mutations into one of the components led to high sensitivity of antibacterial compounds (Elkins & Nikaido, 2002; Tikhonova & Zgurskaya, 2004). It was shown (Elkins & Nikaido, 2002; Murakami & Yamaguchi, 2003) that AcrAB-TolC more likely

captures substrates from the periplasm and/or cytoplasmic membrane making this process favorable for bacteria as the toxic compounds do not reach the cytoplasm.

The substrate specificity of AcrAB-TolC system is strikingly low. It confers resistance to dyes, bile salts, organic solvents and different antibiotics. Even the charge and other chemical properties of the substrates do not play significant role in the recognition, as anionic, cationic, zwitterionic, aromatic or aliphatic chemicals are the substrates being extruded from cell (Eicher *et al.*, 2009; Murakami *et al.*, 2006; Yu *et al.*, 2003). However, there should be some distinction between toxic and vitally important compounds. Consequently, there are a lot of questions to be answered about the substrate recognition (Eicher *et al.*, 2009).

acrA and *acrB* genes are encoded from the same operon, while TolC genes are located in the distinct region of the *E. coli* chromosome (Zgurskaya & Nikaido, 2000). Antibiotic resistance is not the only process where TolC is involved, it acts with different protein exporters as well (Andersen *et al.*, 2001). It was estimated that the exponentially growing *E. coli* cell contains ~5000–7000 copies of AcrA, ~500 copies of AcrB and ~1500 copies of TolC. Moreover, it is assumed that only a small number of AcrAB-TolC system is fully assembled and functional *in vivo* (Tikhonova & Zgurskaya, 2004). It might be assembled only to warrant the balance of the physiological functions of the cell and necessary defense against external compounds (Nishino *et al.*, 2009). Besides, the binding of substrate to AcrB induces conformational changes of the protein followed by assembly of the active efflux system (Fig. 1.4) (Murakami & Yamaguchi, 2003).

1.3. Antibiotic efflux pumps in *P. aeruginosa*

P. aeruginosa was isolated from skin wounds in 1882. This, highly adaptable, Gram-negative bacterium can be found in the soil, plants, costal marine habitats, human and animal skin (Mathee *et al.*, 2008; Wilson &

Dowling, 1998). However, *P. aeruginosa* is one of the major pathogens associated with mortality and morbidity in burn victims, cystic fibrosis patients, AIDS and other immunocompromised patients (Harrison, 2007; Tohidpour *et al.*, 2009). *P. aeruginosa* has high intrinsic and acquired antibiotic resistance. These properties make it difficult and sometimes even impossible to cure infections caused by this microorganism (Bonomo & Szabo, 2006). The wild type *P. aeruginosa* is susceptibile to β-lactams: carboxypenicillins (carbeniclin, ticarcilin), ureidopenicillins (azlocillin, piperacillin), few third generation cephalosporins (ceftazidime, cefsulodine, cefoperazone), all the fourth generation cephalosporins and carbapenems (imipenem, meropenem). However, it is resistant to quinolones, trimethoprim, tetracycline and chloramphenicol (Strateva & Yordanov, 2009). *P. aeruginosa* employs all the antibiotic resistance strategies common to Gram-negative bacteria. However, active efflux plays the major role in the survival under antibiotic pressure (Lomovskaya *et al.*, 2007). Moreover, antibiotic efflux pumps contribute to the resistance in biofilm growing cultures (Zhang & Mah, 2008).

Sequenced genome of *P. aeruginosa* revealed that there are all the five families of MDR efflux pumps encoded in its genome (Stover *et al.*, 2000). Most of the efflux pump genes have been cloned and characterized (He *et al.*, 2004; Li *et al.*, 2003a; Mima *et al.*, 2007; Mima *et al.*, 2009; Poole, 2007). The focus has been given on the RND superfamily efflux systems because of their highly efficient transport activity and enormous range of substrates (Strateva & Yordanov, 2009).

1.3.1. RND pumps in *P. aeruginosa*

There are 12 putative RND efflux systems encoded in the genome of *P. aeruginosa* (www.pseudomonas.com). Mima and colleagues (Mima *et al.*, 2009) characterized the last of them recently. In almost all the cases efflux systems are encoded by one operon, which consist of genes encoding for RND

integral membrane protein (e.g. *mexB*), membrane fusion protein (e.g. *mexA*) and outer membrane channel (e.g. *oprM*). Altogether, these proteins form a tripartite system, typical for all RND efflux systems. Multiple drug efflux (Mex) pumps are the major part of the RND efflux systems in *P. aeruginosa*. There are in total 9 Mex systems: MexAB-OprM (Poole *et al.*, 1996), MexCD-OprJ (Gotoh *et al.*, 1998), MexEF-OprN (Kohler *et al.*, 1997a), MexJK-OprM (Chuanchuen *et al.*, 2002), MexMN-OprM (Mima *et al.*, 2005), MexPQ-OpmE (Mima *et al.*, 2005), MexVW-OprM (Li *et al.*, 2003b), MexGHI-OpmD (Aendekerk *et al.*, 2002), MexXY-OprM (Mine *et al.*, 1999). All these pumps contribute to acquired resistance as a consequence of hyper-expression of the efflux genes due to mutational events. Besides antibiotics, Mex pumps extrude dyes, organic solvents, detergents, metabolic waste products and molecules involved in cell-cell communications (Schweizer, 2003). The expression of Mex efflux pumps is in general strongly regulated and in some cases, hyperexpression of one pump can lead to reduced expression of the other (Lee *et al.*, 2000). In addition to Mex pathway, *P. aeruginosa* possesses multiple drug MuxABC-OpmB (Mima *et al.*, 2009) and triclosan-specific TriABC-OpmH (Mima *et al.*, 2007) efflux systems.

1.3.1.1. Mex efflux systems

MexAB-OprM efflux system is the major pump in the wild type *P. aeruginosa* (Xu *et al.*, 2004). It is a constitutively expressed efflux pump, while other Mex pumps remains unexpressed under normal laboratory conditions (Srikumar *et al.*, 1997). MexAB-OprM plays a physiological role by exporting secondary metabolites, signal molecules and virulence factors into the surrounding niche/media (Li *et al.*, 1995; Sobel *et al.*, 2005; Sugimura *et al.*, 2008). There is considerable data collected about this efflux system, many substrates are defined for it and high resolution structures are available for all the components of this efflux system (MexA (Akama *et al.*, 2004b), MexB (Sennhauser *et al.*, 2009) and OprM (Akama *et al.*, 2004a).

MexB is an integral cytoplasmic membrane RND protein forming 12 TMS (Guan *et al.*, 1999). The calculated monomeric molecular mass for MexB is 113 kDa (Mokhonov *et al.*, 2005). It is assumed that the functional protein forms a trimeric structure bound to the cytoplasmic membrane. MexB is responsible for the drug recognition and energy utilization during the extrusion process. Employing membrane fusion protein MexA (42 kDa), MexB connects to OprM (51 kDa) (Akama *et al.*, 2004a; Yoneyama *et al.*, 2000), where MexA is the central for the MexAB-OprM assembly (Mokhonov *et al.*, 2004). It was shown (Yoneyama *et al.*, 1998) that defect in any component leads to the loss of MexAB-OprM functionality. The subunit stoichiometry is $MexA_6$:$MexB_3$:$OprM_3$ (Akama *et al.*, 2004b; Narita *et al.*, 2003). It is noteworthy that MexAB-OprM system is capable of transporting extremely large particles (up to 80 nm) through the envelope of the cell (Xu *et al.*, 2004).

There are about 400 assemblies of MexAB-OprM per wild type cell (Narita *et al.*, 2003). At least three genes (*mexR*, *nalC*, and *nalD*) control the expression of this efflux system. Mutation in any of these genes leads to increased expression of this efflux system, resulting in decreased susceptibility to antimicrobials (Sobel *et al.*, 2005). The loss of the functional MexAB-OprM efflux system leads to the hypersusceptibility to some antibiotics (e.g. ticarcilin). Such a phenotype has been observed among isolates of *P. aeruginosa* from cystic fibrosis patients. It is assumed that such a phenotype is a result of the adaptation to the ecological surroundings in the lungs of the patients (Vettoretti *et al.*, 2009). Moreover, the loss of this efflux pump may be compensated by expression of an other Mex efflux pump (Li *et al.*, 2000).

MexCD-OprJ, **MexEF-OprN** and **MexXY-OprM** are the main research targets among inducible efflux pumps in *P. aeruginosa*. The expression of these pumps can occur under the presence of an inducer (antibiotic or toxic compound) in the growth medium (Hocquet *et al.*, 2003a; Morita *et al.*, 2001; Morita *et al.*, 2003) or by mutation in genes *nfxB* and *nfxC* for MexCD-OprJ and MexEF-OprN respectively (Gotoh *et al.*, 1998; Kohler *et al.*, 1997a). MexCD-OprJ and MexEF-OprN efflux systems are encoded by

their own operons, however, both MexCD and MexEF may interact with OprM of MexAB-OprM system resulting in the formation of an active efflux systems (Maseda *et al.*, 2000). It has been shown also that the expression of MexCD-OprJ negatively affects the expression level of MexAB-OprM (Gotoh *et al.*, 1998). In addition, a matter of great concern is the MexXY-OprM efflux pump as it confers resistance to aminoglycosides which are broadly used to cure infections caused by *P. aeruginosa* (Hocquet *et al.*, 2003b; Mao *et al.*, 2001). Overexpression of this particular pump is leaded by mutations in the regulatory genes *argZ* or *argW*. The emergence of MexXY-OprM hyperprodusers *in vivo* is rear. However, there is no clear explanation on how such mutants emerge in hospital settings (Hocquet *et al.*, 2008).

1.4. Strategies to overcome antimicrobial resistance

During the past 20 years the antibiotic resistance has steadily increased. Consequently, the need of new ways to combat resistance is crucial. The development of new antibiotic is slow and a long period has passed since new antibiotic entered the market (Norrby *et al.*, 2005). There are a number of methods developed to identify antibiotic resistant microbes and their defense mechanisms, especially those causing active efflux. Those methods are based on antibiotic resistance patterns and genetic identification (Mesaros *et al.*, 2007; Vedel, 2005; Zhu *et al.*, 2007). However, there are no methods employed for monitoring the physiological state of the cell and efflux pump functions in real-time.

The development of new antibiotics takes a small part in the strategies designed to overcome resistance. New targets to combat resistance are being searched for (Coates & Hu, 2007; Norrby *et al.*, 2005). The target in bacteria should be the receptor of a ligand, specific enzyme or metabolic pathway. Target identification might be achieved by the number of ways, but the main principle is that target should not be shared with the human host (Coates & Hu, 2007). Multiple drug efflux pumps are one among such targets. The major

attention has been given to the pump inhibitors, which could be used together with antibiotics to reverse acquired resistance (Lomovskaya *et al.*, 1999). One of the first and the best studied inhibitors is a general RND efflux pump inhibitor phenylalanyl-arginyl-β-naphthylamide (PAβN) (Fig. 1.5), also known as MC-207,110 (Lomovskaya *et al.*, 2001).

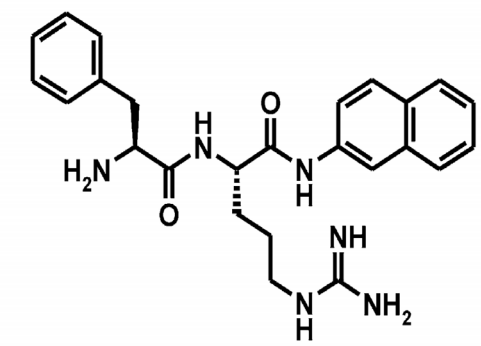

Fig. 1.5. PAβN structure. Addopted with permission from (Renau *et al.*, 2003)

This inhibitor was broadly used in the studies of antimicrobial resistance and invasiveness of *P. aeruginosa*. Moreover, clinical trials have been initiated with one of its analogues (Kumar & Schweizer, 2005; Lomovskaya & Bostian, 2006). However, PAβN is not the only potential inhibitor of efflux pumps in Gram-negative bacteria. There are several compounds (ammonium acetic acid analogue 22 (D13-9001), 1-(1-naphthylmethyl)-piperazine, and resazurin) inhibiting the activities of antibiotic efflux pumps (Kern *et al.*, 2006; Vidal-Aroca *et al.*, 2009; Yoshida *et al.*, 2006) and reducing the virulence in bacteria (Bina et al 2008; Hirakata et al 2009). It is noteworthy, that the D13-9001 has been already tested with laboratory rats and monkeys (Yoshida *et al.*, 2007).

Besides the inhibitors, new antimicrobials are being searched from the natural resources. For this purpose the genomes of sequenced bacteria are searched for putative antimicrobial compounds encoding genes (Clardy *et al.*, 2006; Fischbach, 2009; Lewis & Ausubel, 2006; Sheridan, 2006). Moreover, gene silencing strategy has been recently employed as one of the ways to combat drug resistance in Gram-negative microorganisms (Enne *et al.*, 2006; Woodford & Wareham, 2009).

2. MATERIALS AND METHODS

2.1. Bacterial strains and chemicals

Bacterial strains are listed in the table 2.1, clinical *P. aeruginosa* isolates are listed in the table 3.2. Gramicidin D (GD), polymyxin B (PMB), phenylalanyl-arginyl-β-naphtylamide (PAβN), EDTA and antibiotics (chloramphenicol, ampicillin, streptomycin sulfate and tetracycline hydrochloride) were from Sigma, tetraphenylphosphonium (TPP^+) chloride was from Aldrich, triclosan was from Fluka.

Table 2.1. Bacterial strains used in the study.

Strain	Genotype	Reference
E. coli		
MC4100	F^-araD 139 *lac*ΔU169 thi *relA rpsL* mot^-	(Casadaban, 1976)[a]
IQ86	MC4100 *zdh*::Tn10	(Shiba *et al.*, 1984)[a]
P. aeruginosa		
PAO1	*wt*	
EryR	PAO1 *nfxB*	(Hamzehpour *et al.*, 1995)[a]
MutGR1	PAO1 *mexZ*	(Vogne *et al.*, 2004)[a]
PAO7H	PAO1 *nfxC*	(Kohler *et al.*, 1997a)[a]
PAOdeltaBex	PAO1 Δ*mexB*	[b]
PT629	PAO1*nalB*	(Kohler *et al.*, 1997b)[a]

[a] – original description of the strain
[b] – gift from prof. P. Plésiat's laboratory strain collection

2.2. Bacterial growth and MIC determination conditions

All bacteria were cultivated in Luria-Bertani broth (LB), (Sambrook & Russell, 2001), containing diminished NaCl concentration (0.5 %) for *P. aeruginosa*, 0.9 % of NaCl was used for *E. coli*, at 37 °C with aeration. Cell growth was monitored by measuring optical density: *E. coli* – at 540 nm (OD_{540}) and *P. aeruginosa* – at 550 nm (OD_{550}).

The MICs of antibiotics were determined in LB by the broth microdilution method (Wiegand *et al.*, 2008). Cell concentration in the initial suspension was $5x10^5$ cell/ml, the final concentration of PAβN was 60 μg/ml.

2.3. Cell suspension preparation

To prepare cell suspension overnight cultures were diluted in LB 1/100 and grown to OD_{550} of 1 (in the experiments with *E. coli* OD was evaluated at wave length of 540nm). Then cells were harvested by centrifugation at 6000 g 4°C for 15 min and resuspended in 1/100 of growth medium volume 100 mM Tris-HCl buffer (pH 8.0) (in the case of *E. coli*) or 100 mM (pH 8) sodium phosphate buffer (in the case of *P. aeruginosa*). Cell suspensions were kept on ice until used, maximally 4 hours.

2.4. *E. coli* viability evaluation

For viability measurements the obtained cell suspension (as described in 2.3) was diluted in LB medium to OD_{550} of 0.9 and incubated for 10 min at 37°C with aeration. Then the proper concentration of tetracycline was added and cells incubated for additional 20 min at 37°C with aeration. After incubation cells were diluted in 0.9 % NaCl and plated on solid LB containing plates. Plates were incubated at 37°C for 16-18 h. Obtained colony forming units (CFU) were calculated and the percentage of viable cells was calculated.

2.5. Exposure of *E. coli* to tetracycline

Exposure of the cells to tetracycline was performed when concentration of cultivated cells reached ~3×10^8 cells/ml adding this antibiotic to the final concentration of 100 μg/ml and incubating for additional 15 min at 37°C with aeration. Cells were harvested and utilized as described in 2.3.

2.6. *E. coli* Tris/EDTA treatment

The harvested cells were resuspended in 100 mM Tris buffer containing 2 mM EDTA (pH 8.0) to 1/10 of the original volume, incubated for 10 min at 37°C, pelleted by centrifugation and resuspended in 1/100 of orginal growth volume.

2.7. Clinical isolates of *P. aeruginosa* and antibiotic resistance determination

A total of 63 *P. aeruginosa* isolates from superficial skin infections (leg and foot ulcers and decubitus wounds) were collected in HUSLAB (Laboratory of Helsinki University Central Hospital) in 2007. The culture samples from this area received by HUSLAB are both from these hospitals as well as from outpatients of this geographical areas: City of Helsinki and Uusimaa district in southern Finland. The culture samples of HUSLAB analyzed in this study were from patients treated in 12 different hospitals as well as from 19 different outpatient health centers.

P. aeruginosa strains were cultured in aerobic atmosphere on chocolate and CLED (cysteine lactose electrolyte deficient) agars and incubated at 35°C for 18 h. Antimicrobial susceptibility tests to six antimicrobial agents (ciprofloxacin, ceftazidime, meropenem, piperacillin+tazobactam, tobramycin and amikacin) were performed by disc diffusion method according to the CLSI guidelines (http://www.clsi.org).

2.8. Electrochemical measurements

Construction and characteristics of TPP^+-selective electrode were described previously (Grinius *et al.*, 1980). The Ag/AgCl reference electrodes (Orion Research, Inc., model 9001) were indirectly connected to the vessels through agar salt bridges (Fig. 2.1). The electrodes were connected to potential-amplifying system based on an ultralow input bias current operational amplifier AD549JH (Analog Devices, USA). TPP^+ fluxes in the medium were

monitored using selective electrodes as described previously (Daugelavicius *et al.*, 1997). The cell suspensions were aerated by magnetic stirring. Calibration of the measuring system using standard amounts of TPP^+ chloride (Aldrich) was carried out at the beginning of every measurement when concentrated cell suspension was used. Calibration of the measuring system was done at the end of experiment when samples were directly taken from growing cultures.

To induce ion-permeable pores in the cell envelope and depolarize the plasma membrane, at the end of experiments polymyxin B sulfate was used. This led to efflux TPP^+ ions from the cell to the incubation media. Detailed expereimental conditions are described in the figure legends. Measurements of TPP^+ concentration were performed simultaneously in 4 reaction vessels as described previously (Daugelavicius *et al.*, 2005; Daugelavicius *et al.*, 2007).

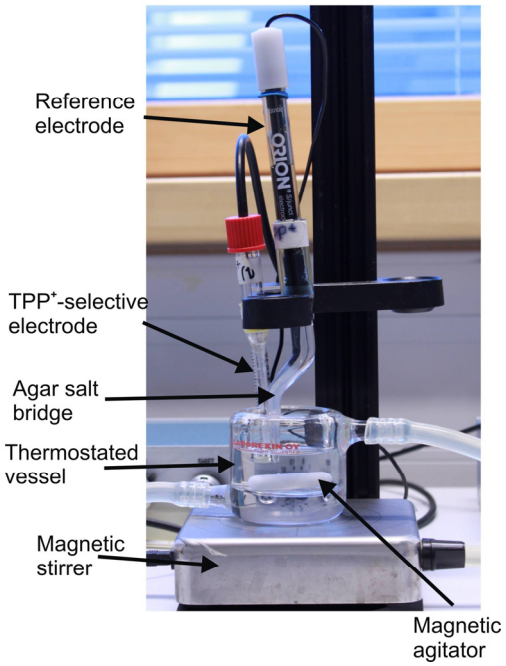

Fig. 2.1. TPP^+ measurement system. All parts are indicated in the figure. Reference and TPP^+-selective electrodes are connected to an amplifier which is further connected to a computer (described above).

The experiments were performed in 10 or 50 ml thermostated vessels at 37°C (if not indicated otherwise) with magnetic stirring (Fig. 2.1). The cell cultures were grown to an appropriate OD_{550} and transferred directly to the vessels or collected by centrifugation (15 min 5000 g rpm, 4ºC). Representative curves from 3 independent measurements are presented in figures.

2.9. Clustering of clinical isolates *P. aeruginosa*

For the evaluation of the initial accumulation of TPP^+ values of 1 to 3 were assigned. The value "1" was given in the case when cells showed no

initial accumulation. The values “2” and “3” were assigned to the cases when cells accumulated less than 1 μM or more than 1 μM of TPP+, respectively. For all other stages of analysis values of 1 to 5 were assigned. If cells responded in a reverse manner than expected (for example, EDTA caused additional leakage of TPP^+ to the medium, Fig. 2.12 I, B) then the value of 1 was assigned. In other cases the values of 3, 4 an 5 were assigned when TPP+ concentration in the medium changed <0.5 μM, 0.5-1 μM and >1 μM, respectively. The value of 2 has been assigned in the cases where no response to the additives was observed (for example see Fig. 2.12, III, GD effect). Such evaluation of bacterial physiological patterns resulted in 32 factors for each analyzed strain. The strain clustering was done with a program MVSP ver2.5 choosing the “Minimum variance” algorithm (Ward’s method). The maximal distance of the dendogram was chosen 30 % of its total distance as in the further distances isolates with significant differences are combined together.

2.10. rep-PCR

DNA was extracted from CLED plates using the UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Solona Beach, CA) and diluted to 35 ng/μl. The DNA was amplified using the DiversiLab *Pseudomonas* kit (Bacterial Barcodes, Inc. cat no PL-PA01, Athens, GA) for DNA fingerprinting following the manufacturer´s instructions. 2 μl of genomic DNA, 18 μl the rep-PCR master mix, 2 μl primer mix provided in the kit, 0.5 μl AmpliTaq polymerase and 2.5 μl 10 X PCR buffer (Applied Biosystems Roche, Branchburg, NJ) were added for a total of 25 μl per reaction. PCR was run on preheated thermal cycler (DNA Engine Tetrad 2, Peltier Thermal Cycler BioRad, Hercules, CA). The thermal cycling parameters were: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 70°C for 1.5 min, and final extension at 70°C for 3 min. The kit specific positive and negative controls were run with each reaction set for the validation of amplification. The

rep-PCR products were detected and the amplicons were separated using microfluidics lab-on-a-chip technology and analysed using the DiversiLab system (Bacterial Barcodes, Inc.).

2.11. Strain discrimination among clinical isolates of *P. aeruginosa*

Further rep-PCR analysis was performed with the web-based DiversiLab software (version 3.4) using the band-based modified Kullback-Leibler distance for the calculation of percent similarities. This calculation places more weight on band presence than intensity variations. The automatically generated dendogram and virtual gel image were used in interpretation. The manufacturer provided guidelines for strain-level discrimination were: similarity more than 97% is considered as indistinguishable (no differences in fingerprints), similarity more than 95% as similar (1-2 band difference in fingerprints) and similarity less than 95% as different.

3. RESULTS

3.1. Evaluation of tetracycline efflux pump TetA(B)

3.1.1. *E. coli* IQ86 and MC4100 viability under different tetracycline concentrations

E. coli isogenic strains were chosen to evaluate the activity of Tn*10* encoded TetA(B) efflux pump. Viability experiment was performed with different tetracycline concentrations. MC4100 capability to form colonies was ~5% compared to control cells when 200 μg/ml tetracycline concentration was used. IQ86 strain showed considerably higher viability, CFU had been detected even after the exposure of cells to 600 μg/ml of tetracycline (Fig).

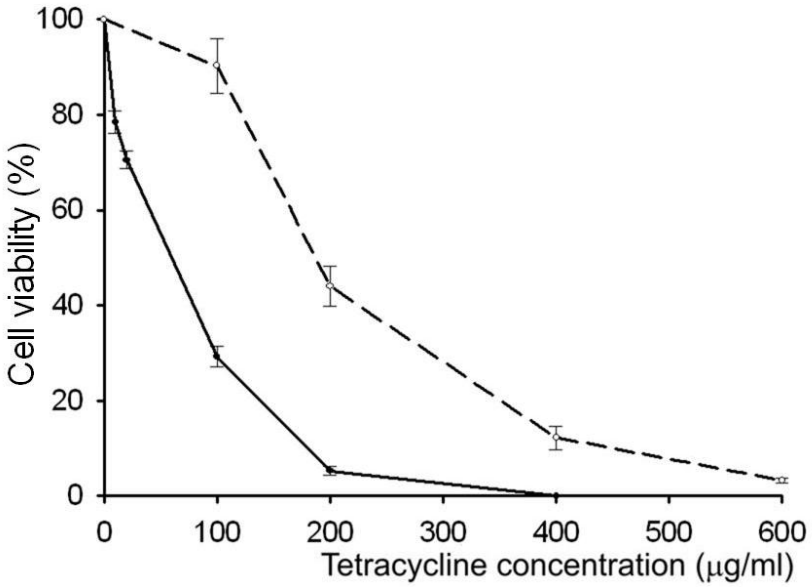

Fig. 2.2. *E. coli* IQ86 (hollow circles) and MC4100 (filled circles) viability under different tetracycline concentrations. Bars indicate standard error.

3.1.2. TetA(B) activity in tetracycline-untreated cells

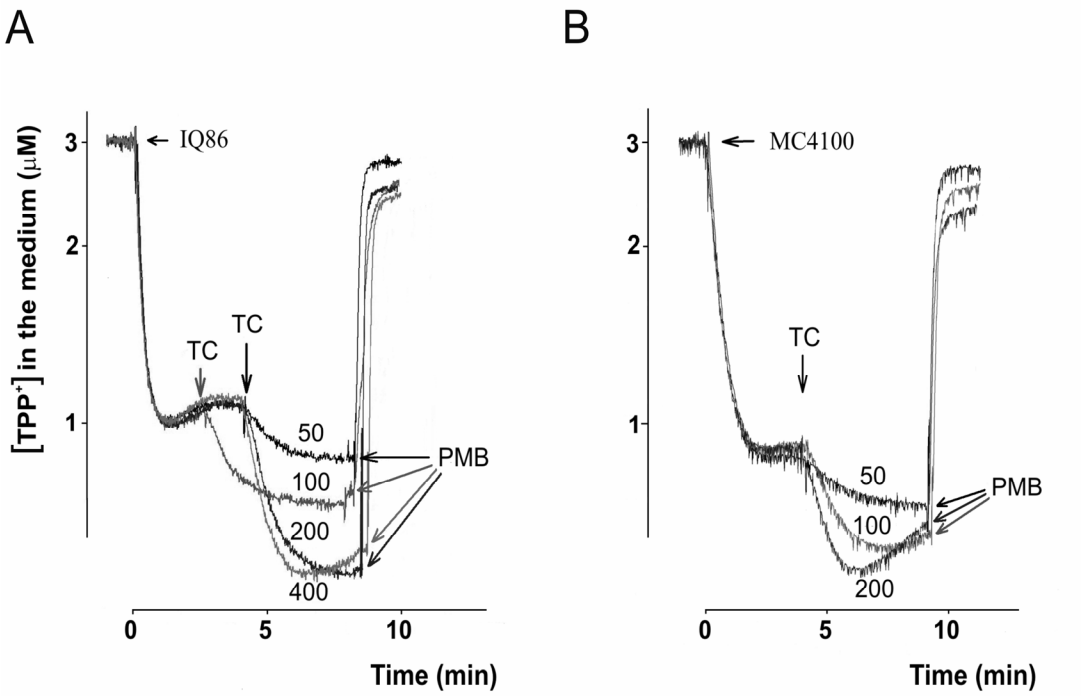

Fig. 2.3. Tetracycline effects on IQ86 (A) and MC4100 (B) cells. The experiments were performed in 100 mM Tris-HCl buffer, pH 8.0, at 37°C. Concentrated Tris/EDTA-treated cell suspension was added to the measurement buffer to obtain ~$8*10^8$ cells/ml; tetracycline (TC) was added to the final concentrations (μg/ml) indicated next to the curves, polymyxin B (PMB) was added to the final concentration of 100 μg/ml. Cells were not induced with tetracycline prior to the analyses .

The initial accumulation of TPP^+ in MC4100 cells was higher compared to IQ86 ones (Fig. 2.3). The final accumulated amount of TPP^+ was distinct between the analyzed strains when tetracycline was added to 50 and 100 μg/ml final concentrations. However, the difference diminished when tetracycline concentration of 200 μg/ml was used. Contrary to IQ86, the depolarization of MC4100 cells was observed when tetracycline was added to the concentration of 200 μg/ml. Minor depolarization of IQ86 cells was also observed only after addition of tetracycline to the concentration of 400 μg/ml.

3.1.3. TetA(B) activity in the tetracycline-treated cells

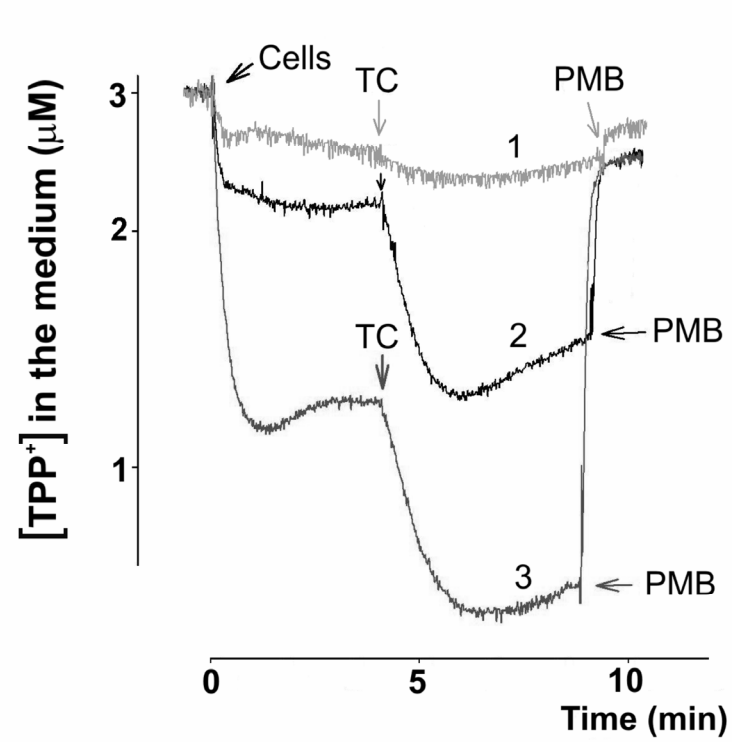

Fig. 2.4. Tetracycline effects on tetracycline-exposed *E. coli* MC4100 (curve 1) and IQ86 (curve 2) cells. The experiments were performed as described in Fig. 2.3. TC was added to the final concentration of 400 μg/ml in all the cases. Curve 3 is taken from Fig. 2.3, A .

Tetracycline-exposed MC4100 cells accumulated only minor amount of TPP^+ during the analysis. Further addition of tetracycline had no effect on accumulation of TPP^+. Tetracycline-exposed IQ86 (Fig. 2.4, curve 2) showed significant (~1.9 times lower) initial TPP^+ accumulation differences when compared to unexposed ones (compare curves 2 and 3 in Fig. 2.4,). The tetracycline addition caused considerable decrement of TPP^+ concentration in the medium. Importanly, tetracycline-exposed IQ86 cells accumulated ~1.7 times more TPP^+ when compared to unexposed ones. However, following effects of tetracycline-induced depolarization of the cells were observed in both tetracycline-exposed and unexposed IQ86 cells.

3.2. RND efflux pumps in *P. aeruginosa*

3.2.1. Contribution of MexAB-OprM efflux system to viability of *P. aeruginosa*

Table 3.1. MIC's of different drugs for *P. aeruginosa* strains with different expression levels of MexAB-OprM efflux system. The concentrations of antimicrobial compouns (AP – ampicillin, TC – tetracycline, CM – chloramphenicol, STR – streptomycin, TRI – triclosan, TPP – tetraphenylphosphonium chloride) are presented in μg/ml. Final concentration of PAβN was 60 μg/ml.

		Strains		
		PAO1	PT629	PAOdeltaBex
Antimicrobial compound	AP	1024	1024	1024
	TC	16	64	16
	CM	16	128	16
	STR	16	16	32
	TRI	>1024	>1024	>1024
	TPP	>8192	>8192	>8192
Antimicrobial compound + PAβN	AP	64	256	128
	TC	4	8	4
	CM	0.5	1	0.5
	STR	32	32	32
	TRI	64	128	64
	TPP	512	1024	1024

Three *P. aeruginosa* strains with different expression of MexAB-OprM were chosen to evaluate the contribution of MexAB-OprM efflux system on the MICs. There was no substantial difference in their resistance levels, except the cases of tetracycline and chloramphenicol, where the resistance of PT629 cells was substantially higher (table 3.1). Combination of antimicrobials with PAβN considerably decreased MICs for most of the tested compounds (except a representative of aminoglycosides streptomycin), indicating them as substrates of PAβN-sensitive RND pumps. The lowest concentration of TPP^+ affecting PAO1 viability was 512 μg/ml (1.36 mM) (table 3.1), while other strains showed higher resistance to this lipophilic compound. This data indicates that TPP^+ concentrations used in electrochemical measurements

(maxium concentration of 3 μM) had no effects on the viability of *P. aeruginosa* cells.

3.2.2. Tris/EDTA treatment effect on *P. aeruginosa*

Possible reasons for the limited TPP^+ accumulation by gram-negative bacteria are: i) low $\Delta\psi$ ii) low permeability of the OM; iii) high activity of MDR pumps. As both, the OM and the pumps prevent TPP^+ accumulation, it is crucial to eliminate or evaluate the impact of the OM barrier on distribution of TPP^+ between the cells and the medium. In other words, it is necessary to establish conditions warranting high and stable permeability of the OM to TPP^+ without affecting the activity of MDR pumps. Tris/EDTA treatment (Daugelavicius *et al.*, 1997) or addition of EDTA to Tris-buffered cell suspension (Daugelavicius *et al.*, 2000) are used to increase the OM permeability to lipophilic compounds and to rapidly equilibrate TPP^+ across the cell envelope of enterobacteria. Tris/EDTA treatment of enterobacterial cells leads to a massive release of LPS into the medium (Alakomi *et al.*, 2003; Nikaido, 2003). It is believed that glycerophospholipids are compensating the reduced amount of LPS in the outer leaflet and creating highly permeable patches in the OM (Vaara, 1992).

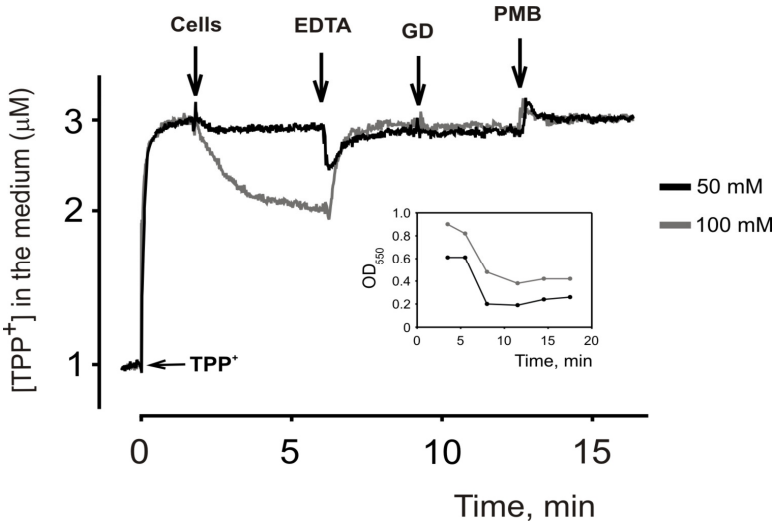

Fig. 2.5. Tris/EDTA effects on *P. aeruginosa* cells. The experiments were performed at 37°C in 5 ml of Tris/HCl, pH 8.0. A concentrated PAO1 cell suspension was added

to the vessels to obtain ~8*10^8 cells/ml. EDTA was added to the final concentration of 0.4 mM, GD and PMB were added 4 μg/ml and 60 μg/ml, respectively. 70 μl of samples were taken from the vessels throughout the experiments (insert) for turbidity measurements.

The OM permeability of *P. aeruginosa* cells was addressed. Addition of 0.4 mM EDTA to PAO1 suspension in 100 mM Tris buffer induced complete release of the cell accumulated TPP^+ (Fig. 2.5, grey curve). EDTA induced some accumulation of TPP^+ in 50 mM Tris buffer, but it was transient and followed by rapid leakage of TPP^+ to the medium (Fig. 2.5, black curve). Leakage of the accumulated TPP^+ to the medium and drop of the suspension turbidity (Fig. 2.5, insert) indicated that the addition of EDTA lysed PAO1 cells.

3.2.3. Phosphate buffer concentration selection

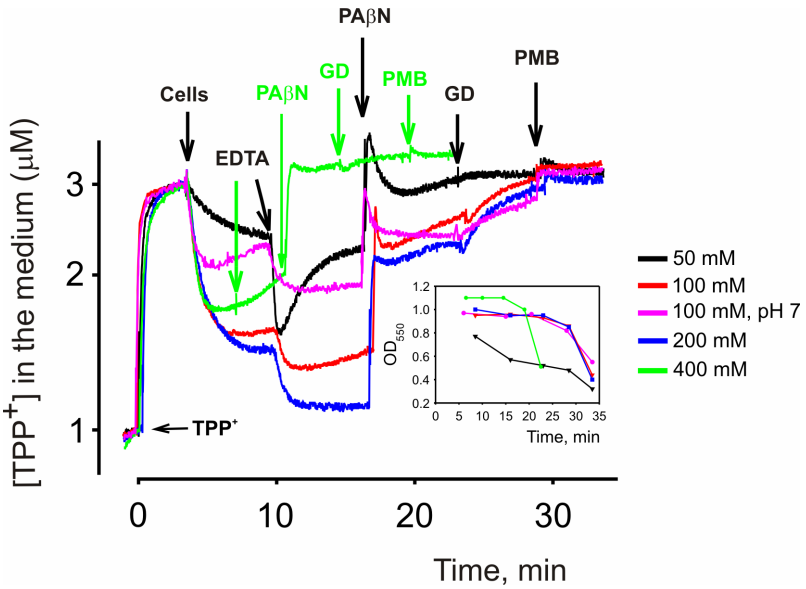

Fig. 2.6. Influence of different phosphate concentrations on efflux pump activity. The experiments were performed at 37°C in 5 ml of sodium phosphate buffer, pH 8.0, if not indicated otherwise. Buffer concentrations are indicated by different colors. All experimental steps were performed as described in (Fig. 2.5). EDTA, PAβN, GD and PMB were added to the concentrations of 0.4 mM, 60 μg/ml, 4μg/ml and 60 μg/ml, respectively.

In contrast to enterobacteria, EDTA permeabilizes the OM of *Pseudomonas syringae* also in phosphate buffer (Daugelavicius *et al.*, 2005).

Consequently as the Tris/EDTA treatment can not be applied to *P. aeruginosa* phosphate-EDTA buffer was used to permeabilize the OM. As the ionic strength of the medium may affect the OM permeability, the $\Delta\psi$, and the activity of MDR pumps, TPP^+ accumulation by PAO1 cells was followed in different phosphate buffer concentrations (Fig. 2.6). At all concentrations studied, except 400 mM, EDTA induced an additional accumulation of TPP^+, whereas PAβN induced a strong release of the accumulated TPP^+ (Fig. 2.6). Parallel measurements indicated, that EDTA induced moderate decrease of the cell suspension OD_{550} only when 50 mM phosphate was used. The addition of PAβN had no effect on the culture turbidity (see Fig. 2.6, insert). Channel-forming antibiotic Gramicidin D (GD) induced an additional leakage of TPP^+, but suspension OD_{550} considerably decreased only after the addition of the polycationic antibiotic polymyxin B (PMB) (Fig. 2.6, insert). Overall, this data indicates that depolarization of the PM is the main reason for the PAβN-induced release of TPP^+. Noteworthy, these results are valid only when the experiments are carried out in phosphate buffer without nutrients.

3.2.4. The effects of nutrient on the activity of MDR efflux pumps

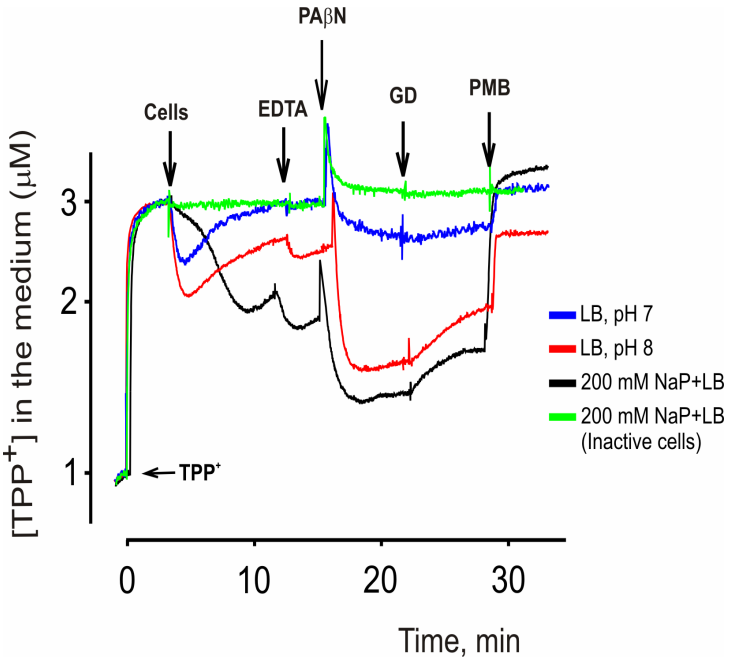

Fig. 2.7. Influence of nutrients on the activity of MDR efflux pumps. Experiments were performed in LB or LB 1:1 diluted with 400 mM sodium phosphate, pH 8. All experimental steps were performed as described in (Fig. 2.6). Green curve represents TPP^+-selective electrode responses to additives in inactivated cell suspension (inactivation was done by incubating the concentrated cell suspension at 70°C for 15 minutes).

If TPP^+ is a substrate and PAβN is an inhibitor of RND pumps, blockage of the pump activity should increase accumulation of TPP^+. Bacterial MDR pumps are usually activated by nutrients, such as glucose (Lomovskaya *et al.*, 2001; Martins *et al.*, 2009; Pannek *et al.*, 2006). However, TPP^+ accumulation in *P. aeruginosa* was not sensitive to glucose (not shown). PAO1 cells in LB did not accumulate significant amounts of TPP^+. However, when PAβN was added, a strong accumulation of TPP^+ occurred indicating the suppression of the pump activities. GD had a weak effect on the distribution of TPP^+ between the cells and the LB medium, but subsequent additions of PMB released most of the cell taken lipophilic cations, indicating that accumulation of TPP^+ was the $\Delta\psi$-dependent process.

The difference in the initial accumulation of TPP^+ by PAO1 cells in phosphate buffer and LB medium suggests that LB components have a considerable effect on the efficiency of MDR pumps and/or on the OM permeability to TPP^+. The accumulation of TPP^+ was also pH dependent (compare Fig. 2.6, pink and red curves; Fig. 2.7, blue and red curves). To increase the bufferic capacity, LB was diluted with 400 mM sodium phosphate (1:1). Initial accumulation of TPP^+ in the buffered LB (Fig. 2.7, black curve) proceeded considerably slower compared to the measurements in 200 mM phosphate buffer (Fig. 2.6, blue curve) or in unbuffered LB (Fig. 2.7, red curve). Low amplitude of EDTA-induced accumulation of TPP^+ was a common for all cases where fresh LB-containing media were used (see below). Although the addition of EDTA was rather inefficient, PAβN induced a considerable accumulation of TPP^+ by the cells. The following addition of GD had a weak effect, but the subsequent additions of PMB caused an effective release of accumulated TPP^+. Experiments with heat-inactivated cells (Fig. 2.7, green curve) indicated that the initial effect of PAβN on the potential of TPP^+-selective electrode is not connected to the activity of MDR pumps. Consequently, ratio of the cell-accumulated amounts of TPP^+ before and after addition of this inhibitor can be considered as a measure of the total activity of PAβN-sensitive MDR pumps.

3.2.5. PAβN effects on *P. aeruginosa* PAO1

To evaluate PAβN action to the capacity of the cells to accumulate TPP^+, gradually increasing concentrations of the inhibitor were used (Fig.12).

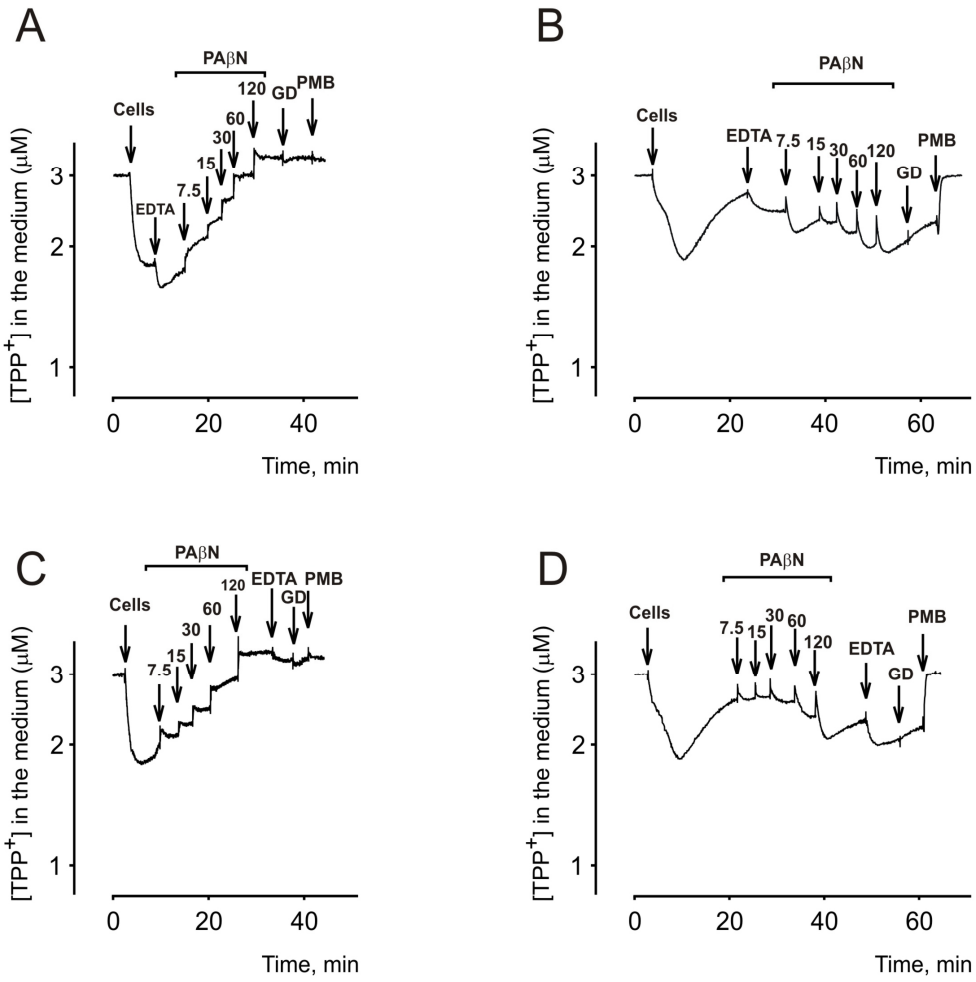

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Fig. 2.8. Effects of PAβN on *P. aeruginosa* PAO1. Measurements were performed in 200 mM sodium phosphate buffer, pH 8.0 or LB medium 1:1 diluted with 400mM pH 8.0 sodium phosphate buffer. Concentrations (μg/ml) of PAβN are indicated in the figure. A concentrated cell suspension was added to obtain OD_{550} 1 in the measurement vessels. EDTA, GD and PMB were added to the final concentrations of 0.4 mM, 4 μg/ml and 60 μg/ml respectively.

The lowest PAβN concentrations tested induced only release of cell-accumulated TPP^+ in phosphate buffer (Fig. 2.8, A and C). PAβN was ineffective up to concentration of 30 μg/ml when cells were not permeablized

with EDTA (Fig. 2.8, D). Further concentrations of the inhibitor induced additional accumulation of TPP^+. However, EDTA addition induced additional accumulation of TPP^+ into the cells indicating that PAβN is not effective permeabilizer of OM. When EDTA was added in the beginning of experiment (Fig. 2.8, B) low (≥7.5 μg/ml) concentrations of PAβN induced accumulation of TPP^+. The second phase of the inhibitor-induced TPP^+ accumulation was observed at 30 μg/ml and higher concentrations of PAβN. In both cases, very high concentrations of PAβN (≥120 μg/ml) induced TPP^+ release from the cells.

3.2.6. Accumulation of TPP^+ by *P. aeruginosa* at different growth phases

Experiments utilizing the concentrated cell suspensions are useful for analysis of factors affecting the MDR pump activity as it is possible to perform several series of measurements on the same cell suspension kept on ice. However, the energy state of the cells might be affected during procedures such as pelleting of bacteria, resuspending to high densities and keeping the cells on ice. To avoid such procedures 2.5 ml samples of growing indicator strain cultures were transferred to small measuring vessels containing equal volumes of 400 mM sodium phosphate.

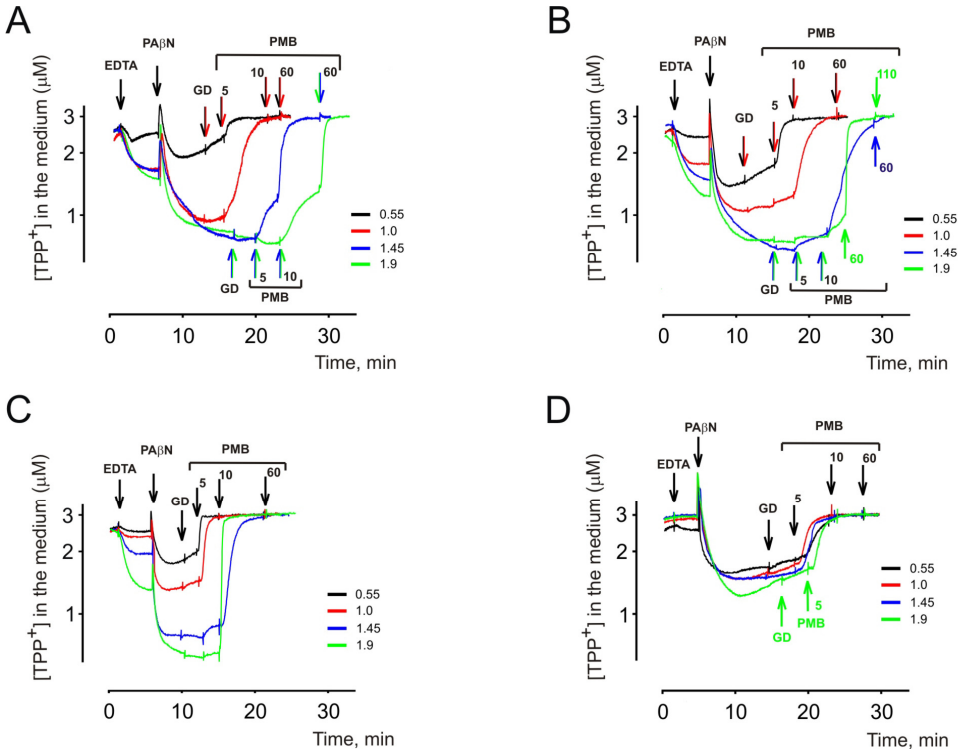


Fig. 2.9. Interaction of TPP^+ with cell cultures of different growth phases. PAO1 (A), PAOdeltaBex (B) and PT629 (C) cells were grown in LB medium at 37°C with aeration to densities indicated in the figure and 1:1 diluted with 400 mM *(continued)*sodium phosphate, pH 8.0. In (D) PAO1 cell cultures from different growth phases were diluted with fresh LB medium, pH 8.0, to obtain OD_{550} 0.45. EDTA, PAβN and GD were added to the cell suspensions to the final concentration of 0.4 mM, 60 μg/ml and 4 μg/ml, respectively. PMB was added to the final concentrations indicated in the figure (μg/ml).

It has been shown (Evans & Poole, 1999) that expression of the MexAB-OprM components increases when the culture density increases. In our experiments EDTA added to phosphate buffer-diluted PAO1 suspensions of different culture densities had weak effect on TPP^+ accumulation by the cells at the lowest culture density studied (0.55), but induced accumulation of this indicator cation at OD_{550} 1 or higher (Fig. 2.9, A). In all cases addition of PAβN caused a strong additional accumulation of TPP^+. At all densities tested PAO1 cultures had low sensitivity to GD, but at lower culture densities (OD_{550} 0.55 - 1) the cells were more sensitive to PMB than the ones grown further.

PM protein MexB, substrate-binding and energy-employing component of the MexAB-OprM pump (Guan & Nakae, 2001; Middlemiss & Poole, 2004;

Sennhauser *et al.*, 2009), transfers its substrates using the proton electrochemical gradient and can extrude a very broad range of compounds such as antibiotics, biocides, dyes, organic solvents, and detergents (Li *et al.*, 1995; Li *et al.*, 2003a; Tikhonova *et al.*, 2002). We compared the accumulation of TPP^+ by *wt* PAO1 and MexAB mutant cells - PAOdeltaBex (Fig. 2.9, B) and PT629 (Fig. 2.9, C). Addition of EDTA to early exponential cell cultures (OD_{550} 0.55) did not change the concentration of TPP^+ in the medium, but the cells accumulated this indicator cation after addition of PAβN. The accumulated amount was not stable and a slow increase of TPP^+ concentration in the medium was observed. After addition of EDTA to cultures of OD_{550} 1.0 - 1.45, MexAB overproducing PT629 cells accumulated considerably lower amount of TPP^+ compared to PAOdeltaBex or *wt* strains. However, this difference became negligible when the cultures were grown to OD_{550} 1.9. It is worth noticing that at all growth stages studied the TPP^+ equilibration was faster with PT629 cells compared to other two analyzed strains.

Different growth phases or different cell concentration may affect the level of TPP^+ accumulation and the cell sensitivity to PMB. This was addressed by growing PAO1 cells to variable densities followed by dilution to LB to obtain OD_{550} 0.5. In such cases, the accumulated amounts of TPP^+ as well as the sensitivity of the cells to PMB were alike (Fig. 2.9, D). This implies that functions of the pumps are considerably depended on the cell incubation conditions and indicates an important role of the changes in medium composition in relationship between the pump activity and the culture growth phase.

3.2.7. Effect of temperature on the activity of MDR pumps

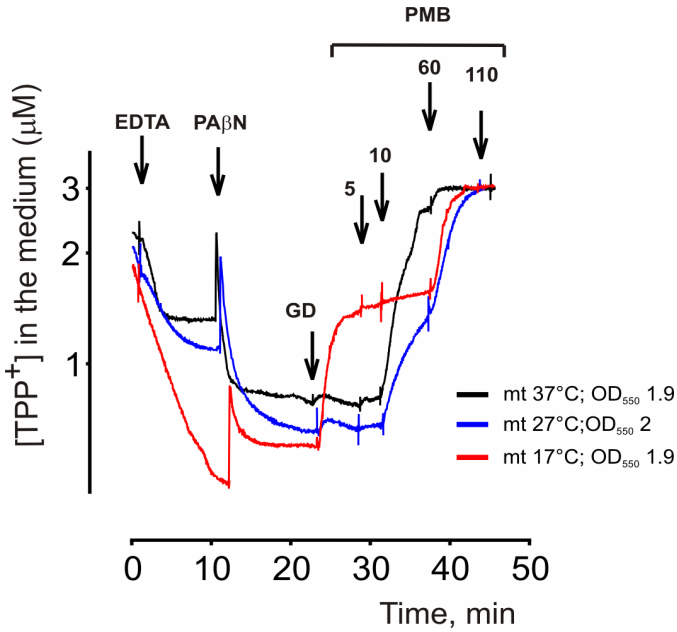


Fig. 2.10. Temperature influence on efflux pumps of *P. aeruginosa* PAO1. Different measurements temperatures are indicated in the figure. EDTA, PAβN and GD were added to the final concentration of 0.4 mM, 60 μg/ml and 4 μg/ml respectively. PMB final concentrations (μg/ml) are indicated in the figure.

MDR pump activities are usually measured at room temperature (RT) or at 37°C. Accumulation of TPP^+ by PAO1 cells grown overnight at 37°C were assayed at 37°C, 27°C and 17°C (Fig. 2.10). Usually the permeability of biological membranes to lipophilic compounds increases with the increase of temperature (Bhatti *et al.*, 1999; Rahmati-Bahram *et al.*, 1995). Surprisingly, PAO1 cells very efficiently accumulated TPP^+ at 17°C and the addition of EDTA had no effect on the rate of uptake (Fig. 2.10, red curve). In these conditions the effect of PAβN on the accumulation of TPP^+ was weak, but the depolarizing efficiency of GD was high. There were no considerable differences in the accumulated amounts of TPP^+ and the sensitivity to GD when the measurement temperatures were 27°C and 37°C (compare blue and black curves in Fig. 2.10). These results indicate that the low depolarizing

activity of GD at 27 and 37°C may be the result of MDR pump(s) excluding it from effecting the PM.

3.2.8. Aeration influence on the activity of efflux pumps of *P. aeruginosa*

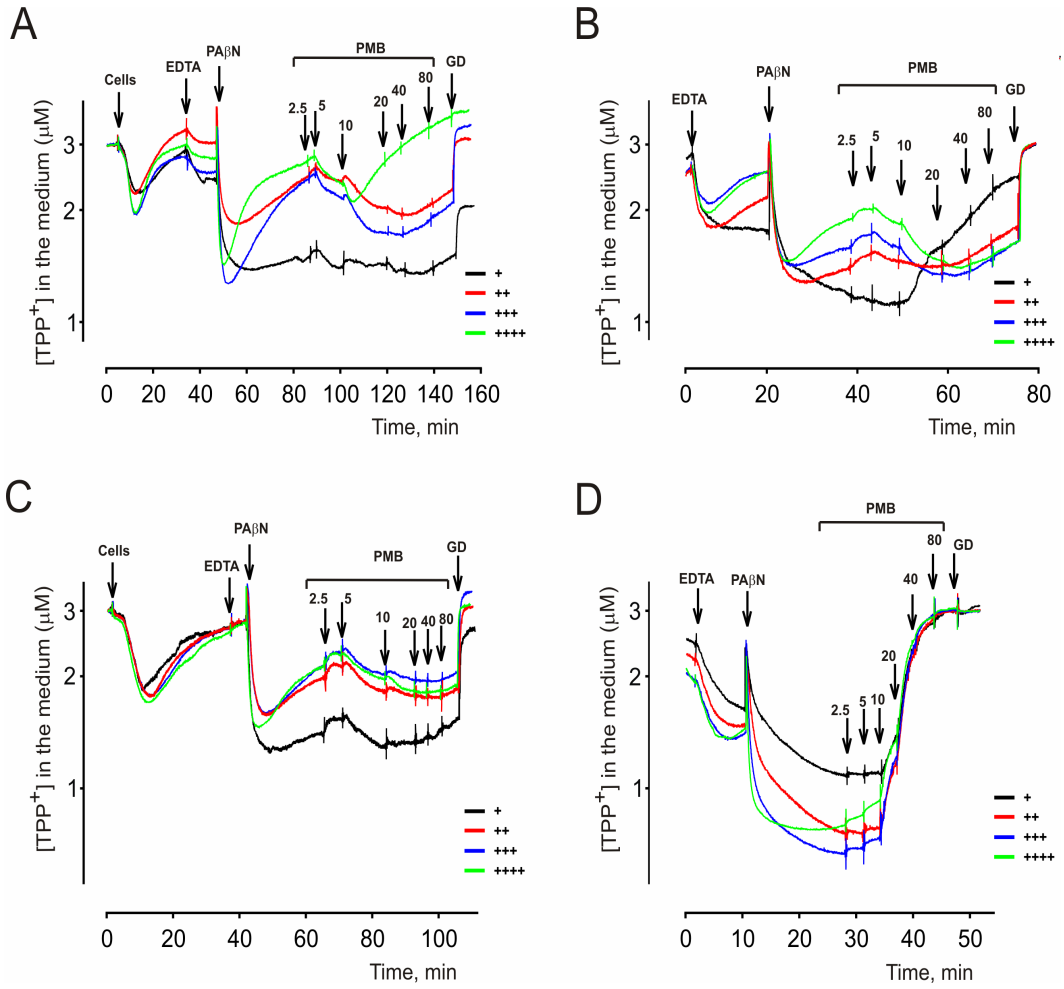


Fig. 2.11. Aeration impact on the interaction of TPP^+ with PAO1 cells. The experiments were performed at 37°C. The cells were grown to OD_{550} 1 (A, B) or overnight (C, D). In A and C the measurements were performed by adding concentrated cell suspension to buffered LB medium, in B and D – direct growing cultures were diluted 1:1 with 400 mM sodium phosphate, pH 8.0. The intensity of aeration was controlled by magnetic stirring using four levels: "high" (green curves), "intermediate" (blue), "low" (red), and "very low" (black). EDTA, PAβN and GD were added to the cell suspension to the final concentration of 0.4 mM, 60 μg/ml and 4 μg/ml, respectively. PMB was added to the final concentrations indicated in the figure (μg/ml).

After a short initial accumulation of TPP^+, the cells extruded most of the accumulated indicator. The consecutive addition of EDTA only slightly increased the accumulation. The following addition of PAβN induced a strong but reversible accumulation of TPP^+. The extent of reversibility increased with the increase of the intensity of aeration. At low concentrations PMB induced an additional accumulation of TPP^+ and only at high concentration it depolarized the PM. The depolarizing activity of PMB was also related to the intensity of aeration. At the highest level of aeration 10 μg/ml was enough to induce the depolarization, but ~80 μg/ml was needed when using the lowest aeration level. It should be mentioned that the consecutive increase of PMB concentration did not lead to faster depolarization of the PM. The subsequent addition of GD depolarized the PM in all aeration levels studied (Fig. 2.11, A). In fresh buffered LB the TPP^+ accumulation increased with increasing PMB concentration indicating that the OM permeability to TPP^+ was low even in the presence of 2 mM EDTA (not shown).

When the PMB effect was studied, PAO1 cells (exponentional growth phase) from the culture acted differently compared to those pelleted and resuspended (compare Fig. 2.11, A to Fig. 2.11, B). When pelleted and resuspended cells were measured at the highest aeration conditions 10 μg/ml PMB caused depolarization but no such effect was observed in the lowest aeration conditions (Fig. 2.11, A). On the contrary, when cell culture samples were used the outcome was the opposite (Fig. 2.11, B). When the depolarizing concentration was reached, no further PMB concentration-dependent leakage of TPP^+ accured. This data indicates that procedures such as pelleting and resuspension have a considerable effect on the physiological state of the cells.

When the same experiments were carried out using overnight pelleted cultures the only differences observed were: decreased dependence on aeration and the lack of EDTA-caused permeabilization (compare Fig. 2.11, A and Fig. 2.11, C). However, when direct culture samples were utilized the system was less sensitive to differences in the aeration levels. When the lowest aeration

was used, PAβN addition caused reduced TPP^+ accumulation when compared to other aeration levels (Fig. 2.11, D).

Based on all these results it appears that the aeration level as well as the sampling method are very important in respect to the efflux pumps activity measurements. Our results also indicate that the change of media composition can be the main reason of the culture growth phases-related changes of the pump activities.

3. 3. Analysis of clinical isolates of *P. aeruginosa*

3.3.1. Similarities in total MDR efflux pump activity in *P. aeruginosa* clinical isolates

All the *P. aeruginosa* clinical isolates and control strains were analyzed for their interactions with TPP^+ during different growth phases as described in 3.2.6. Each isolate displayed a specific physiological pattern as judged from the interactions with TPP^+ and indicator compounds. All the stages (initial accumulation of TPP^+, EDTA, PAβN, GD and PMB effects) of each growth phase were evaluated according to the changes in TPP^+ concentration in the measurement medium. The numeric values for each of evaluation points of evaluation were assigned as described in 2.7. The obtained grouping of the isolates corresponded to their physiological properties based on the interaction with TPP^+. The physiological patterns for representatives from each group are shown in Fig. 2.12.

There are four major groups in the acquired clustering named I, II, III and IV (Fig. 2.13). Resistance to antibiotics is presented next to the name of a particular strain. Numbers presented with the names of the strains (e.g. "ET0309_cipR_1") represent the patient who was sampled several times for *P. aeruginosa*. Clinical isolates from the same patients distributed within the same groups, except isolates MX2069 and MX1987 that showed different efflux pump activities and were assigned to the groups II and IV respectively.

The number of isolates was different within each group (Fig. 2.13): I – 8; II – 18; III – 14; IV – 28. In the set of isolated *P. aeruginosa* strains ,17 of

them had resistance to one or more antibiotics tested (piperacillin, ceftazidime, meropenem, ciprofloxacin and tobramycin). Six ciprofloxacin resistant isolates fall into the group I. Group II contains five ciprofloxacin resistant isolates, where ME1343 and KK2373 are additionally resistant to tobramycin and TK0352 has additional resistance to meropenem. Four antibiotic resistant isolates are assigned to the group III. Isolate K3221 harbors resistance to meropenem, isolates KK0680 and ME2726 have resistance to both ciprofloxacin and tobramycin, and isolate TK0469 has resistance to ciprofloxacin and meropenem. Group IV contains the least resistant isolates. There are only two ciprofloxacin resistant isolates (TK0432 and KK1981) in this group. However, the last group is the largest compared to other three. In addition, groups I and IV does not contain any control strains.

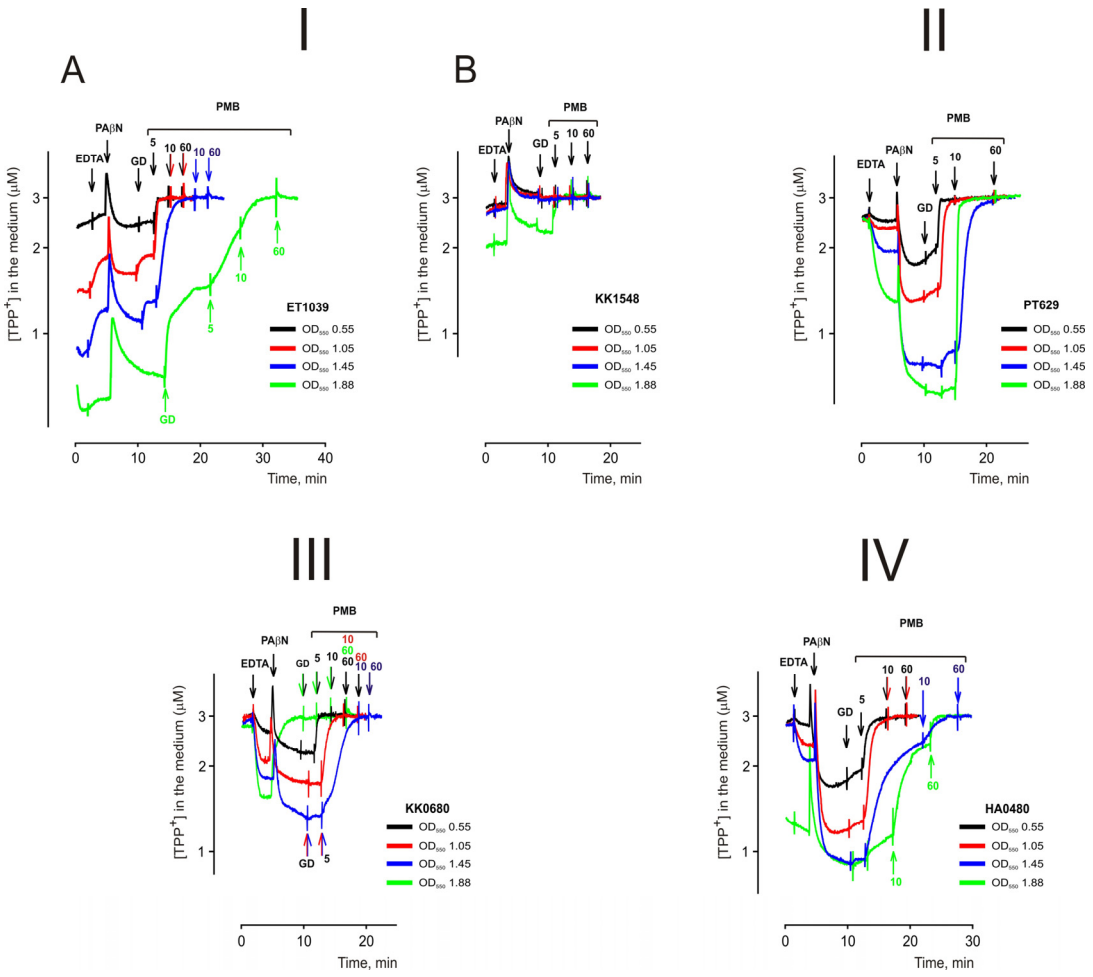


Fig. 2.12. Representative isolates for each clustering group. A and B represents examples from both sub-groups of group I.

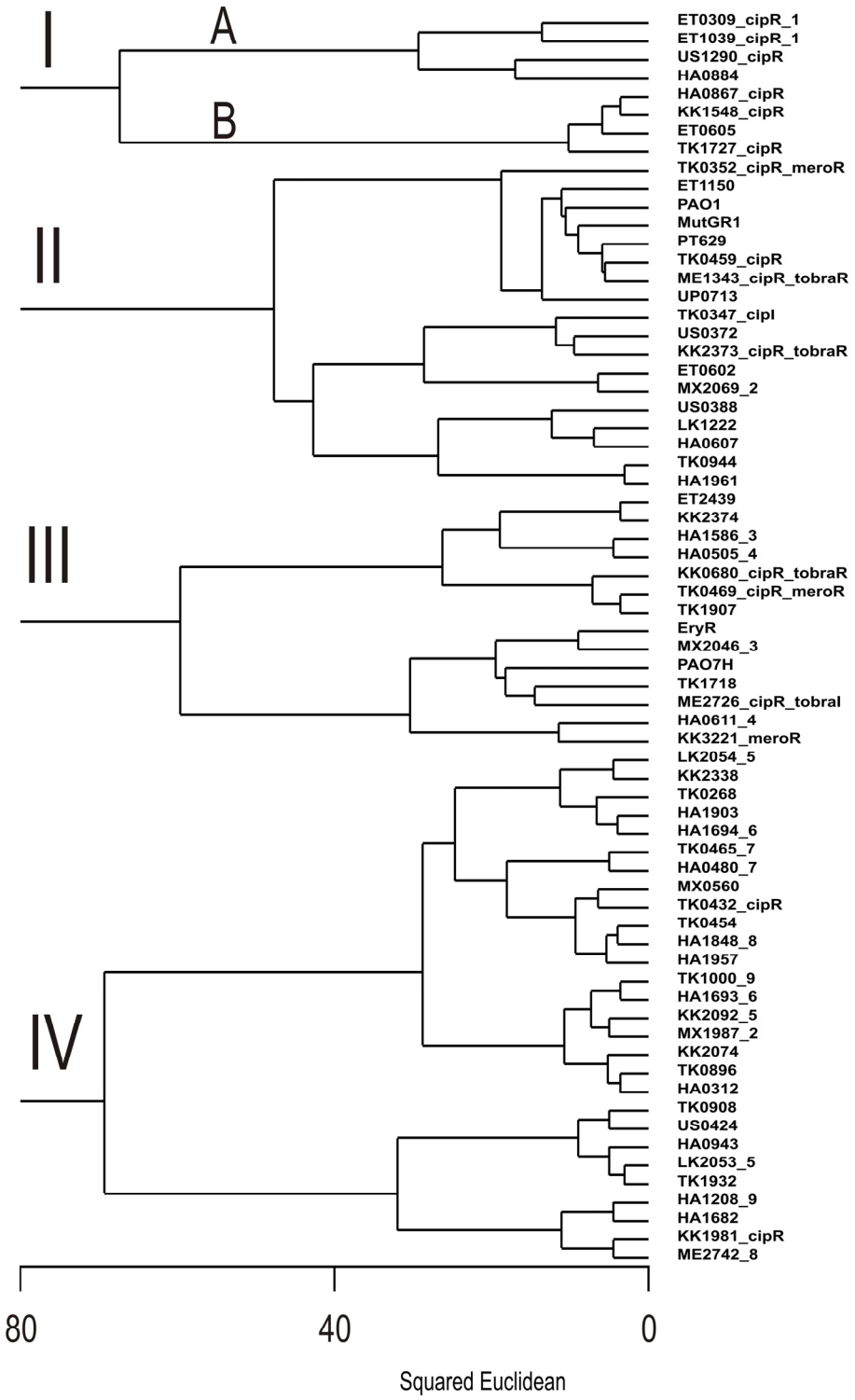


Fig. 2.13. A dendogram of clinical isolates of *P. aeruginosa* (clustered using the "Minimum variance" algorithm).

Group I is the most diverse group. Both sub-groups within group I (A and B) have major difference in TPP^{+} accumulation throughout the assays (Fig.

2.12 A, B). Isolates from the "A" subgroup have high initial accumulation of TPP^+ and respond to the addition of indicator compounds during the analysis. The unique property is the negative response to addition of EDTA (contrary to induced accumulation, the leakage of TPP^+ to the medium is observed). In contrast to A, B sub-group isolates showed no considerable initial accumulation of TPP^+ and no responses to indicator compounds in all growth phases and analysis steps (Fig. 2.12, I B).

Standard strains of *P. aeruginosa* were distributed between the two groups: MutGR1 (MexXY-OprM), PAO1 (*wt*) and PT629 (MexAB-OprM) were assigned to group II; EryR (MexCD-OprJ) and PAO7H (MexEF-OprN) were assigned to group III. In spite of other factors, the main differences between these groups were observed in the initial steps of analyses. Contrary to group II, the typical properties of group III were the significantly higher initial accumulation of TPP^+ and sensitivity to EDTA in all growth stages of the cells. Moreover, PAβN had either no or minor effect on the cells of this group or it was negligible (Fig. 2.12, III).

Table 3.2. Features of analyzed isolates and standard strains of *P. aeruginosa*. Pump activity group is from Fig. 2.13, genotyping key is from Fig. 2.14.

Sample ID/strain	Resistance to antibiotics	Pump activity group	Genotyping key number	Patient number*	Isolation date
ET0309	cipR	IA	21	1	31 01 2007
US0372	S	II	35	-	31 01 2007
US0388	S	II	34	-	31 01 2007
TK0268	S	IV	38	-	31 01 2007
US0424	S	IV	28	-	31 01 2007
HA0312	S	IV	42	-	06 02 2007
TK0347	cipI	II	44	-	06 02 2007
TK0352	cipR, meroR	II	13	-	06 02 2007
KK0680	cipR, tobraR	III	10	-	06 02 2007
TK 0432	cipR	IV	24	-	12 02 2007
TK 0454	S	IV	57	-	12 02 2007
TK 0459	cipR	II	29	-	12 02 2007
TK 0465	S	IV	61	7	12 02 2007
TK 0469	cipR, meroR	III	14	-	12 02 2007
(continued in page 63)					

(continued from page 62)					
HA 0480	S	IV	62	7	20 02 2007
HA 0505	S	III	59	4	20 02 2007
MX 0560	S	IV	53	-	20 02 2007
ET 0602	S	II	26	-	20 02 2007
ET 0605	S	IB	30	-	20 02 2007
UP 0713	S	II	9	-	20 02 2007
HA 0607	S	II	68	-	27 02 2007
HA 0611	S	III	60	4	27 02 2007
LK 1222	S	II	3	-	13 03 2007
HA 0867	cipR	IB	63	-	20 03 2007
HA 0884	S	IA	25		20 03 2007
TK 0896	S	IV	4	-	20 03 2007
ET 1039	cipR	IA	22	1	20 03 2007
US 1290	cipR	IA	27	-	20 03 2007
ME 1343	cipR, tobraR	II	11	-	20 03 2007
KK 1548	cipR	IB	6	-	20 03 2007
TK 0908	S	IV	19	-	27 03 2007
HA 0943	S	IV	65	-	27 03 2007
TK 0944	S	II	46	-	27 03 2007
TK 1000	S	IV	32	9	27 03 2007
ET 1150	S	II	12	-	27 03 2007
HA 1208	S	IV	33	9	25 04 2007
KK 1981	cipR	IV	31	-	25 04 2007
LK 2053	S	IV	39	5	25 04 2007
LK 2054	S	IV	40	5	25 04 2007
KK 2074	S	IV	18	-	25 04 2007
KK 2092	S	IV	41	5	25 04 2007
KK 2338	S	IV	43	-	08 05 2007
KK 2373	cipR, tobraR	II	16	-	08 05 2007
KK 2374	S	III	5	-	08 05 2007
HA 1586	S	III	67	3	22 05 2007
HA 1682	S	IV	64	-	22 05 2007
HA 1693	S	IV	7	6	22 05 2007
HA 1694	S	IV	8	6	22 05 2007
TK 1718	S	III	37	-	22 05 2007
TK 1727	cipR	IB	36	-	22 05 2007
MX 1987	S	IV	56	2	22 05 2007
MX 2046	S	III	66	3	22 05 2007
MX 2069	S	II	55	2	22 05 2007
ME 2726	cipR, tobraI	III	17	-	22 05 2007
ME 2742	S	IV	1	8	22 05 2007
HA 1848	S	IV	2	8	13 06 2007
KK 3221	meroR	III	47	-	13 06 2007

(continued in page 64)					
(continued from page 63)					
HA 1903	S	IV	45	-	19 06 2007
TK 1907	S	III	23	-	19 06 2007
TK 1932	S	IV	54	-	19 06 2007
HA 1957	S	IV	15	-	19 06 2007
HA 1961	S	II	58	-	19 06 2007
ET 2439	S	III	20	-	19 06 2007
EryR	S	III	48	-	
PAO7H	S	III	49	-	
PT629	S	II	50	-	
MutGR1	S	II	51	-	
PAO1	S	II	52	-	

***** – "-" means isolates from different patients

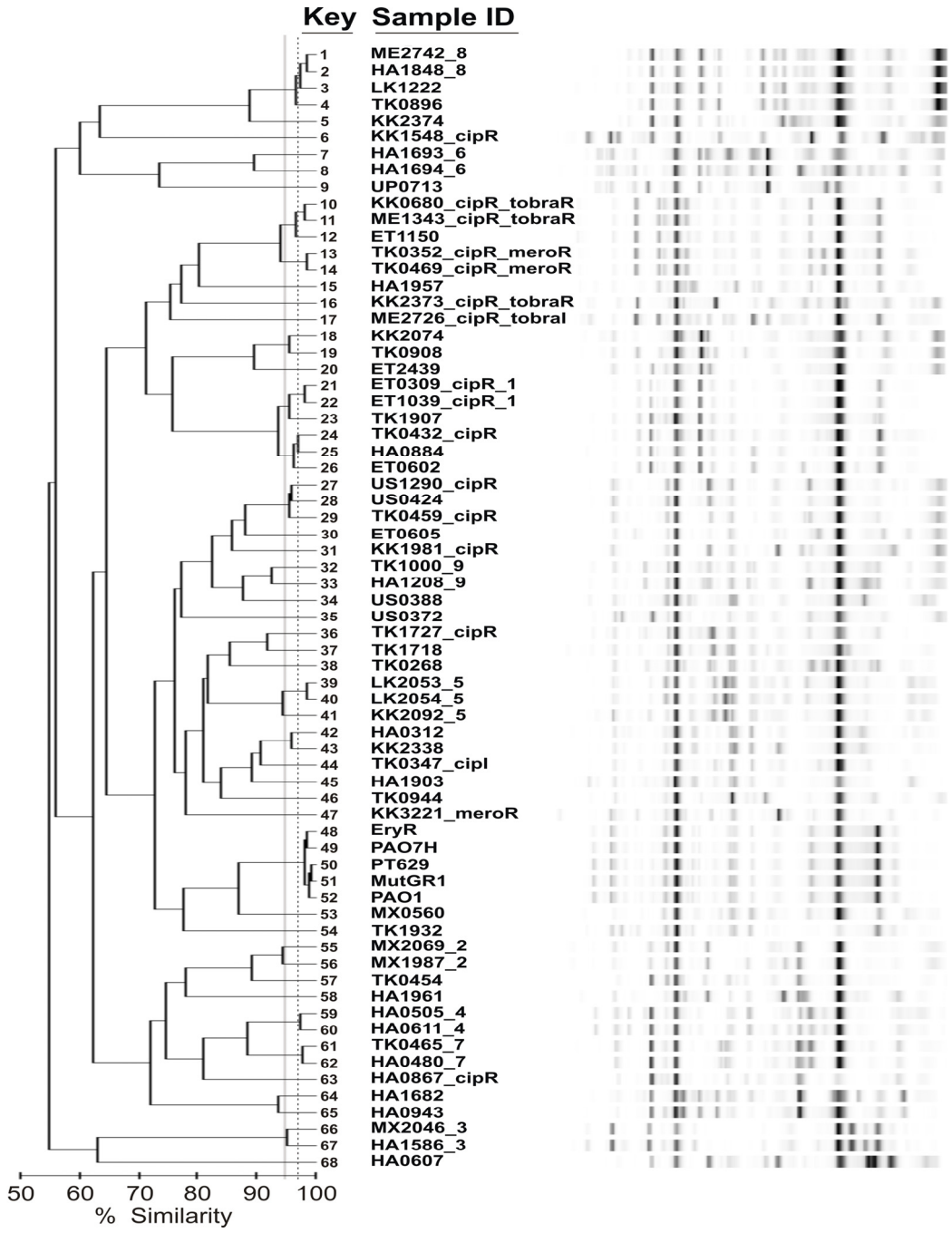
3.3.2. Genotypic similarities of clinical *P. aeruginosa* isolates

To evaluate the genotypic similarities/differences among clinical *P. aeruginosa* isolates the rep-PCR has been performed.

Genotyping results indicate that the collection of clinical *P. aeruginosa* isoltes is very heterogeneous. Thirty strains displayed little similarity to other strains in the collection (e.g. Fig. 2.14, keys 16 and 17), while other strains were more similar to each other, forming tight clusters (Fig. 2.14, key numbers): 1, 2 and 3; 10 and 11; 13 and 14; 21 and 22; 24 and 25; 39 and 40; 48-52; 59 and 60; 61 and 62. As expected, all indicator strains accumulated into a single cluster (Fig. 2.14, key nr. 48-52). Isolates collected from the same patient were considered as similar or indistinguishable, except isolates from patients 2, 6, 5 (strain KK2092) and 9. The genetic similarities/differences showed no connection with isolation periods (Table 3.2).

DiversiLab genotyping is used to evaluate differences among infection outbreak-causing pathogens (Ratkai *et al.*, 2010). Isolates (Fig. 2.14, key numbers 10 and 11, 13 and 14) resistant to more than one antibiotic tested were identified as indistinguishable. However, less than 95% similarity was observed between both pairs indicating their different origin.

The relationship between the genotypic and phenotypic (evaluation of efflux pumps activities) properties of *P. aeruginosa* isolates were explored. Significant correlation between the results of the two analyses was observed for the isolates originating from the same patient. Isolated strains MX1987 and MX2069 (patient nr. 2) showed remarkable differences in efflux pump



activities and were clustered into different clusters (Fig. 2.13; table 3.2). Accordingly, genotypic patterns of those isolates were also recognized as different (Fig. 2.14).

Fig. 2.14. Genotypic dendogram and virtual gel of clinical *P. aeruginosa* isolates. Dotted grey line – 97% similarity, straight grey line – 95% similarity. rep-PCR and genotypic dendogram were obtained in collaboration with Laboratory of Helsinki University Central Hospital.

4. DISCUSSION

We present here the results on the activity of the tetracycline-specific efflux pump TetA(B) and the RND-type MDR pumps of *E. coli* and *P. aeruginosa*, respectively. The total efflux pump activity was assayed and monitored in real-time using the nonradiolabeled TPP^+ – a popular $\Delta\psi$ indicator and a universal substrate of MDR pumps – is used for electrochemical real-time monitoring of the total efflux pump activity. During the assay, cell suspensions are aerated and thermostated, reagents are added to the reaction mixtures and samples are taken for additional analyses without interrupting the experiment or changing the culture conditions.

4.1. Evaluation of TetA(B) activity

Tn*10* encodes a tetracycline inducible efflux pump TetA(B), which is widely used as a tetracycline-resistance genetic marker in molecular microbiology. In this work TetA(B) influence on the interactions of TPP^+ with *E. coli* cells was analyzed. Both, MC4100 (wild type) and IQ86 (harboring Tn*10*) cells accumulated similar amounts of TPP^+ when added to the measurement medium. However, the effects of tetracycline were different. IQ86 and MC4100 cells displayed different responses to different tetracycline concentrations. It indicates that tetracycline-inducible *tetA* at some level might be expressed constitutively. The differences (initial TPP^+ accumulation and the effects of added tetracycline) between the tetracycline-exposed versus unexposed IQ86 cells indicates that TPP^+ is a substrate of TetA(B) efflux pump. Tetracycline induced remarkable additional TPP^+ accumulation only when concentrations higher two orders of magnitude compared to TPP^+ were

used. This indicates that TPP^+ is more preferable substrate for this pump. In this part of the work, we have utilized TPP^+ to monitor of the activity of the substrate-specific efflux pump activity as well as its competitive inhibition in real-time. Our results illuminate the broad substrate range of the antibiotic-specific efflux pumps. Moreover, our assay can be successfully applied to study the TPP^+-extruding MDR pumps, especially when screening for novel inhibitors or combinations of antibiotics to overcome the antibiotic resistance conferred by active efflux.

4.2. Factors affecting MDR efflux pump activities.

Penetration of the MDR pump substrates through the cell envelope is generally dependent on the OM permeability in Gram-negative bacteria. The equilibrium distribution of the charged compounds also depends on $\Delta\psi$. TPP^+ has several advantages as an indicator substrate to study the activity of the MDR pumps: (i) the interactions with bacterial envelope components and cytosol are well understood (Flewelling & Hubbell, 1986; Hockings & Rogers, 1996; Lolkema *et al.*, 1983); (ii) the interference with the cellular functions is significantly lower when compared to that of intercalating agents, such as ethidium and has no effect on cell viability at concentrations used in these measurements (Table 3.1); (iii) TPP^+ sensitively reflects the PM depolarization induced by other substrates and/or inhibitors of MDR pumps, e.g. PAβN or GD; (iv) the relatively simple chemical structure of TPP^+ makes it easily amenable to extensive chemical modifications (Murphy, 2008). This feature might be useful for designing TPP^+ analogs specific to a particular MDR pumps.

One of the main factors, determining the distribution of TPP^+ between the interior of Gram-negative bacteria and the incubation medium is the OM permeability to lipophilic compounds. As the common Tris-EDTA treatment lysed PAO1 cells (Fig. 2.5), we adapted an alternative way to permeabilize *P.*

aeruginosa by adding EDTA to cells in phosphate buffer or buffered LB medium (see Fig. 2.7). There are essentially two pathways that might enable water-soluble lipophilic compounds, like TPP^+, to cross the OM: a lipid-mediated pathway for the hydrophobic compounds and the OM porin mediated pathway ensuring the penetration of hydrophilic compounds. In spite of its low molecular mass (339 Da), TPP^+ efficiently crosses the OM of enterobacteria only when the LPS layer is disturbed (Daugelavicius *et al.*, 1997; Daugelavicius *et al.*, 2000). TPP^+ accumulation-inducing effects of EDTA and PMB indicate that this lipophilic cation crosses the OM of *P. aeruginosa* through the lipid-mediated pathway. It is worth noting that the efficiency of these two permeabilizers (EDTA and PMB) is different. At cell growth supporting conditions – when *P. aeruginosa* cells are incubated in well-aerated LB-containing medium – the OM-permeabilizing efficiency of EDTA is low, but PMB effectively increased the accumulation of TPP^+ (Fig. 2.11). However, the hydrophilic pathway of TPP^+ entry into *P. aeruginosa* cells cannot be ignored. In contrast to the *E. coli* porin channels permeable to hydrophilic compounds up to 600 Da, the main porin of *P. aeruginosa* OprF allows diffusion of polysaccharides of 2,000–3,000 Da (Hancock & Brinkman, 2002; Nikaido, 2003). Besides this, the size of OprF channel depends on the medium temperature (De *et al.*, 1997; Jaouen *et al.*, 2004). This could be the reason for the fast accumulation of TPP^+ and the high efficiency of GD on *P. aeruginosa* cells incubated at 17°C (Fig. 2.10). It is necessary to take into account that in contrast to water-soluble compounds, the hydrophobic ones might use other protein channels to bypass only the hydrophilic LPS layer (Hearn *et al.*, 2009).

It is possible to abolish the activity of certain MDR pumps by selective inhibitors, i.e. PAβN for RND-type pumps as it was done here. We discovered that in phosphate buffer PAβN causes depolarization of the PM and, as a consequence, leakage of the accumulated TPP^+ (Fig. 2.6). It is worth noting that in the case of negatively charged pump substrates PAβN-induced depolarization of the PM cannot be detected because both the direct interaction of the inhibitor with the pump or the blockage of energy supply for the pump

activity (depolarization of the PM) lead to the same result – additional accumulation of the anions inside the cell. The depolarizing effect of PAβN can be prevented by supplementing the buffer with nutrients of the LB medium (Fig. 2.7) leading to additional TPP^+ accumulation. This additional TPP^+ accumulation – an indicator of the PAβN-sensitive MDR pump activity – is the highest under conditions at which the recreation of EDTA-disturbed OM barrier is abrogated: at low aeration conditions (Fig. 2.11, A) and/or in the exhausted growth medium (Fig. 2.11, D).

The RND-type efflux pumps use the energy of the proton gradient across the PM for drug extrusion and the drug efflux is accompanied by the influx of H^+ (Murakami *et al.*, 2006; Nikaido & Takatsuka, 2009). It has been shown (Zgurskaya & Nikaido, 1999) that the drug transport is dependent on the transmembrane pH gradient and is assumed to be dependent on $\Delta\psi$ as well. The structural information available for the RND type pumps AcrAB-TolC and MexAB-OprM also suggests that drug extrusion is coupled to H^+ influx (Seeger *et al.*, 2006; Sennhauser *et al.*, 2009). However, the molecular mechanism of such transport still remains to be determined. In alkaline incubation medium, used for our experiments (pH 8.0), chemical gradient of H^+ (transmembrane pH gradient) is negligible. According to the amount of accumulated TPP^+, the levels of $\Delta\psi$ of *P. aeruginosa* cells in 200 mM phosphate buffer and in the same buffer containing LB are very similar. Consequently, the level of proton motive force should be also very similar and therefore it cannot be the only factor influencing the interaction of TPP^+ and PAβN with the MDR pumps.

It has been shown that the mode of action of PAβN does not rely on deenergization of the PM, but rather on direct interaction with the pumps (Lomovskaya *et al.*, 2001; Yu *et al.*, 2003). It cannot be excluded that PAβN-induced depolarization of the PM in the phosphate buffer is due to a very intensive, $\Delta\psi$-exhausting, extrusion of PAβN from the cells or due to the inhibitor-induced short-circuiting of the membrane voltage. On the other hand, results in the same buffer obtained in the presence of LB indicate the

importance of the nutrient-induced intensification of metabolism. It is possible that the high level of proton motive force is not enough to keep the H^+ flux-driven MDR pumps active and it is necessary to guarantee high concentration of protons (low local pH) in the near vicinity of the drug-extruding pumps. According to Koch (1986), active bacterial proton pumps can increase the local proton concentration at the outer surface of the PM by two orders of magnitude. This factor could also explain the need of glucose for activation of MDR pumps in the cases of enterobacteria (Lomovskaya *et al.*, 2001; Martins *et al.*, 2009; Pannek *et al.*, 2006) and the coupling between proton and substrate binding to the pumps in the case of isolated MDR systems (Aires & Nikaido, 2005; Schuldiner, 2009). Beside this, it was shown (Chen *et al.*, 2008) that oxidation of *P. aeruginosa* Mex efflux pump's expression represor MexR leads to its dissociation from the promoter DNA. Respiration-induced derepression of the drug efflux pump operons could also explain the highly effective extrusion of TPP^+ from *P. aeruginosa* cells incubated in LB-containing medium at high aeration conditions (Fig. 2.11).

The total MDR pump activity varies significantly at the growth-promoting conditions. We have shown here that interaction of TPP^+ with the efflux pumps in *P. aeruginosa* depends not only on the $\Delta\psi$, but also on the physiological state of the cells, medium composition, intensity of aeration as well as temperature. All these experimental factors should be optimized when choosing the conditions for the evaluation of the MDR pump activities. Under optimal growth conditions, cells respond to the absence or the inhibition of the main MDR pumps and increase the efficiency of pumping by reinforcing the OM permeability barrier, and/or actuating alternative MDR pumps. Our description of the variables affecting the activity of the efflux systems in *P. aeruginosa* can be utilized to set up conditions for robust and reproducible measurements of the MDR pump activities.

4.3. Properties of clinical isolates of *P. aeruginosa*

The clustering of clinical isolates of *P. aeruginosa* was undertaken to analyze the heterogeneity of the isolated samples. In addition, the clinical isolates of *P. aeruginosa* were analyzed for the relationship between the total activities of efflux pumps and the antibiotic resistance.

In addition to the clustering results obtained using the "Minimum variance" algorithm other clustering algorithms ("Farthest neighbour" and average linkage variants) were tested. Contrary to the "Minimum variance", other dendograms showed only low correlations among the strains when physiological patterns were compared. Such misalignment is likely to be due to different methods used to compare the strains. The "Minimum variance" algorithm focuses on the variation with-in the cluster of strain factors, where in the further steps clustering is performed determining which two of the clusters give the least difference increase in with-in clustering variation. Other mentioned algorithms are based on the comparison of the single points ("Farthest neighbour") or the mean of distances (average linkage) of two clusters. Such clustering methods "overweight" some values that have only minor significance in some in a data cluster. Consequently, such a clustering leads to more dispersed data when compared to "Minimum variance" algorithm. Initial TPP^+ accumulation was considered to be evaluated in a less accurate manner (as described above) as it depends on both the ability of the cell surface to bind indicator cation and the OM permeability to lipophilic compounds, physiological properties of bacteria that cannot be reliably distinguished using this method. The Euclidean distance was restricted to the 30% of total length as different physiological properties of the analyzed strains begin to overlap in further grouping (e.g. group III and IV).

Ciprofloxacin belongs to the fluoroquinolones group of antibiotics. Ciprofloxacin is one of the last resort medicine used to treat the infections treatments caused by *P. aeruginosa*. Fluoroquinolones are antibiotics targeting the DNA gyrase (also known as topoisomerase II). The main resistance

mechanisms to fluoroquinolones in *P. aeruginosa* are active efflux of this antibiotic and/or modification of the target (mutations in the *gyrA/gyrB* genes) (Strateva & Yordanov, 2009). Isolates falling into group I showed no significant interactions with RND-type efflux pump inhibitor PAβN leading to the assumption that particular MDR efflux pumps had negligible impact on antibiotic resistance. It is possible that numerous ciprofloxacin-resistant isolates in group I belong to the resistant strains with modified targets for this antibiotic.

MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM efflux pumps have a wide range of transported antibiotics (Masuda *et al.*, 2000b). Moreover, several different Mex efflux pumps can be expressed in the same cell. The latter property confers the resistance to an even broader spectrum of antibiotics. Flourquinolones (e.g. ciprofloxacin) are the universal substrates for all above mentioned efflux pumps (Strateva & Yordanov, 2009). Such type of resistance could be assigned to the groups II, III and IV. However, the impact of target modification-based resistance cannot be excluded. Additional resistance to tobramycin (aminoglycoside) in isolates from the group II may be assigned to the activity of MexXY-OprM as standard strain MutGR1 is assigned to this group. This assumption was made as MexXY-OprM efflux system is the major factor contributing to the resistance to aminoglycosides in clinical isolates of *P. aeruginosa* (Poole, 2005b).

The second factor contributing to the resistance of clinical isolates to aminoglycosides is the enzymatic antibiotic inactivation. Aminoglycosides can be phosphorylated, acetylated or adenylated by aminoglycoside phosphoriltransferase, acetyltransferase and nucleotidyltransferase, respectively (Poole, 2005b). This type of resistance could be assigned to the additional resistance to tobramycin in isolates belonging to the group III. All group III isolates share physiological similarities with MexCD-OprJ and MexEF-OprN overproducers EryR and PAO7H, respectively. These efflux pumps do not contribute to the resistance to aminoglycosides (Morita *et al.*, 2001). According to this data it can be assumed that additional resistance to

tobramycin is conferred due to active enzymatic inactivation of this antibiotic in the isolates from this group.

Resistance to β-lactam antibiotic meropenem cannot be defined by particular resistance mechanism as β-lactam antibiotics can be expelled from *P. aeruginosa* by active efflux pumps, inactivated by β-lactamases (Strateva & Yordanov, 2009) or the entry of antibiotic into the cell could be restricted by low permeability of the cell envelope.

The physiological properties of bacteria observed in this study reflect remarkable differences among the members of a particular strain collection and/or population. It was shown that registration of MDR efflux pumps can be used for characterization of clinical *P. aeruginosa* isolates. Such characterization could be used to monitor physiological changes of isolates obtained from the same patient in different treatment periods. Physiological monitoring of bacteria is of high importance, as genotyping does not allow evaluation of the activity of MDR efflux pumps in physiologically active bacterial cells. Such difference, for example, is reflected in genetically indistinguishable strains KK0680 (cipR, tobraR) and ME1343 (cipR, tobraR) showing remarkably different efflux pumps activities. The same antibiotic resistance pattern could be explained by the different Mex efflux pump expression. It is known that Mex efflux pumps in *P. aeruginosa* extrude similar compounds and different pumps can contribute to the same antibiotic resistance pattern (Masuda *et al.*, 2000b). However, physiological patterns of those isolates are significantly different.

The best of our knowledge, the evaluation and grouping of clinical isolates based on their efflux pump activities has not been reported previously. We note that all described antibiotic resistance mechanisms cannot be fully corroborated by this type of classification. However, evaluation of the clinical isolates of *P. aeruginosa* using the real time efflux pump monitoring assay offers a more comprehensive understanding of the individual comparable physiological properties of each isolate analyzed.

CONCLUSIONS

1. Tetraphenylphosphonium ions can be successfully utilized as a probe for the real-time assessment of TetA(B) and MDR efflux pumps in *E. coli* and *P. aeruginosa*, respectively;

2. TetA(B) has a higher selectivity for tetraphenylphosphonium than for tetracycline;

3. The activity of RND-type efflux pump inhibitor PAβN depends on medium composition. PAβN depolarizes the plasma membrane of *P. aeruginosa* cells in experimental medium with no nutrients;

4. Lower than room temperature abolishes the activities of efflux pumps in *P. aeruginosa*;

5. *P. aeruginosa* cells restore the envelope barrier affecting the monitoring of efflux pumps activities when growth-optimal conditions are provided;

6. Described method can be successfully applied for evaluation of total MDR efflux pumps activities in clinical isolates of *P. aeruginosa*.

Acknowledgements

I would like to thank all the people who helped me during thesis period:

My supervisor Rimantas Daugelavičius for interesting and challenging PhD studies.

My scientific advisor Dennis Bamford for enormous help in both science and life.

Elenutė Bakienė for her sincere support and care.

Virga, Mart and Matas Krupovič for being the best friends and immeasurable help and support.

Inga Griškova-Bulanova and Oleg Bulanov for consultations.

Simona Bandzinaitė for the "new breath".

All the people who was working together with me during my PhD studies.

Professor Patrick Plésiat for sending standard strains of *P. aeruginosa*.

Professor Martti Vaara and Tanja Pasanen for genetic data on clinical isolates of *P. aeruginosa*.

My parents for their love and never doubting in me.

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